



# The effects of phycocyanin on bleomycin-induced pulmonary fibrosis and the intestinal microbiota in C57BL/6 mice

Yuanyuan Xie<sup>1,2,3</sup> · Wenjun Li<sup>1,3</sup> · Chenyang Lu<sup>4</sup> · Limeng Zhu<sup>5</sup> · Song Qin<sup>1,3</sup> · Zhenning Du<sup>2</sup>

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## Abstract

Phycocyanin (PC) is a light-harvesting protein isolated from *Spirulina* and has health benefits for a range of diseases including pulmonary fibrosis (PF). In this study, a bleomycin-induced pulmonary fibrosis model was used to determine whether PC attenuates PF and modulates the intestinal microbiota. The results showed that PC intervention attenuated the pulmonary fibrosis, demonstrated by hematoxylin-eosin staining (HE), Masson's trichrome staining, and lung dry-wet weight ratio, and PC significantly inhibited the production of interleukin-1 beta (IL-1 $\beta$ ), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), and lipopolysaccharide (LPS). Additionally, intestinal microbiota analysis revealed that PC intervention significantly increased the bacterial diversity and richness. Correlation analysis indicated that 9 families and 17 genes were significantly associated with at least 1 physiological index. And PC intervention significantly decreased the bacteria which is related to inflammation and dramatically increased the SCFAs-producing bacteria and probiotics. These data indicated that PC can decrease the pro-inflammatory cytokines and regulate the intestinal microbiota in BLM-induced PF mice.

**Keywords** Phycocyanin · Pulmonary fibrosis · Cytokines · Intestinal microbiome

## Introduction

Idiopathic pulmonary fibrosis (IPF) is a disease characterized by macrophages, lymphocytes, and other inflammatory cells in the interstitial infiltration, fibroblast proliferation, and fibrous connective tissue deposition, which is the ultimate

outcome of a series of chronic lung diseases and it causes great harm to human health with median survival of 2–3 years (Shappell et al. 1998). Pulmonary fibrosis is a complex pathophysiological process. In recent years, the incidence of pulmonary fibrosis has been increasing and there is still no effective treatment in clinic (Noble et al. 2012). Therefore, the

Yuanyuan Xie and Wenjun Li contributed equally to this work.

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✉ Song Qin  
sqin@yic.ac.cn

✉ Zhenning Du  
zhenning.du@163.com

Yuanyuan Xie  
1434589950@qq.com

Wenjun Li  
wjli@yic.ac.cn

Chenyang Lu  
cy\_lu1988@163.com

Limeng Zhu  
limengzhu@yic.ac.cn

- <sup>1</sup> Key Laboratory of Biology and Bioresource Utilization, Yantai Institute of Coastal Zone Research, Chinese Academy of Sciences, Yantai 264003, China
- <sup>2</sup> School of Pharmacy, Yantai University, Yantai 264005, China
- <sup>3</sup> Center for Ocean Mega-Science, Chinese Academy of Sciences, Qingdao 266071, China
- <sup>4</sup> School of Marine Sciences, Ningbo University, Ningbo 315211, China
- <sup>5</sup> Institute of Process Engineering, Chinese Academy of Sciences, Beijing 100190, China

current treatment of this devastating disease is basically unsuccessful (Raghu et al. 2013; Wilson et al. 2014), resulting in an average survival time of only 3–5 years after diagnosis (Yao et al. 2016). The search for effective treatment of pulmonary fibrosis has become an urgent need for current medicine (Lissi et al. 2000).

Phycocyanin (PC) is a light-harvesting protein isolated from *Spirulina platensis* and participates in algal photosynthesis. The role of PC in promoting health has been widely accepted in the past two decades (Wu et al. 2016). Many researches have shown that PC has significant antioxidant, anti-inflammatory, liver-protecting, anti-arthritic, neuroprotective, and anti-tumor effects (Cherng et al. 2007; Li et al. 2005; Romay et al. 2003; Sathyaikumar et al. 2007). Previous study showed that PC could attenuate acute lung injury in rats by reducing oxidative stress damages and inhibiting NF- $\kappa$ B-mediated cytotoxicity. And the effect of PC in attenuation lung injury was also detected in lipopolysaccharide (LPS)-induced rats (Sun et al. 2011).

In recent years, with the deepening of research on lung diseases (Ndj and Marsland 2017; Samuelson et al. 2015), more and more studies have proved that there are important crosstalk between intestinal microbiota and lung, which has an important role in maintaining host health. Numerous studies have shown that many lung diseases are often accompanied by changes in intestinal microbiota, which in turn can affect lung immunity (He et al. 2017).

In this study, we use bleomycin (BLM) to induce pulmonary fibrosis mice model. And the effects of PC on pulmonary fibrosis were evaluated by pathological section staining of the lung, the levels of pro-inflammatory and intestinal microbiota in mice. We are the first to report the improvement and regulation of PC in pro-inflammatory cytokines and intestinal microbiota in PF mice.

## Materials and methods

### Materials

PC was purchased from King Dnarmse Spirulina Company (Fuqing, China). The mouse feed was purchased from the Jinan Pengyue Animal Breeding Center (Jinan, China). Table S1 shows the daily dietary components of mice. IL-1 $\beta$  and TNF- $\alpha$  enzyme-linked immunosorbent assay (ELISA) kits were purchased from proteintech™. HE and MASSON stain kits were purchased from Solarbio (Beijing, China).

### Animals and sample collection

In this study, 72 6-week-old SPF male C57BL/6 mice were used. The mice were acclimated for 1 week under conditions controlled by temperature ( $23 \pm 2$  °C) and humidity

(50–60%), maintaining a 12 h/12 h light/dark cycles and allowing mice to freely harvest energy. Then we use SPSS software to divide the mice into four groups ( $n = 18$  for each group): control group (NC), bleomycin group (BL), bleomycin + 50 mg/kg phycocyanin group (PC), and bleomycin + pirfenidone group (PFD). And mice were placed in an IVC closed cage in a sober condition and 0.25% BLM was atomized with a nebulizer and sprayed into the cage through a spray tube for 30 min each time for six times. The control mice were treated with the same method; only BLM was changed to deionized water. Then, mice in PC group were intragastrically administered with 50 mg/kg PC per day, mice in PFD group were intragastrically administered with 50 mg/kg PFD per day, and the other two groups were given with the same volume of deionized water per day. At 7 and 28 days, nine mice were euthanized. Mice serum, lung, and gut tissue were excised and stored in liquid nitrogen until use.

At the beginning and end of the experiment, each mouse was placed in a separate sterile cage and 150 mg of fresh stool samples were collected in sterile cryopreserved tubes and immediately stored in liquid nitrogen until use.

### Pathological changes in lung tissue of mice

Pathological examination in lung tissue was performed using an optical microscope. In our study, the left lung of the mice were perfused and fixed with 4% formaldehyde and routinely processed to embed the samples in paraffin. Lung tissues were sectioned and subjected to HE and MASSON stain.

Sections were observed using BX-51M fluorescence microscope (Olympus, JP). Lung injury scores 0, 1, 2, 3, and 4 indicate no injury, mild injury, moderate injury, severe injury, and very severe histological changes respectively. And fibrosis was scored using Ashcroft score (Hubner et al. 2008).

### Determination serum and lung tissues IL-1 $\beta$ , TNF- $\alpha$ , and lipopolysaccharide levels

To detect IL-1 $\beta$ , TNF- $\alpha$ , and LPS levels in serum and lung tissue, the IL-1 $\beta$ , TNF- $\alpha$ , and LPS ELISA kits were used. Absorbance values were measured at 450 nm using an enzyme labeling instrument according to the instructions.

### Determination of lung dry-wet weight ratio

Seven days after, the bleomycin was atomized. The right lung of the mouse was taken and the right lower lobe was blotted to its “wet” weight. It was then placed in an oven at 60 °C and after 72 h weighed to obtain a “dry” weight.

## Extraction of genomic DNA, amplicon generations, and Illumina sequencing

The QIAamp DNA Stool Mini Kit (Qiagen, Germany) was used to extract the total genomic DNA in the feces samples. The 16S rRNA gene V3-V4 hypervariable region was selected and the barcode-specific primers (338F and 806R) were used for amplification. Gel electrophoresis was used to detect the presence of the amplicons and AxyPrep DNA Purification Kit (Axygen Biosciences, Union City, CA, USA) was used to purify the amplications, and then the PCR product was quantified using a QuantiFluor-ST fluorometer (Promega, USA) and analyzed using the MiSeq platform (Illumina, Novogene, Beijing, China) according to standard protocols for paired end sequencing ( $2 \times 300$ ).

## Data accessibility

The raw data were uploaded to the National Center for Biotechnology Information, and SRA database under the accession number PRJNA546446.

## Data analysis

The paired-end-sequenced fragments were spliced with FLASH and 0.97 was set as the operational classification units (OTUs) and species classification threshold. The representative sequence of each OTU was annotated by the RDP classifier, and then the corresponding species information and species-based abundance distribution were obtained. The sequence was analyzed using QIIME software. The alpha ( $\alpha$ ) diversity was analyzed by mothur. The difference between different groups was analyzed based on the principal coordinate analysis (PCoA) of UniFrac. And the correlation between different physiological indexes and the intestinal microbiota was analyzed by SPSS.

## Statistical analysis

Before the difference analysis, we performed the homogeneity test of variance. All data are expressed as mean  $\pm$  SDs. Comparisons between groups were tested by one-way ANOVA (Tukey's test);  $p < 0.05$  was considered significantly different. Correlations were calculated by Spearman's correlation analyses with 95% confidence intervals.

## Results

### Pathological changes of lung tissue in mice after PC intervention

HE staining and Masson staining was used to analyze lung injury and fibrosis. Compared with the NC group, obvious

inflammatory cell infiltration in the lung tissue, blood cells, and tissue edema in the alveoli and a small amount of blue collagen fibers appeared in mice 7 days after the BLM was atomized (Fig. 1a). Alveolar structural disorder, abnormal thickening of the alveolar wall, massive deposition of matrix, amount of blue collagen fibers, and fibroblasts appeared in mice 28 days after the BLM was atomized (Fig. 1b). Obviously, PC and PFD treatment groups significantly reduced lung injury and fibrosis induced by BLM at 7 and 28 days. The PC group and the PFD group have no significant differences.

### The effect of PC on lung dry-wet weight ratio

The lung dry-wet weight ratio (D/W) was used to assess the extent of pulmonary edema. Compared with the NC group, the lung dry-wet weight ratio was significantly increased in the BL group ( $p < 0.05$ ), and the PC and PFD treatments significantly decreased the ratio (Fig. 2). The results indicate that PC might attenuate the pulmonary fibrosis by decreasing the lung edema.

### The effect of PC on BLM-induced cytokines and LPS release

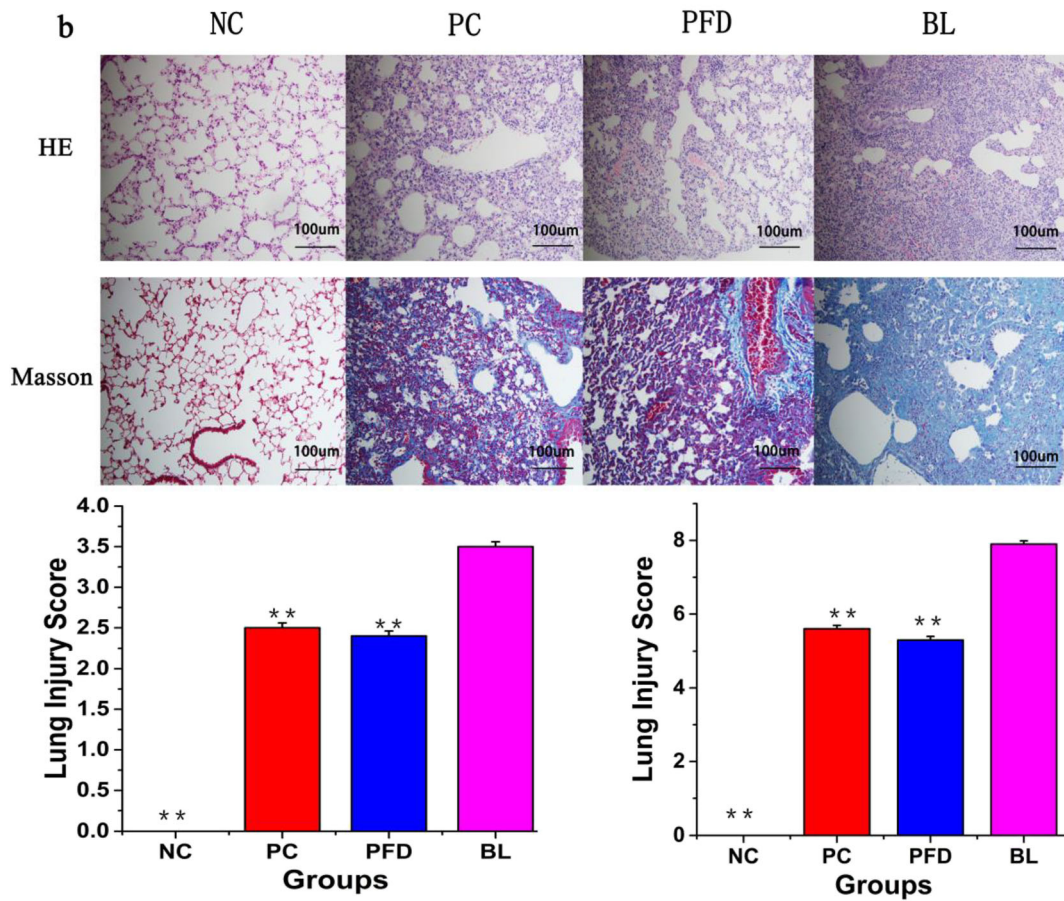
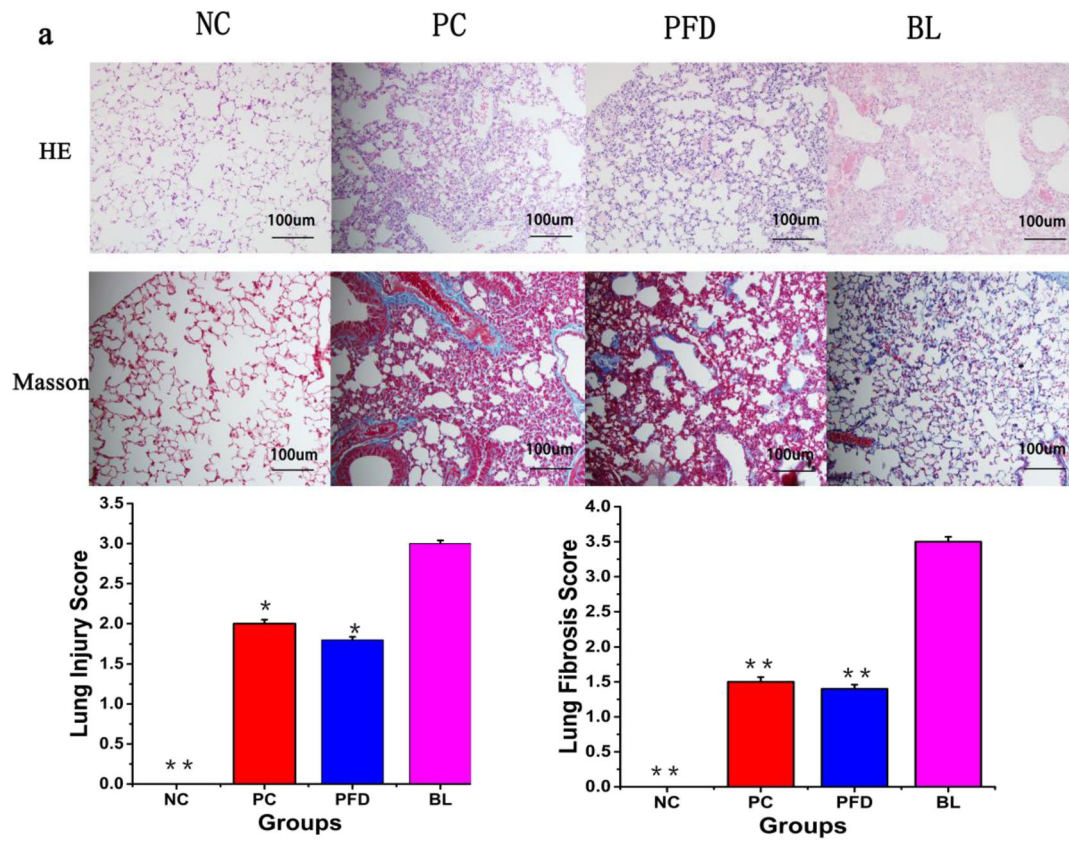
The levels of IL-1 $\beta$ , TNF- $\alpha$ , and LPS in lung serum and gut of mice were measured by ELISA kit after 7 days of BLM nebulization (Fig. 3). The ELISA results showed that compared to the control group, the levels of IL-1 $\beta$ , TNF- $\alpha$ , and LPS in lung tissue, serum, and gut tissue of the BLM group were significantly increased on day 7 after BLM atomization, and the inflammatory cytokines were significantly decreased after PC and PFD treatment ( $p < 0.05$ ).

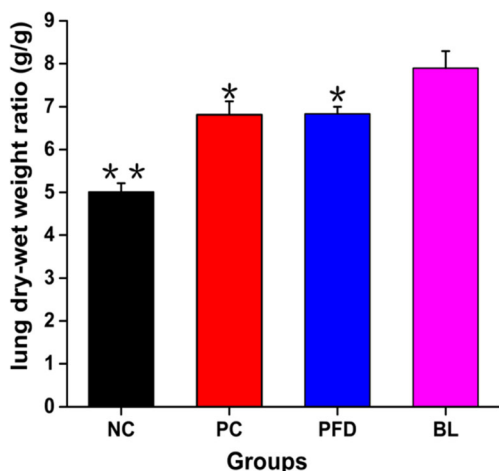
### The effects of PC on modulation of intestinal microbiota structure

After high-throughput sequencing, we obtained 32,179 effective sequences. The sample rarefaction curve tended to be stable, indicating that sequencing data was sufficient (Fig. 4a). The richness and diversity of bacterial in the intestinal microbiota were determined. Compared to NC group, the index of Shannon and Simpson were lower in the BL group (Fig. 4b, c). And the index of Chao1 and ACE was also lower in the BL group (Fig. 4d, e). The diversity and richness in

**Fig. 1** The effects of PC on the pulmonary fibrosis tissue morphology on day 7 and 28 after nebulization. **a** Observation of lung morphology by hematoxylin-eosin (HE) staining and Masson's trichrome staining on the 7th day after nebulization. **b** Pulmonary morphology observed with HE and MASSON staining on the 28th day after nebulization. (HE and Masson stained sections were imaged at  $\times 200$  magnification). *NC* blank control group, *PC* phycocyanin treatment group, *PFD* Pirfenidone treatment group, *BL* bleomycin model group







**Fig. 2** The effect of PC on lung dry-wet weight ratio. Data was represented as the mean  $\pm$  SD ( $n=6$ ). One-way ANOVA (Tukey's test) was used to test the difference in different groups,  $p < 0.05$  (\*) was considered significantly different compared with the BL group ( $p < 0.05$ )

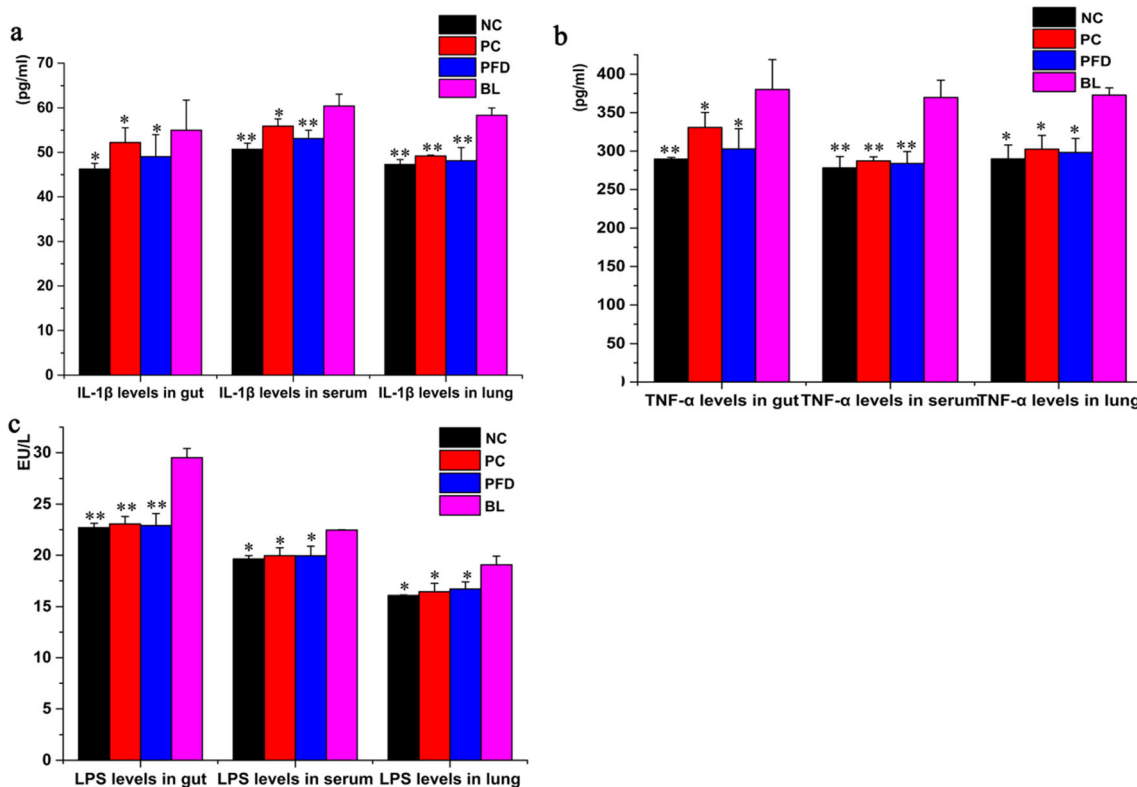
intestinal microbiota was significantly increased after PC and PFD intervention ( $p < 0.05$ ). The overall structure of intestinal microbiota was analyzed using weighted UniFrac PCoA (Fig. 4f). The result indicated that the structure of the overall intestinal microbiota was significantly changed after BLM intervention, and the overall structure of the intestinal

microbiota was shifted toward the NC group after PC and PFD treatment.

At the phylum level, the most abundant phyla in intestinal microbiota were *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Deferribacteres*, *Actinobacteria*, and *Tenericutes*, and the dominant bacteria were *Firmicutes* and *Bacteroidetes*, accounting for more than 90% in all bacteria (Fig. 5a). Compared to PC group, the abundance of *Firmicutes* and *Proteobacteria* was increased and the abundance of *Bacteroidetes* was decreased after BLM treatment; PC and PFD treatment inverse the effects (Fig. 5a and Table S2).

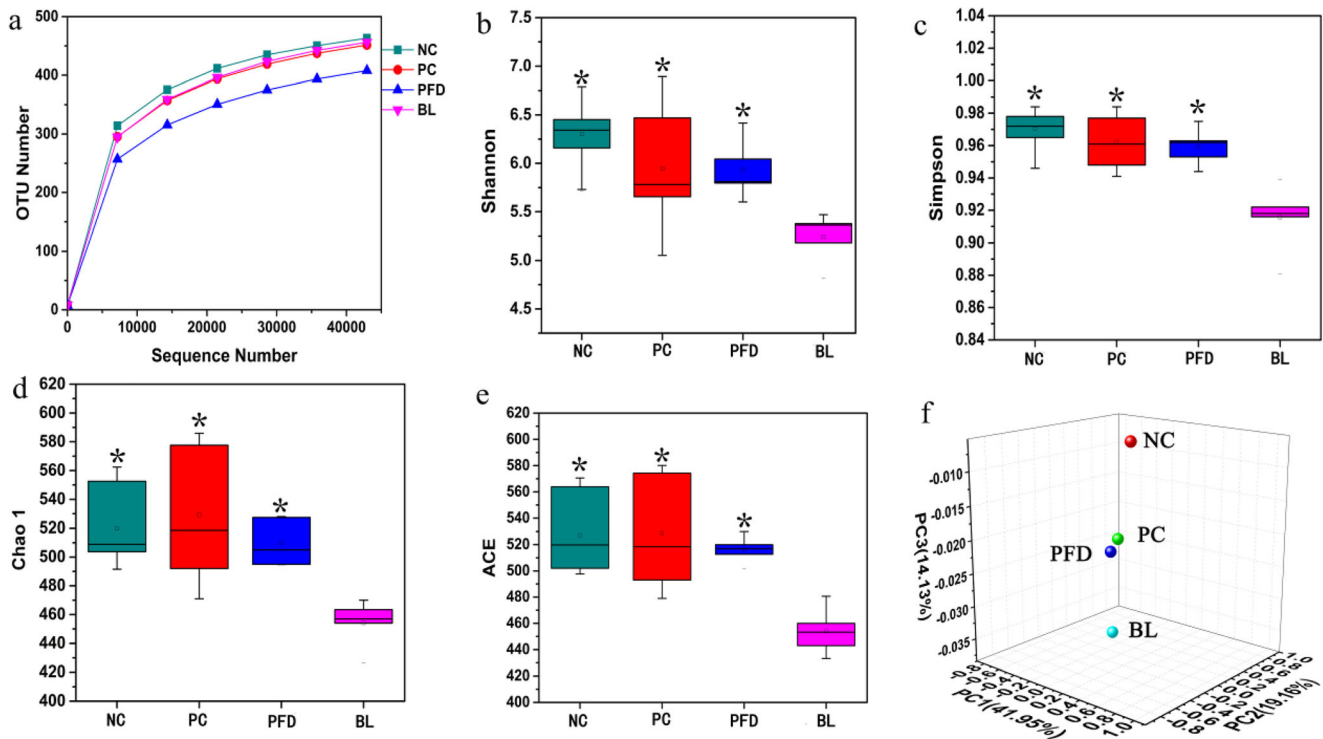
At the family level, high abundance of the intestinal microbiota in all groups was shown in Fig. 5b. Compared to NC group, the relative abundance of *Muribaculaceae*, *Lachnospiraceae*, *Lactobacillaceae*, *Ruminococcaceae*, *Rikenellaceae*, *Akkermansiaceae*, and *Bacteroidaceae* was significantly reduced and the relative abundance of *Erysipelotrichaceae*, *Helicobacteraceae*, and *Staphylococcaceae* was significantly increased after BLM treatment. The treatment of PC and PFD significantly inverted the effects (Fig. 5b and Table S3).

The correlation coefficients of the differential abundance of the bacterial families and the ratio of lung dry-wet weight or expression of pro-inflammatory cytokines in lung and serum were calculated by spearman's correlation



**Fig. 3** The effect of PC on BLM-induced cytokines release. **a** The levels of IL-1 $\beta$  in lung, serum, and gut of mice. **b** The levels of TNF- $\alpha$  in lung, serum and gut of mice. **c** The levels of LPS in lung, serum, and gut of mice. Data were represented as mean  $\pm$  SD ( $n=5$ ). One-way ANOVA

(Tukey's test) was used to test the difference in different groups,  $p < 0.05$  (\*) was considered significantly different compared with the BL group ( $p < 0.05$ )



**Fig. 4** Analysis of  $\alpha$  diversity and overall structure of mouse intestinal microbiota. **a** Rarefaction curve of intestinal microbiota in each group of mice. **b** Shannon index of intestinal microbiota in each group of mice. **c** Simpson index of intestinal microbiota in each group of mice. **d** Chao1 value of intestinal microbiota in each group of mice. **e** ACE value of

intestinal microbiota in each group of mice. **f** Principal coordinates analysis (PCoA) of mice based on weighted UniFrac distances. Data were represented as mean  $\pm$  SD ( $n = 5$ ). One-way ANOVA (Tukey's test) was used to test the difference in different groups.  $p < 0.05$  (\*) was considered significantly different compared with the BL group ( $p < 0.05$ )

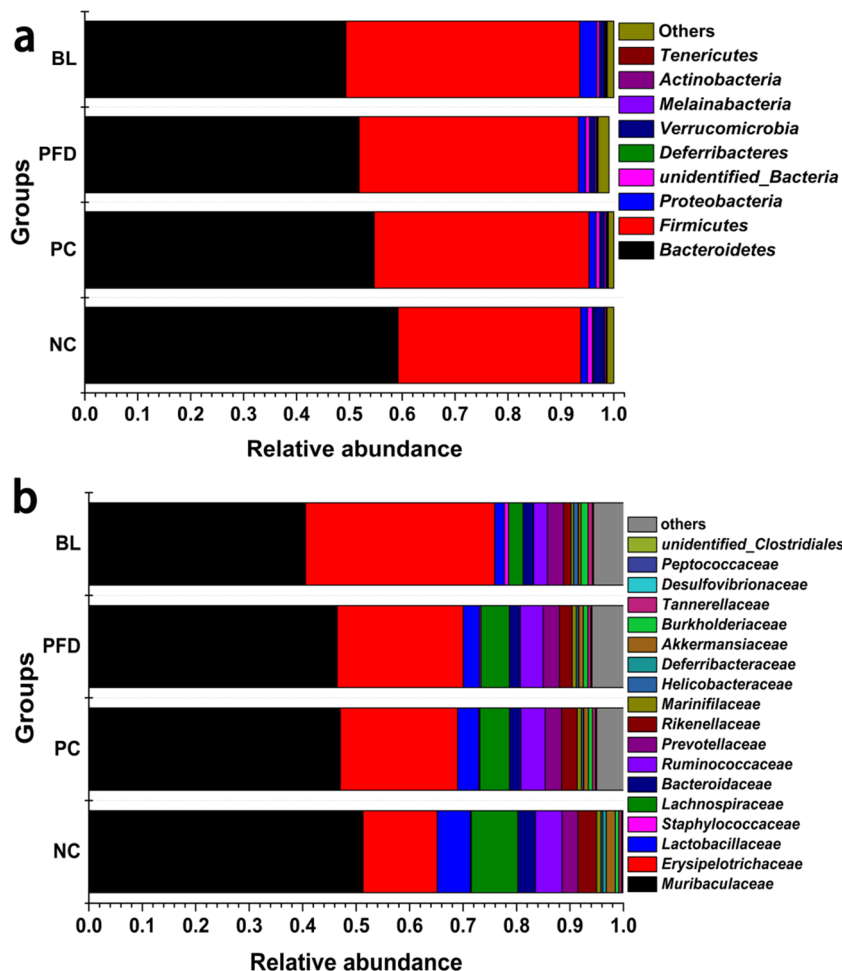
analyses in mice ( $n = 5$ ). The results (Fig. 6) showed that the abundance of the bacterial families *Muribaculaceae*, *Lachnospiraceae*, *Lactobacillaceae*, *Ruminococcaceae*, *Rikenellaceae*, and *Akkermansiaceae* were negatively correlated with the ratio of lung dry-wet weight and expression of pro-inflammatory cytokines, while the community abundance of *Erysipelotrichaceae*, *Helicobacteraceae*, and *Staphylococcaceae* were positively correlated with the ratio of lung dry-wet weight and expression of pro-inflammatory cytokines. In addition, high abundance of the intestinal microbiota in all groups of 22 genus were identified after PC intervention. Spearman's correlation analyses of the differential abundance of the 22 genus and the ratio of lung dry-wet weight or expression of pro-inflammatory cytokines in lung and serum were calculated in mice ( $n = 5$ ). The results (Fig. 7) showed that 17 genus were significantly correlated with at least 1 of the physiological index. These results indicated that intestinal microbiota might be associated with inflammation in the mice and anti-inflammation effects of PC were associated with the specific intestinal microbiota in a certain degree. The effect of intestinal microbiota changes on pulmonary fibrosis caused by BLM was shown in Fig. 8. The result showed that changes in intestinal microbiota might influence the lung through the blood.

## Discussion

The study showed that PC attenuated BLM-induced pulmonary inflammatory and fibrosis demonstrated by HE and MASSON. And the PC intervention significantly decreased the levels of lung, serum, and gut pro-inflammatory cytokines and LPS. Additionally, BLM treatment significantly increased the abundance of some harmful bacteria, while the abundance of some beneficial bacteria was significantly reduced, and PC treatment can effectively reverse this phenomenon.

As one of the chemotherapeutic drugs, BLM is widely used for the induction of mouse IPF model. The drug causes a time-dependent increase in tissue infiltration of pro-inflammatory cells and cytokines (Aono et al. 2012). In this study, HE and MASSON stain were used to analyze lung injury and fibrosis. Under the influence of BLM, alveolar structure and interstitial lung presented different degrees of injury and fibrosis (Fig. 1a, b). PC and PFD intervention significantly attenuated the injury and fibrosis, which indicated that PC can reduce the degree of pulmonary fibrosis to a certain extent. Pro-inflammatory cytokines directly damage lung tissue cells, resulting in extensive damage and permeability of pulmonary vascular endothelial cells and alveolar epithelial cells, pulmonary edema, and microthrombus formation,

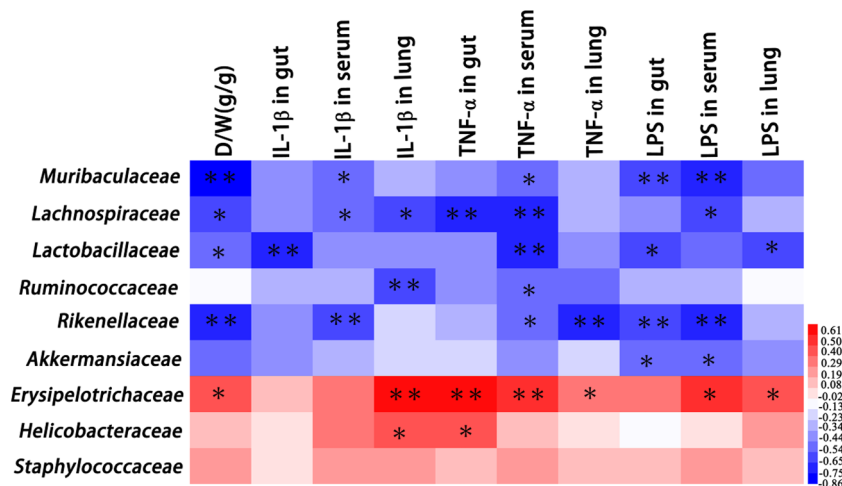
**Fig. 5** Effects of PC on the phylum level (a) and family level (b) of intestinal microbiota



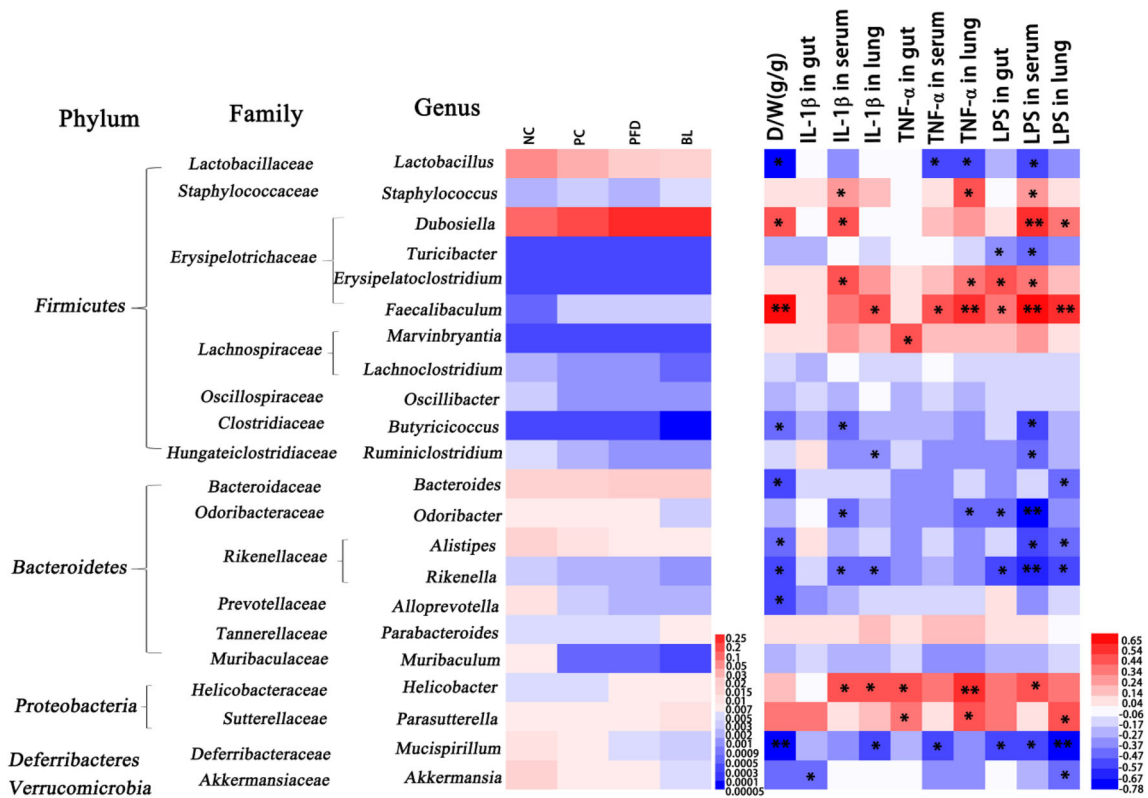
which finally lead to the progress of pulmonary fibrosis. Pro-inflammatory cytokines are also associated with a variety of pathways, such as NF-κB. Effective inhibition of the production of pro-inflammatory cytokines can reduce the progression of pulmonary fibrosis. Numerous studies have shown that the production of pulmonary fibrosis is associated with increased inflammation, mainly as an

increase in IL-1β and TNF-α (Meziani et al. 2018; Shih et al. 2009). In this study, PC intervention significantly reduced the levels of IL-1β, TNF-α, and LPS in BLM-treated mice. The protective effect of PC on pulmonary fibrosis might be highly related to the reduction of inflammation and LPS. Moreover, PC reversed the increasing of lung dry-wet weight ratio in BLM-treated mice, that may

**Fig. 6** Correlation analysis between the bacteria of family and mouse physiological index (dry-wet weight ratio (g/g), IL-1β level, and TNF-α level). A positive correlation was expressed as red and a negative correlation was expressed as blue. The degree of correlation between the family abundant and the physiological index of the hos was expressed by intensity of the colors. \**p* < 0.05; \*\**p* < 0.01







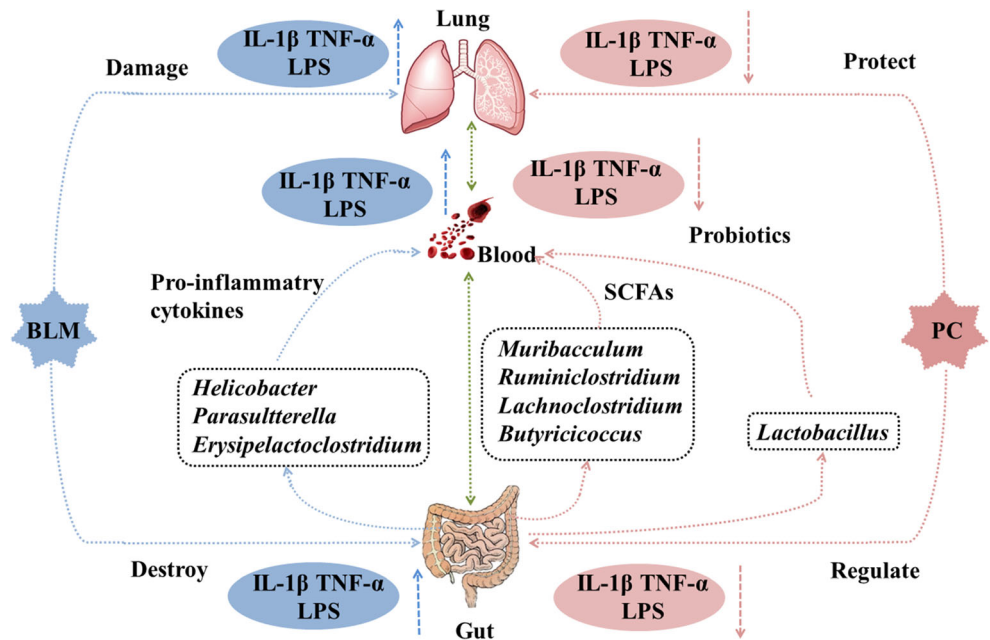
**Fig. 7** Effects of PC on the genus level of intestinal microbiota. **a** The abundance of 22 OTUs was shown as Heatmap. **b** Correlation analysis of genus abundance and mouse phenotypes (dry-wet weight ratio (g/g), IL-1β level, and TNF-α level). A positive correlation was expressed as red

and a negative correlation was expressed as blue. The degree of correlation between the genus abundant and the physiological index of the hos was expressed by intensity of the colors. \* $p < 0.05$ ; \*\* $p < 0.01$

reduce the lung injury. Studies have shown that PC significantly inhibits TNF-α, IL-1β, IL-6 release, and neutrophil infiltration in experimental hyperalgesia inflammation sites. PC can reverse the increase of TNF-α, IL-1β,

and IL-6 levels after microglia LPS induced (Chen et al. 2012). In addition, PC administration also exerts significant protective effects on inflammatory bowel disease, including a reduction in weight loss and inflammatory

**Fig. 8** The effect of intestinal microbiota changes on pulmonary fibrosis caused by BLM





lesions in the colon, and inhibition of macrophage activation by inhibition of nuclear translocation (Budden et al. 2017). These results indicate that PC can alleviate a variety of diseases by inhibiting the inflammatory response, including BLM-induced pulmonary fibrosis.

Not only the lung functions were changed but also the intestinal microbiota were dysbiosis after BLM treatment. The intestinal microbiota structure analysis showed that the diversity and richness of intestinal microbiota increased after PC and PFD treatment compared to BL group, which indicated that PC and PFD treatment can significantly improve the reduction of intestinal microbiota in mice caused by BLM. Additionally, the structure of the intestinal microbiota in BLM-treated mice was significantly changed compared to the NC group mice, and the overall structure of the intestinal microbiota was shifted toward the NC group after PC and PFD treatment.

On the basis of the analysis of the correlation between intestinal microbiota and pulmonary fibrosis physiological indicators, we determined the possible regulation of PC on intestinal microbiota.

First, numerous studies have shown that many bacteria in the intestinal microbiota were related to inflammation (Dziarski et al. 2016; Lavelle et al. 2015; Loy et al. 2017). For example, the genes of *Helicobacter*, which is represented by *Helicobacter pylori*, can induce a variety of cytokines and chemokines, such as TNF- $\alpha$ , IL-6, IL-8, and histamine (Kountouras et al. 2015). *Helicobacter pylori* harms the intestinal wall by producing ammonia and some biochemicals. Ammonia, which used to regulate pH, is toxic to epithelial cells, and biochemicals such as protease, vacuolating cytotoxin A (*VacA*), and some phospholipases, which are produced by *Helicobacter pylori*, can destroy epithelial cells, disrupts tight junctions, and leads to apoptosis (Smoot 1997). Compared to BL group, PC intervention significantly decreased the relative abundance of *Helicobacter*. And other bacterial associated with inflammation include *Erysipelactoclostridium* and *Parasultterella*, which followed the same trend with *Helicobacter* in our study.

Second, a large number of studies have shown that many bacteria in the intestine were associated with the production of short-chain fatty acids (SCFAs), such as *Muribaculum*, *Ruminiclostridium*, *Lachnoclostridium*, and *Butyrivicoccus* (Chen et al. 2011; Meehan and Beiko 2014). SCFAs are produced by fermentation of the intestinal microbiota, which can directly supply energy to the intestinal epithelium, improve intestinal digestion, nutrient absorption, and intestinal immunity (Vital et al. 2017). SCFAs could regulate at least two signaling molecular systems, which are histone deacetylase (HDACs) and G protein coupled receptors (GPCRs). SCFAs are natural inhibitors of HDACs and GPCRs (Tan et al. 2014). HDACs are involved in traumatic injury, and GPCRs are involved in lung diseases (Edwards 2010). Compared to BL group, PC intervention significantly increased the relative abundance of

*Muribaculum*, *Ruminiclostridium*, *Lachnoclostridium*, and *Butyrivicoccus*, which increased the synthesis of SCFAs and enhance its inhibitory effects of HDACs and GPCRs.

In addition, studies have shown that many of the microbiota in the gut are probiotics, such as *Lactobacillus* (Daughtry et al. 2018). Probiotics have an important regulatory effect on human health; it can increase the immunity of human mucous intestinal tract by increasing the amount of LgA(+)(Ashraf and Shah 2014). And probiotics help to treat the disease by regulating the balance of the intestinal microbiota in the body, when the level of opportunistic pathogens in the intestinal microbiota is abnormally elevated (Ford et al. 2014). Additionally, *Lactobacillus salivarius* interacts with many pathogens by producing salivary hormone B (a bacteriocin) (ten Brink et al. 1994). Compared to BL group, the relative abundance of *Lactobacillus* was significantly increased after PC intervention, which may increase the immunity and balance of the intestinal microbiota.

In conclusion, pulmonary fibrosis mouse model induced by BLM was used in this study, which has been shown that PC intervention (i) significantly attenuated pulmonary fibrosis scores demonstrated by HE and MASSON staining; (ii) significantly reduced pro-inflammatory cytokine and LPS levels demonstrated by reducing IL-6, TNF- $\alpha$ , and LPS levels in lung, serum, and gut; and (iii) significantly shifted the overall structure of the intestinal microbiota toward the NC group. And the changes of IL-6, TNF-, and LPS levels in lung, serum, and gut demonstrated that there was a significant crosstalk between the intestines and the lungs. However, the crosstalk mechanism between PC attenuate pulmonary fibrosis and intestinal microbiota needs further exploration.

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## Compliance with ethical standards

The study design was approved by the Animal Care and Maintenance Committee of Shandong International Biotechnology Park (SCXK 20140007) (Yantai, China), and complied with NIH guidelines for the care and use of laboratory animals. All authors confirm that ethical principles have been followed in the experiments.

**Conflict of interest** The authors declare that they have no conflict of interest.

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