Doctor of Philosophy

Gut microbial modulation attenuates emphysema development via suppression of inflammation and apoptosis

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Gut microbial modulation attenuates emphysema development via suppression of inflammation and apoptosis

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This certifies that the dissertation of Se Hee Lee is approved

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I am deeply thankful to my parents for their love, support, and prayers. Also, I express my thanks to my brother for his support and encouragement. Finally, I thank all the people who have supported me to finish the research work directly or indirectly.

Abstract

Background: Chronic obstructive pulmonary disease (COPD) is the treatable, but hardly curable disease associated with significant morbidity. Recent work has suggested a microbial dysbiosis association between the lung and gut in respiratory diseases. However, the therapeutic implication of the gut microbiome for COPD remains unclear. Here, we demonstrated that gut microbiome modulation attenuated emphysema development.

Methods: To modulate the gut microbiome, fecal microbiota transplantation (FMT), diet modification, or both were used in mice exposed to smoking and poly I:C for an emphysema model. We analyzed emphysema severity using mean linear intercept (MLI) and apoptosis using TUNEL assay. Microbiome analyses were also performed in feces and fecal extracellular vesicle.

Results: MLI was significantly increased with smoking exposure. FMT or high-fiber diet (HFD) attenuated the increase. Weight loss, combined with smoking exposure, was not noted in mice with FMT. HFD significantly decreased macrophages and lymphocytes in bronchoalveolar lavage fluid. Furthermore, IL-6 and IFN- γ were decreased in the bronchoalveolar lavage fluid and serum. The TUNEL score was significantly lower in mice with FMT or HFD, suggesting decreased cell apoptosis. In microbiome analysis, *Bacteroidaceae* and *Lachnospiraceae* increased with FMT and HFD. The fecal SCFA concentration was also notably higher with FMT and HFD.

Conclusions: FMT and HFD attenuated emphysema development via local and systemic inhibition of inflammation and changes in gut microbiota composition, which could provide a new paradigm in COPD treatment.

Keywords: Dysbiosis, Emphysema, Microbiota, Prebiotics, Probiotics.

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Introduction

Chronic obstructive pulmonary disease (COPD) is a chronic progressive disease with significant worldwide morbidity and mortality[1, 2]. Despite progress in management, the overall therapeutic strategy has not changed in recent decades; smoking cessation and bronchodilators are still the main treatment. Pulmonary and systematic inflammation persists in patients with established COPD even after smoking cessation[3]. Bronchodilators improve symptoms, lung function, and quality of life, but they hardly normalize pulmonary function in most cases, and their ability to control inflammation is limited. Therefore, new COPD treatment modalities are needed.

Although cigarette smoking (CS) is the most important risk factor for COPD, only some smokers progress to COPD, suggesting that there are individual differences in CS susceptibility[4, 5]. The maintenance of immune homeostasis may be a critical contributor to the susceptibility of a smoker, and this could be due to interactions between the host immune system and microbes[6, 7]. Recently, intimate interactions between gut microbes and the lung were called the gut-lung axis (**Fig. 1**), and microbial dysbiosis in this axis is related to chronic respiratory diseases[8-10], relatively well described in allergic airway diseases[11, 12]. Modulation of gut microbial dysbiosis using diet (prebiotics) and microbe metabolites (postbiotics) demonstrated a beneficial role in the asthma model[11]. Accumulating evidence supports the diverse role of dietary metabolites from microbes in host immune homeostasis[13-15].

Currently, the functional role of the gut-lung microbial axis in COPD pathogenesis remains poorly understood. Moreover, the potential impact of its modulation as a novel therapeutic strategy for COPD has not been adequately addressed. Here, we used a murine emphysema model that has been widely used for COPD research[16, 17] to demonstrate that gut microbiome modulation would attenuate the development of emphysema.

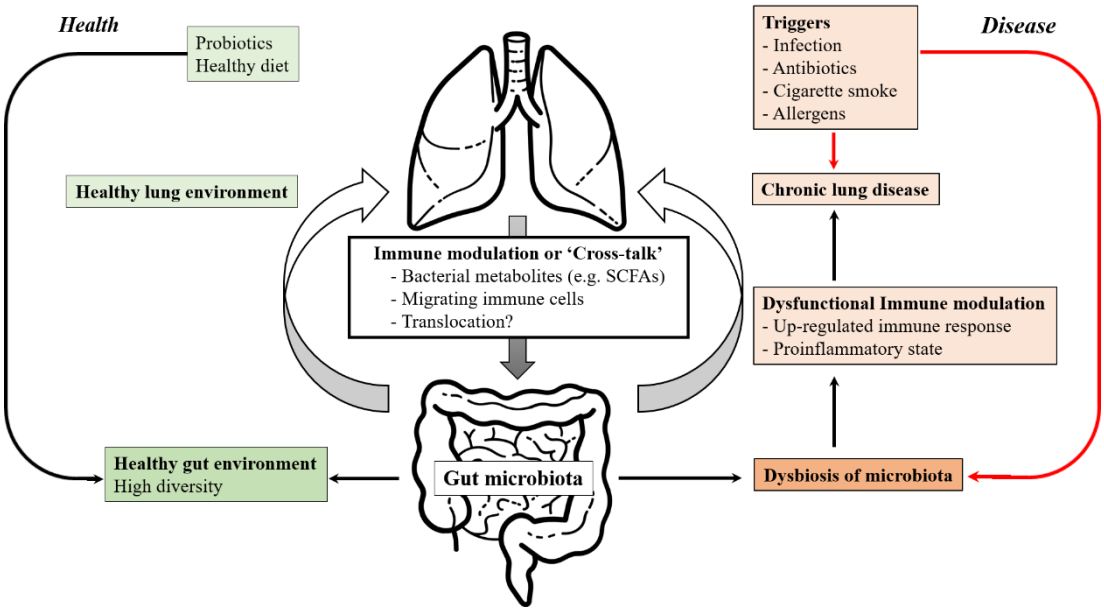

Figure 1. The proposed concept of the gut-lung axis in health and disease.

In healthy individuals, healthy gut microbiota maintains local and systemic immune homeostasis through microbiota-derived metabolites or various host immune cells. There are triggers, such as infection, antibiotic use, and cigarette smoking, which disturb normal gut microbiota. Dysbiosis of gut microbiota results in dysfunctional immune response, which can lead to chronic systemic inflammation, including lung tissue. This concept can be adopted to explain Individual susceptibility to cigarette smoke exposure in the development of chronic inflammatory lung disease.

Methods and Materials

1. Emphysema mouse model

Eight-week-old inbred female C57BL/6 mice (Orient Bio, Seongnam, Republic of Korea) were maintained at room temperature (25°C) with a 12/12 h light/dark cycle. The mice were exposed to cigarette smoke 5 days/week for 4 weeks with administration of 50 μg (1 μg/μL) of poly(I:C) via nasal aspiration twice a week at 3 weeks and 4 weeks[18]. Cigarette smoke exposure was performed using 12 commercial cigarettes per day (4 cigarettes/session, 3 sessions/day, 8.0 mg of tar/cigarette, and 0.70 mg of nicotine/cigarette, Camel) according to a protocol previously described with modifications[18, 19]. Control animals inhaled only clean room air in the cages. This study complied with the recommendations in the 8th edition of the Guide for the Care and Use of Laboratory Animals. The experimental protocols were approved by the Institutional Animal Care and Use Committee (2018-01-0027).

2. Experimental design

Study 1. The effect of FMT on emphysema development was determined in three groups (n = 5 mice/group): control, CS exposure only, and CS exposure with FMT.

Study 2. The effect of different dietary supplements on emphysema development was determined in five groups (n = 4 - 6 mice/group): control, CS exposure only, CS exposure with high-fat diet, CS exposure with high-protein diet, and CS exposure with high-fiber diet.

Study 3. To further investigate the effect of a high-fiber diet and FMT on the development of emphysema, mice were randomly allocated into five groups (n = 6 mice/group): control, CS exposure only, CS exposure with high-fiber diet, CS exposure with FMT, and CS exposure with high-fiber diet and FMT.

Study 4. The effect of SCFAs on emphysema development was determined in three groups (n = 5 mice/group): control, CS exposure only, CS exposure with SCFAs.

3. Diet modifications

Mice were fed *ad libitum* with diets based on the purified AIN-76A diet supplied by Daehan Biolink Co., LTD (Chungbuk, Korea). The AIN 76-A diet was modified to study the effect of different diets. The high-protein diet (40% protein) was modified with the increment of casein; high-fat diet (40% fat) with corn oil; high-fiber diet (20% fiber) with 20% cellulose (Study 2); high-fiber diet (20% fiber) with 10% cellulose and 10% pectin (Study 3).

4. Fecal microbiota transplantation

For FMT, 200 mg of fresh feces were collected from control mice or mice receiving the high-fiber diet immediately after defecation before being resuspended in 5 mL of PBS. Homogenates were passed through 40 μm pore-size nylon filters to remove large particulate and fibrous matter. The suspension was centrifuged for 2 min and immediately transplanted into recipient mouse using oral gavage with 200 μL resuspended feces twice a week at 3 weeks and 4 weeks.

5. Short chain fatty acid administration

The SCFAs acetate, propionate, and butyrate (Sigma-Aldrich St. Louis, Mom USA) were administered in drinking water at 76 mM, 29 mM, and 45 mM, respectively, for the latter 3 weeks of experiment period.

6. Separation and preparation of samples

After 4 weeks, the animals were anesthetized using isoflurane inhalation and blood samples were collected by heart puncture. Spleens were harvested after acquiring the blood from the right atrium. The trachea was catheterized and perfused with 1.5 mL of PBS. The cellular and liquid fractions of BALF were separated by centrifugation at 2200 rpm for 5 mins at 4°C. The cell pellet was suspended in PBS, seeded onto a slide, and stained with Diff-Quick (Sysmex, Kobe, Japan). After ligating the right main bronchus, the left lung lobe was inflated with 0.5% low melt point agarose at a constant pressure of 15 cmH_2O. The left lung lobe was sectioned and fixed in 10% formalin for histological examination. All specimens were collected, fixed, immediately frozen, and stored at -80°C for analysis.

7. Histomorphological assessment

Thick sections (5 μm) of paraffin-embedded lung lobe were prepared and stained with hematoxylin and eosin (H&E). Emphysematous changes were assessed by measuring the MLI, which is a measurement of the mean inter-alveolar septal wall distance determined by the number of interruptions in 1 mm lines of the alveolar wall. Four lines were drawn in each field, and at least five random fields were examined per mouse.

8. Cytokine level quantification

IFN-γ and IL-6 levels in the serum and BALF were measured using a commercially available ELISA kit (R&D systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

9. Quantitative real-time PCR analysis

Total RNA was extracted from lung tissue using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. cDNA was synthesized from total RNA (1 μg) using the SSIV VILO Master Mix (Thermo Fisher Scientific). Transcript levels were measured using real-time PCR with sequence-specific primers for TNF-α, TGF-β, IL-18, IL-8, MMP-12, MMP-9, IRF-5, Cathepsin S, and IFN-γ (Supplementary Table 1). Amplification reactions were performed with SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) in an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems) according to the manufacturer's instructions. Data were analyzed using SDS 2.2.2 software (Applied Biosystems). The expression levels of the target genes were normalized to actin as an endogenous control gene. Relative changes were calculated using the equation $2^{-\Delta\Delta Ct}$.

10. TUNEL assay

End-labeling of the exposed 3'-OH ends of DNA fragments in paraffin-embedded lung tissue was performed using the TUNEL In Situ Cell Death Detection Kit (Roche Diagnostics) according to the manufacturer's instructions. Cell nuclei were counterstained with VECTASHIELD mounting medium containing 4',6-diamino-2-phenylindole (DAPI) (Vector

Laboratories, Burlingame, CA, USA). Fluorescent images were observed under a laser scanning confocal microscope (LSM-880, Carl Zeiss, Germany). After staining, the number of TUNEL-positive cells (apoptotic cells) was evaluated in ten random fields per mouse. The percentage of TUNEL-positive cells against total nuclei was computed for each image, and a mean value was obtained for each mouse.

11. Fluorescence-assisted Cell Sorting (FACS) analysis

Lung tissue was chopped and digested with collagenase D (Roche Diagnosis) for 30 min at 37°C with agitation. Next, the chopped lung or spleen tissue were passed through a 40 μm cell strainer to obtain single cell suspensions before RBC lysis. Cells were incubated with LIVE/DEAD Aqua (Thermo Fisher Scientific). Cells were stained with the following monoclonal antibodies: anti-mouse CD45-PerCP-Cy5.5 (BioLegend, San Diego, CA, USA), CD4-PE-Cy7 (BioLegend), CD25-PE (eBioscience), and Foxp3-APC (eBioscience, Waltham, MA, USA). Flow cytometry analysis was performed using a BD FACSCanto II (BD Bioscience, San Jose, CA, USA), and the results were analyzed using FlowJo software (TreeStar, Ashland, OR, USA).

12. Quantitative SCFA measurement

Standard metabolites and internal standards were purchased from Sigma-Aldrich. 10-20 mg of feces were freeze-dried for 12 h using a Benchtop manifold freeze drier and stored at -80°C with harvested serum until analysis. For sample preparation, the fecal sample was vortexed vigorously with 150 μL of internal standard solution (1 mM of Propionic acid ((C3)-d6) in water and centrifuged at 13,200 rpm for 10 min at 4°C. The supernatant was then collected. For mouse serum analysis, 20 μL of serum was mixed with 150 μL of internal standard solution (1 mM of Propionic acid(C3)- d6) in water and mixed well. The solution was centrifuged at 13,200 rpm for 10 min at 4°C, and the supernatant was collected. 100 μL of 20 mM AABD-SH in dichloromethane, 100 μL of 20 mM TPP in acetonitrile, and 100 μL of 20 mM DPDS in acetonitrile were added to the supernatant. The solution was incubated for 10 min at room temperature while vortexing and dried under a vacuum. The dried matter was reconstituted with 20 μL methanol to prepare for liquid chromatography-tandem mass spectrometry (LC-

MS/MS) analysis.

An LC-MS/MS system equipped with 1290 HPLC (Agilent Technologies, Glostrup, Denmark), Qtrap 5500 (ABSciex, Framingham, MA), and a reverse-phase column (Pursuit 5 C18 150 × 2.0 mm, Agilent Technologies) were used. Mass spectrometry was operated in the positive ion mode with turbo ion-spray voltage of 5500 V using 20 psi curtain gas, 50 psi nebulizer gas, and 50 psi drying gas at 400°C. The LC separation used mobile phase A (0.1 % formic acid in water) and mobile phase B (0.1 % formic acid in acetonitrile), and proceeded at 500 µl/min and 40 °C. The separation gradient was as follows: 30% of B at 0 min, 50% of B for 30 min, 50 to 30% of B for 0.1 min, and 30% of B for 4.9 min. 15 V of collision energies were used for multiple reaction monitoring (MRM) of each SCFA. LC-MS/MS data were analyzed with Analyst 1.5.2 software (AB Sciex).

The extracted ion chromatogram (EIC) corresponding to the specific transition for each metabolite was used for quantitation. Area under the curve of each EIC was normalized to the EIC of the internal standard. The peak area ratio of each metabolite to internal standard was normalized using serum volume in a sample before being used for relative comparison. The internal standard for mouse feces was not detected, thus the results were presented as the analyte peak area.

13. Isolation of bacteria derived EVs and DNA extraction

Bacteria EVs were isolated from the feces using a previously described procedure[20]. Briefly, the feces samples were filtered through a cell strainer after being diluted in 10 mL of PBS for 24 hours. EVs in feces samples were isolated using differential centrifugation at 10,000 x g for 10 min at 4°C. DNA from EVs were extracted as previously described[21]. Tissue DNA extraction was performed immediately after the sample was cut without boiling or centrifuging. DNA was extracted using a DNA isolation kit (PowerSoil DNA Isolation Kit, MO BIO, USA) following the manufacturer's instructions. Isolated DNA from each sample was quantified using the QIAxpert system (QIAGEN, Germany).

14. Bacterial metagenomic analysis using DNA

Bacterial genomic DNA was amplified with 16S_V3_F (5´ -

TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG -3′) and 16S_V4_R (5′ - GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC -3′) primers specific for the V3-V4 hypervariable regions of the 16S rDNA gene. The libraries were prepared using PCR products according to the MiSeq System guide (Illumina, USA) and quantified using QIAxpert (QIAGEN, Germany). Each amplicon was quantified, an equimolar ratio was, pooled, and sequenced on a MiSeq (Illumina, USA) according to the manufacturer's recommendations.

15. Analysis of bacterial composition in the microbiota

Paired-end reads that matched the adapter sequences were trimmed using cutadapt version 1.1.6[22]. The resulting FASTQ files containing paired-end reads were merged with CASPER and quality filtered for Phred (Q) score as described by Bokulich[23, 24]. A reference-based chimera detection step was conducted to identify the chimeric sequences with VSEARCH against the SILVA gold database[25, 26]. Next, the sequence reads were clustered into Operational Taxonomic Units (OTUs) using VSEARCH with a de novo clustering algorithm under a threshold of 97% sequence similarity. The representative OTU sequences were finally classified using SILVA 128 database with UCLUST (parallel_assign_taxonomy_uclust.py script on QIIME version 1.9.1) under default parameters[27]. The Chao Indices, an estimator of the richness of taxa per individual, were estimated to measure the diversity of each sample.

16. Statistical Analysis

Data were analyzed using the Kruskal-Wallis H test, the Manne-Whitney U test, and one-way ANOVA, followed by a Tukey's test with SPSS software version 24.0 (IBM, Armonk, NY, USA). All values are expressed as mean ± standard error (SE). Values of $p < 0.05$ were considered statistically significant.

Results

1. FMT attenuates body weight loss and alveolar destruction in emphysema mice

To address whether gut microbiota influences emphysema, the first experiment analyzed the effect of FMT on CS-exposed emphysema. Fresh feces from control mice were prepared in a suspension and transplanted into a recipient mouse using oral gavage. The emphysema with FMT group did not exhibit decreases in relative body weight, but the relative body weight of the emphysema group gradually decreased during the study period compared to the control group (**Fig. 2a**). CS caused lung parenchymal destruction and airspace enlargement, leading to an increase in the mean linear intercept (MLI), a measurement of the mean interalveolar septal wall distance. Histological analysis of lung tissue sections from each group showed that the alveolar destruction area was greater in the emphysema group than in the control group (**Fig. 2b**). Importantly, the emphysema with FMT group showed relatively preserved alveoli compared to the emphysema group (**Fig. 2b, 2c**).

In accordance with this, the levels of the cytokines, interleukin-6 (IL-6), and interferon- γ (IFN-γ), which were significantly increased in emphysema mouse bronchoalveolar lavage fluid (BALF) and serum compared to control, were significantly decreased in FMT-treated emphysema compared to the emphysema group (**Fig. 2d, 2e**). Moreover, the mRNA expression of other representative pro-inflammatory mediators, including IL-1β, tumor necrosis factor-α (TNF-α), IL-8, IL-18, and immune modulators including interferon regulatory factor-5 (IRF-5) and transforming growth factor-β (TGF-β), was increased in emphysema mice. Importantly, the mRNA levels of IL-1β, TNF-α, IL-8, IRF-5, IL-18, IFN-γ, TGF-β, matrix metalloproteinase-9 (MMP-9), and MMP-12 were decreased in the FMT-treated emphysema group compared to the emphysema group (**Fig. 2f**). Taken together, these results demonstrate that the transplantation of gut microbiota attenuated the pathological changes in lungs and influenced the inflammatory response associated with CS-exposed emphysema, suggesting that transfer of eubiotic feces could counterbalance the local and systemic effects of emphysema.

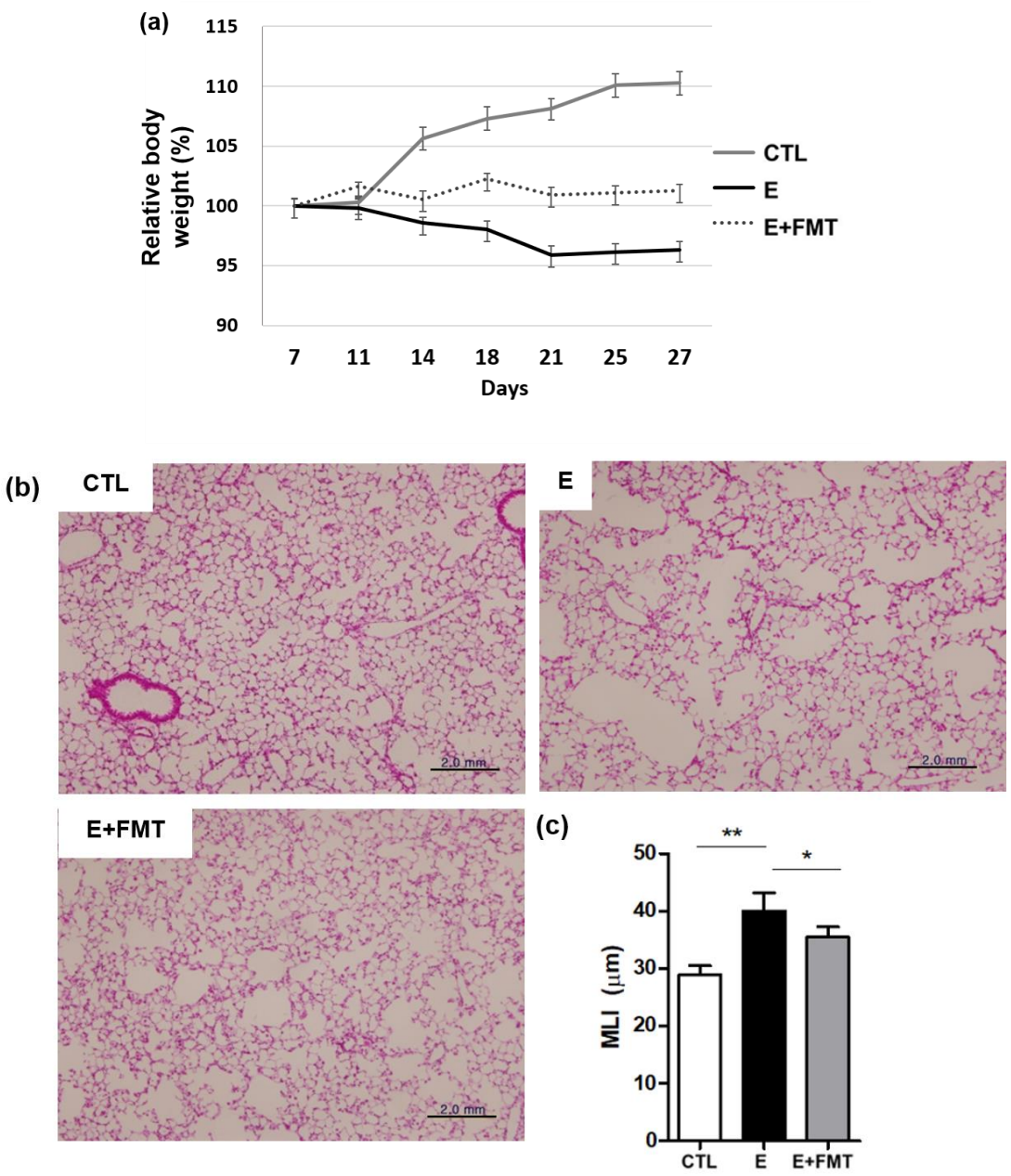

Figure 2. The effect of fecal microbial transplantation on emphysema development (Experiment 1). (a) Relative body weight change during 4 weeks of experimental period. **(b)** Representative H&E-stained lung tissues from control, emphysema, emphysema with FMT mice (magnification: 100x). **(c)** Mean linear intercept (MLI) of lung tissues from each group. *Continued.*

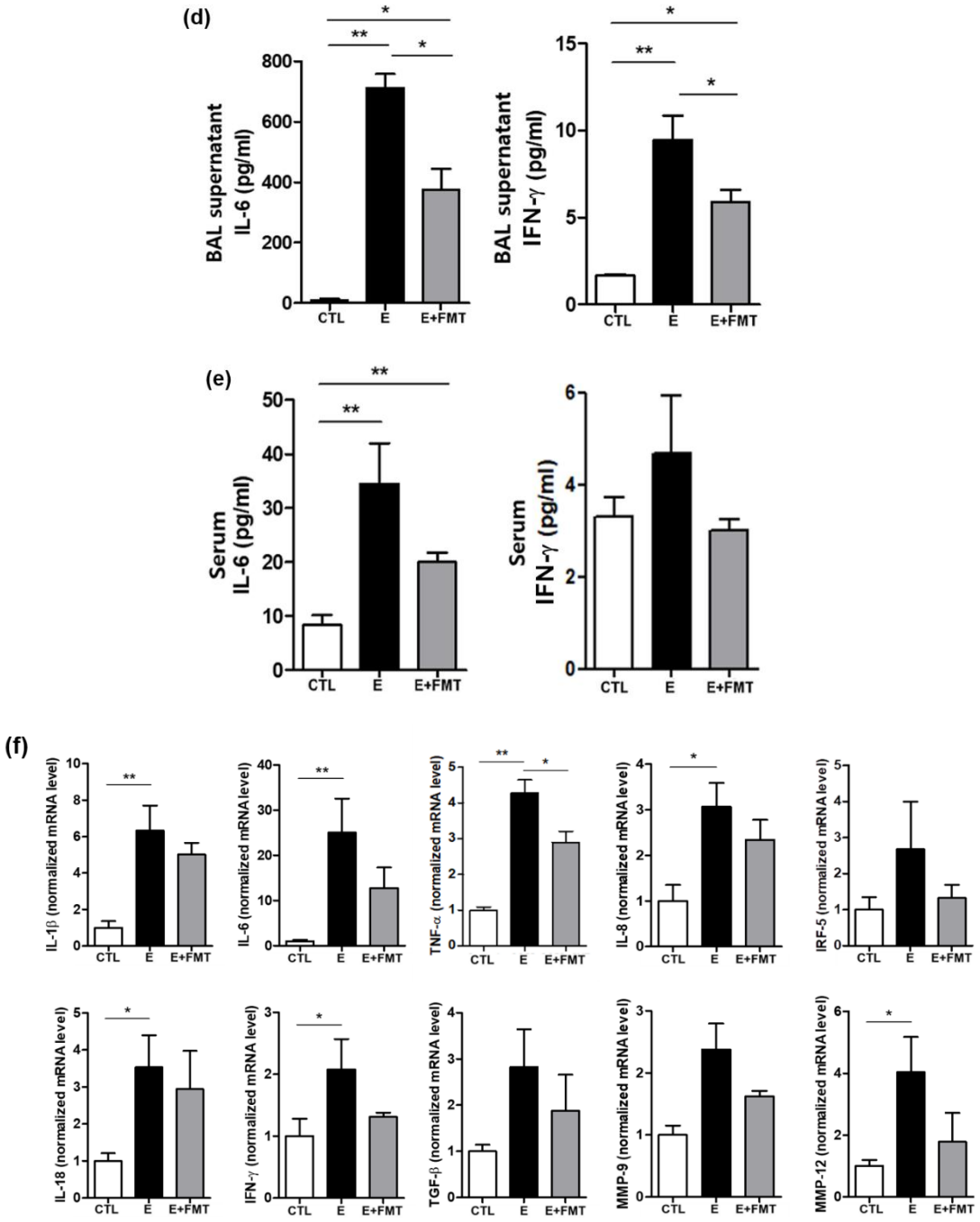

Figure 2. The effect of fecal microbial transplantation on emphysema development (Experiment 1). (d, e) Levels of IL-6 and IFN-γ cytokines in the BALF **(d)** and serum **(e)** measured by ELISA. **(f)** The relative mRNA levels of IL-1β, IL-6, TNF-α, IL-8, IRF-5, IL-18, IFN-γ, TGF-β, MMP-9, and MMP-12 in lung tissues (n = 5 mice per group). Values are expressed as the mean ± SE. *P < 0.05 and **P < 0.01. CTL, control; E, Emphysema; FMT, Fecal microbial transplantation.

2. High-fiber and high protein diet have a protective role in emphysema mice

Previous studies reported that the diet has a major influence on gut microbiota and immune responses in inflammatory diseases[28-30]. Hence, we investigated whether dietary modification could affect the pulmonary inflammatory response in mice with CS-exposed emphysema. Mice were fed either a high-fat diet (40% fat), a high-protein diet (40% protein), or a high-fiber diet (20% fiber). Detailed composition of each diet is described in the Materials and Methods section.

Mice exposed to CS with a high-fat diet displayed increased body weight until day 19, but their weights gradually decreased to baseline (**Fig. 3a**). The body weights of mice receiving high-fiber or high-protein diets gradually increased after day 19. Histological analysis showed that lung alveolar destruction was the most severe in the emphysema group (**Fig. 3b**). Mice receiving a high-fiber diet or high-protein diet presented less severe forms of histological emphysema compared to the emphysema group (**Fig. 3b**). This observation was further confirmed by the MLI measurement, which was significantly lower in mice receiving high-fiber or high-protein diets compared to emphysema mice (**Fig. 3c**).

Inflammatory cell infiltration was increased in BALF of all CS-exposed groups (**Fig. 3d**). This inflammation was characterized by increased macrophage infiltration, which was the highest in the emphysema group fed a high-fat diet. The number of macrophages and lymphocytes in BALF was significantly lower in mice fed high-fiber diet than emphysema mice (**Fig. 3e**).

The levels of the cytokines IL-6 and IFN-γ were increased in BALF and serum of emphysema mice presumably in association with increased macrophage infiltration[31]. IL-6 and IFN-γ levels in BALF were the lowest in the high-fiber diet group (**Fig. 3f**). Moreover, IL-6 and IFN-γ serum levels decreased in mice receiving modified-diets (**Fig. 3g**). The relative mRNA levels of IL-1β, TNF-α, IFN-γ, IL-6, IL-8, IL-18, IRF-5, TGF-β, and MMP-12 were the lowest in the mice fed a high-fiber diet compared to other CS-exposed mice (**Fig. 3h**). Here, we showed that a high-fiber diet attenuates the overall magnitude of the inflammatory response, protecting CS-exposed emphysema development. Moreover, these results indicate that a high-fiber diet may confer the immune-modulating effects derived from the byproducts of dietary fiber.

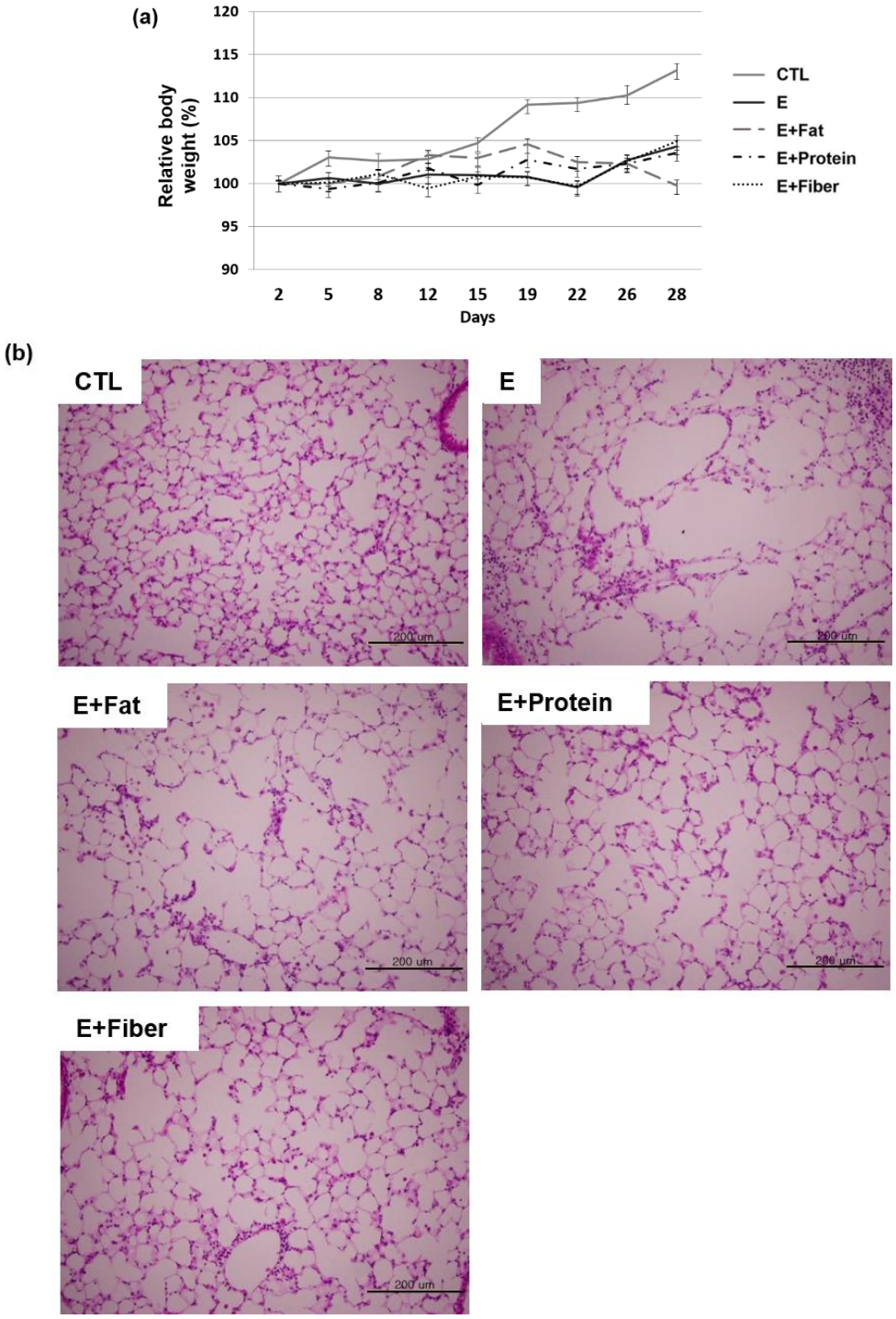

Figure 3. Dietary modification alters inflammation and degree of alveolar destruction (Experiment 2). (a) Relative body weight change during 4 weeks of experimental period. **(b)** Representative H&E-stained lung tissues from mice in the control, emphysema, emphysema with high-fat diet, emphysema with high-protein diet, and emphysema with high-fiber diet (magnification: 200x).

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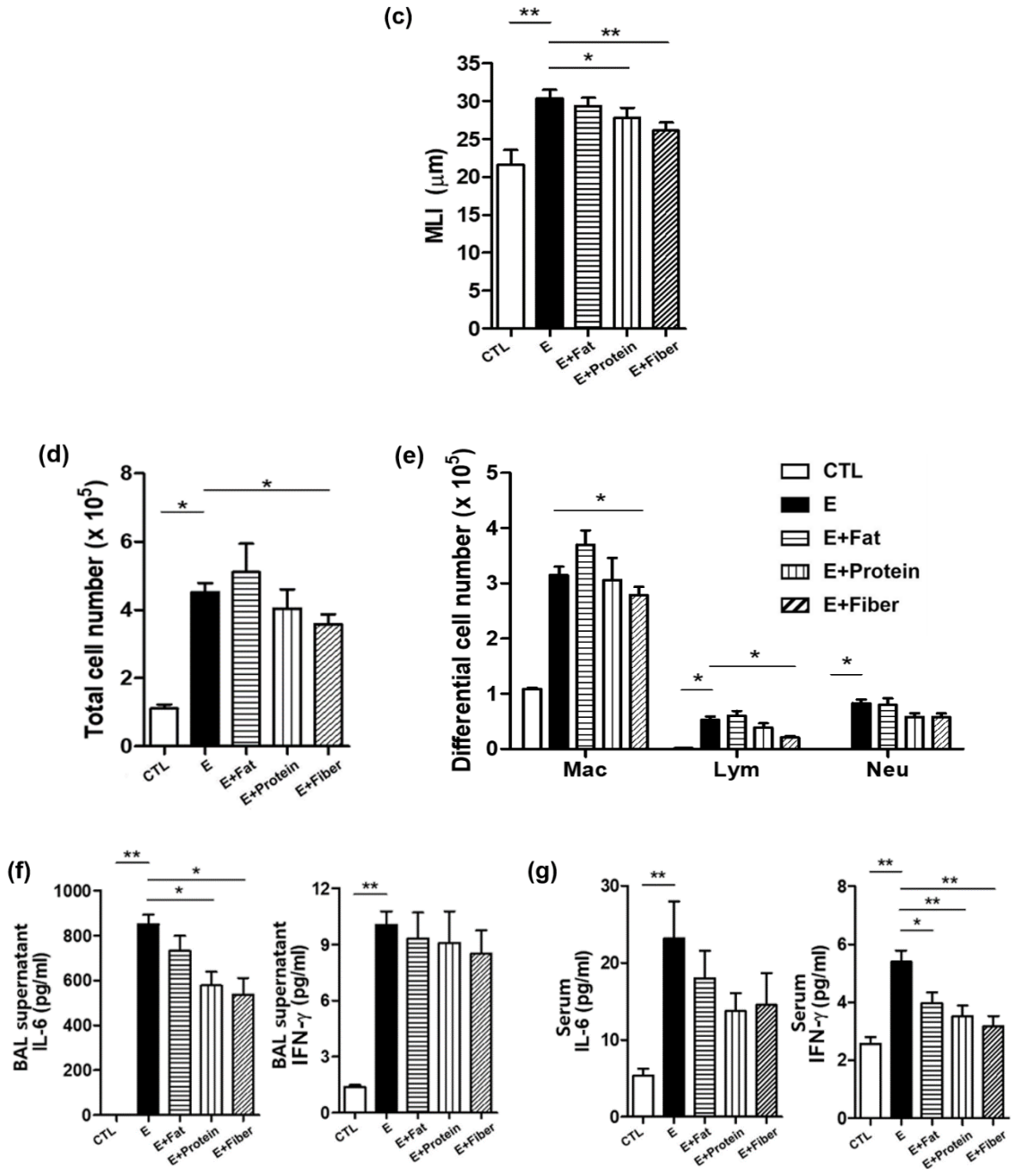

Figure 3. Dietary modification alters inflammation and degree of alveolar destruction (Experiment 2). (c) MLI of lung tissues from each group. **(d)** Total number of cells in the BALF infiltrating the airways. **(e)** Differential cell numbers of BALF in each group. **(f, g)** Levels of the cytokines IL-6 and IFN-γ in the BALF **(f)** and serum **(g)** measured using ELISA. *Continued*

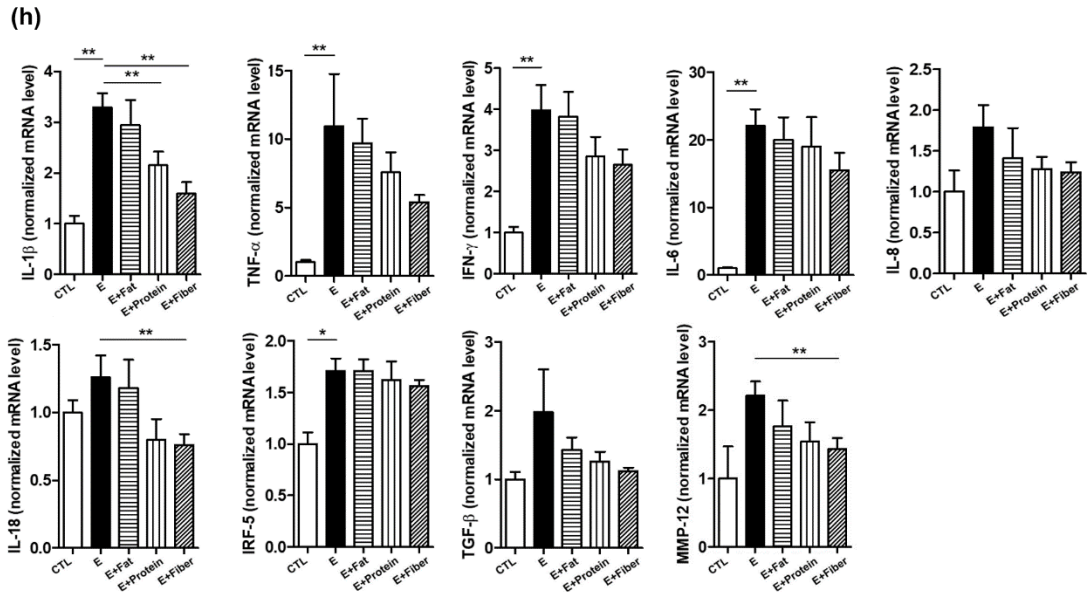

Figure 3. Dietary modification alters inflammation and degree of alveolar destruction (Experiment 2). (h) The relative mRNA levels of IL-1β, TNF-α, IFN-γ, IL-6, IL-8, IL-18, IRF-5, TGF-β, and MMP-12 in lung tissues. (n = 4 control mice, n = 6 emphysema mice, n = 6 emphysema with high-fat diet mice, n = 6 emphysema with high protein diet mice, n = 6 emphysema with high-fiber diet mice). Values are expressed as the mean ± SE. *P < 0.05 and **P < 0.01. CTL, control; E, Emphysema

3. A combination of FMT and high fiber-diet further reduces inflammation

Both probiotics and prebiotics influence gut microbiota composition. The next experiment was performed to address the combined effect of FMT and high-fiber diet on gut microbiota and emphysema. Fresh feces from mice fed high-fiber diet were prepared for FMT and transplanted into recipient mice using oral gavage.

The relative body weight of CS-exposed mice decreased after day 20, but the control mice showed no changes (**Fig. 4a**). The decrease in body weight was gradual in mice with FMT and mice with high-fiber diet. In emphysema mice, the decrease in body weight was relatively rapid after day 21.

Similar to the previous results, alveolar destruction was most severe in emphysema mice. Alveolar structures were relatively preserved in other CS mice compared to emphysema mice (**Fig. 4b**). Gut microbiome modulation using FMT, high-fiber-diet, or both significantly lowered MLI (**Fig. 4c**).

As in the above results, IL-6 and IFN-γ cytokine levels in BALF and serum were lower in both the FMT and high-fiber diet groups compared to the emphysema group. A combination of FMT and high-fiber diet further reduced IL-6 and IFN-γ levels in both BALF and serum (**Fig. 4d**, **4e**). The relative mRNA levels of IL-1β, TNF-α, IFN-γ, IL-6, IRF-5, MMP-12, and Cathepsin S were the lowest in mice with both FMT and high-fiber diet (**Fig. 4f**). In accordance with our previous results, we confirmed that FMT and fiber diet attenuated CS-exposed emphysema. Moreover, this study indicated that combined FMT and high-fiber diet had more potent preventive effects on the development of emphysema compared to FMT or high-fiber diet.

We next determined whether CS exposure influences cell apoptosis and whether a combination of FMT and high-fiber diet would affect the pathogenesis of this cell death response. Cell apoptosis was the most prominent in emphysema mice (**Fig. 4g**), and it was significantly lower in both the FMT and high-fiber diet groups than the emphysema group (**Fig. 4h**).

Since regulatory T cells (T_{reg}) are important for immune homeostasis, we hypothesized that T_{reg} cells may play a role in immune response attenuation. We examined the production of $CD4^+CD25^+Foxp3^+$ T cells in the spleen (**Supplementary Fig. 1a, Fig. 1b**). The mice with FMT and high-fiber diet showed reduced production of $CD4^+CD25^+Foxp3^+$ T cells compared

to emphysema mice (**Supplementary Fig. 1a**), suggesting a correlation between inflammatory symptoms and T_{reg} cell number. These results demonstrated that gut modulation using both FMT and high-fiber diet had additive effects in attenuating both local and systemic inflammation, thereby attenuating emphysema development.

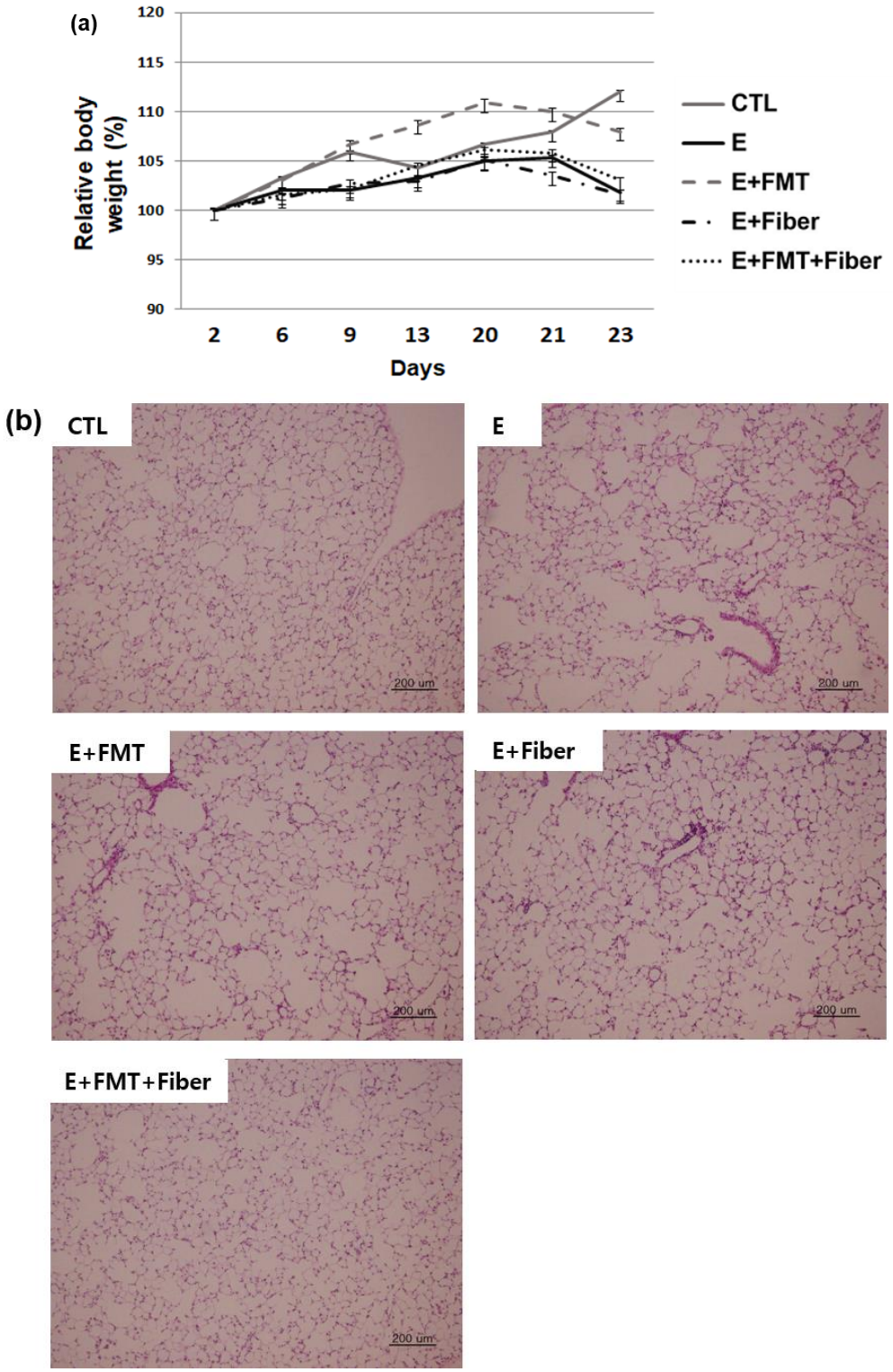

Figure 4. The effect of FMT and dietary modification on emphysema (Experiment 3). **(a)** Relative body weight change during experimental period. **(b)** Representative H&E-stained lung tissues from mice in the control, emphysema, emphysema with FMT, emphysema with high-fiber diet, and emphysema with both FMT and high-fiber diet groups (magnification: 100x). *Continued*

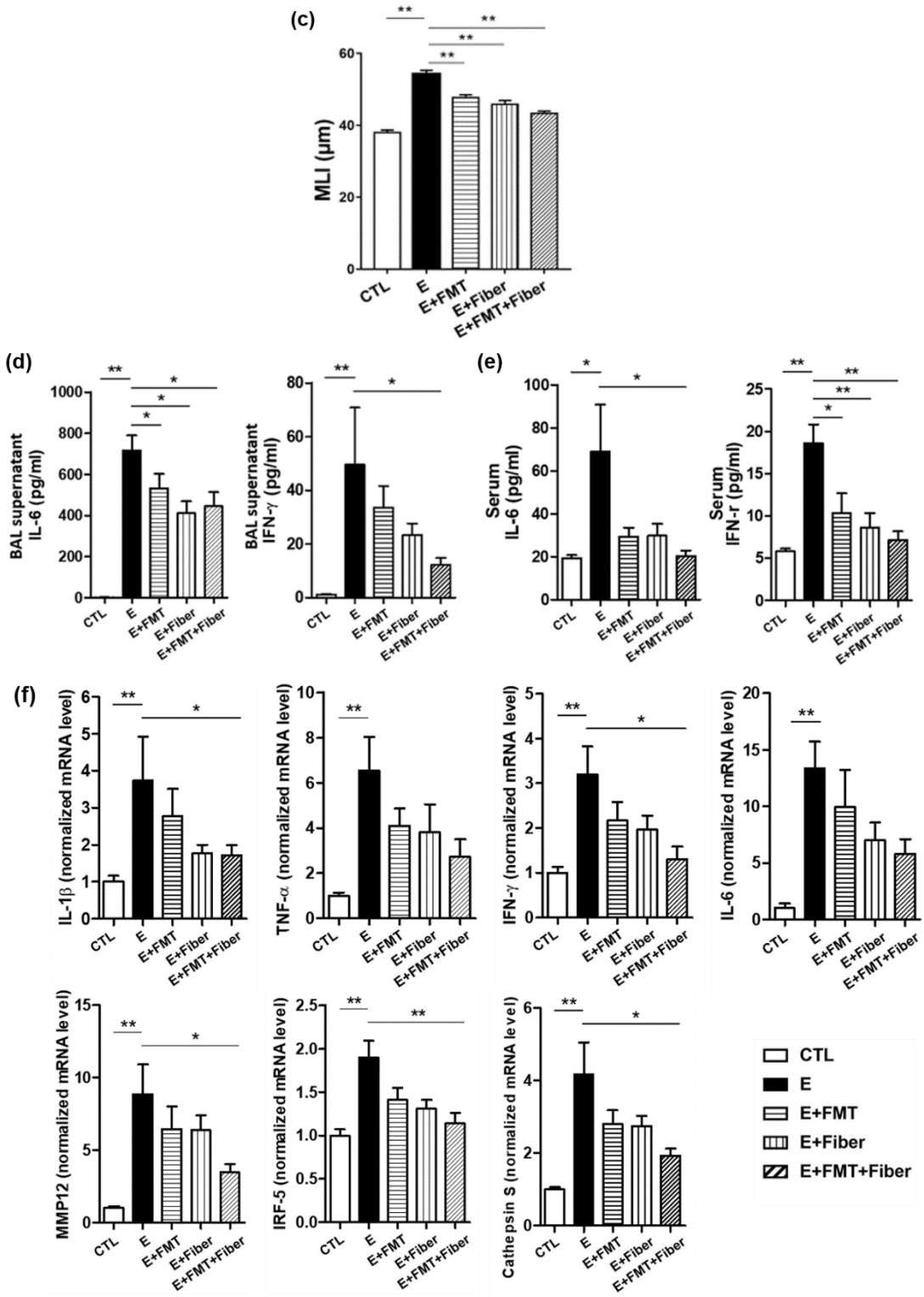

Figure 4. The effect of FMT and dietary modification on emphysema (Experiment 3). **(c)** MLI of lung tissues from each group. **(d, e)** Levels of IL-6 and IFN-γ cytokines in the BALF **(d)** and serum **(e)** measured using ELISA. **(f)** The relative mRNA levels of IL-1β, TNF-α, IFN-γ, IL-6, MMP-12, IRF-5, and Cathepsin S in the lung tissue. *Continued*

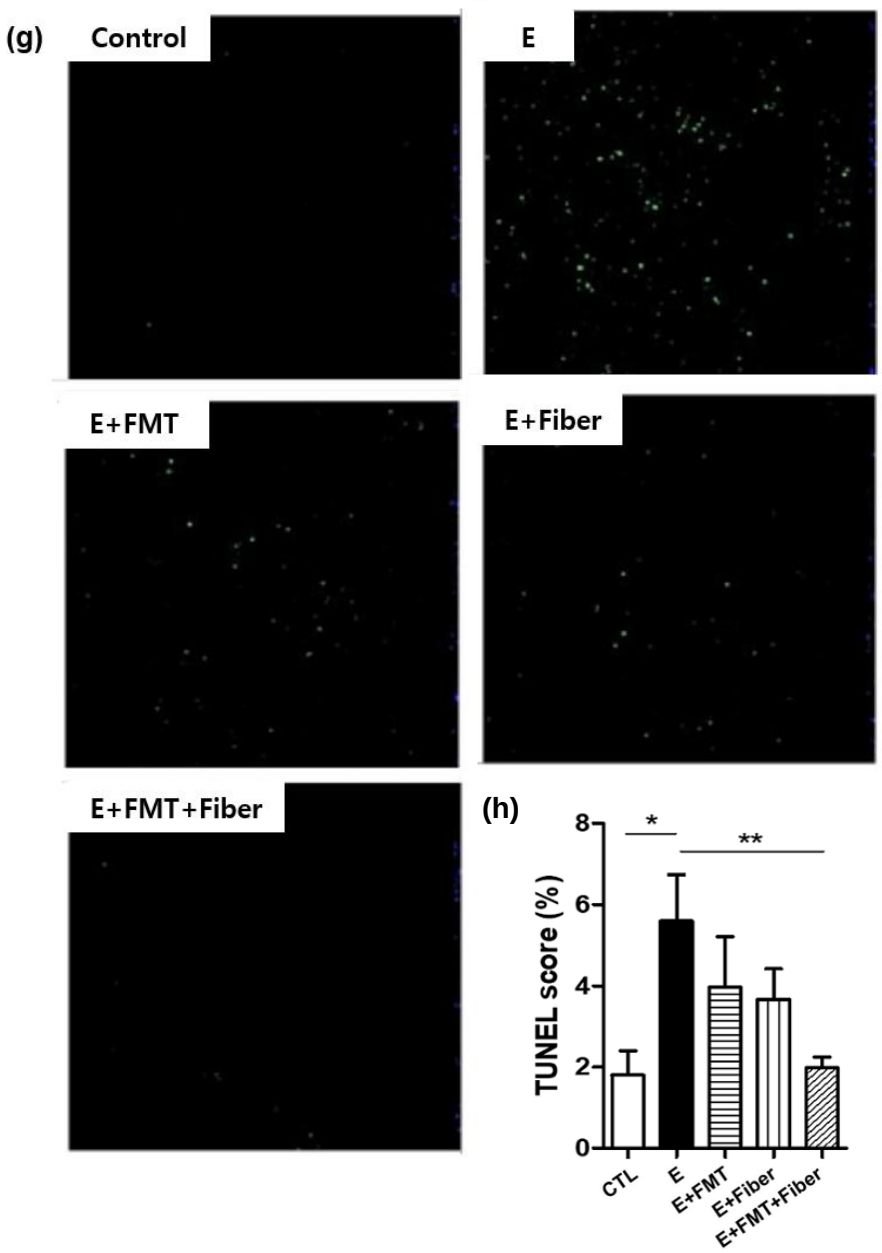

Figure 4. The effect of FMT and dietary modification on emphysema (Experiment 3). **(g)** Representative TUNEL images of lung tissue from mice in the control, emphysema, emphysema with FMT, emphysema with high-fiber diet, and emphysema with both FMT and high-fiber diet groups. **(h)** TUNEL score (%) of each group. TUNEL score (%) = TUNEL cell x 100 / total cell. (n = 6 mice per group; n = 4 control mice for IFN-γ and IL-6 analysis in BAL and serum). Values are expressed as the mean ± SE. *P < 0.05 and **P < 0.01. CTL, control; E, Emphysema; FMT, Fecal microbial transplantation.

4. FMT and dietary modification alter the gut microbiota and local SCFA concentration

The above results enabled us to hypothesize that the beneficial effects of FMT combined with a high-fiber diet would induce a shift in microbial composition, resulting in changes to gut-microbiota derived metabolites. SCFAs have been extensively investigated among various metabolites for their beneficial immunomodulatory role[15, 32]. To verify the hypothesis, fecal samples from each group in the second and third experiment were retrieved for microbial analysis. Bacteria extracellular vesicle (EV) was additionally isolated from the feces of mice in the third experiment. We also analyzed representative SCFA concentrations in fecal samples and serum from the third experiment.

Principal component analysis (PCA) showed that the control mice, emphysema mice, and emphysema mice with assigned intervention exhibited distinct microbial community structures in feces and fecal EVs, but not in lung tissue (**Fig. 5a**). FMT and high-fiber diet were significantly associated with different microbial community structures in the gut. The microbial composition was further analyzed at the phylum and family levels. At the phylum level, Firmicutes and Bacteroides were major microbiota in feces and fecal EV (**Fig. 5b**). Firmicutes species were the most dominant in fecal samples from emphysema mouse feces. FMT and high-fiber diet led to an increase in the abundance of Bacteroides phyla, reducing the Firmicutes/Bacteroides (F/B) ratio. In fecal EV, Bacteroides was the major phylum in both emphysema mice and emphysema mice with FMT and high-fiber diet. However, the intervention similarly increased the Bacteroides phyla, decreasing the F/B ratio. The decreased F/B ratio in mice treated with FMT and high-fiber diet was also observed in lung tissue microbiota, where the microbial composition differed from gut microbiota.

Pyrosequencing at the family level showed that emphysema mice generally had increased *Lactobacillus* family and decreased *Bacteroidaceae* family compared to control mice. Comparatively, high-fiber diet and FMT led to a decrease in *Lactobacillus* family and an increase in *Bacteroidaceae* family (**Fig. 5c**). At the bacterial specific level, *Bacteroidaceae* family was decreased in emphysema mice and increased in emphysema mice treated with FMT and high-fiber diet in both fecal samples and fecal EVs. *Lachnospiraceae* family in the Firmicutes phylum, which allegedly produces SCFAs including propionate like the *Bacteroidaceae* family, had a similar change in relative abundance in feces and fecal EV (**Fig.**

5d). The *Ruminococcus* family, which also metabolizes dietary fiber into SCFAs, showed similar trend in the fecal sample of the second experiment.

There were no significant differences between emphysema mice and emphysema mice with FMT and high-fiber diet in the serum concentrations of SCFAs (**Fig. 5e**). However, the local concentration of SCFAs was notably higher in emphysema mice with FMT and high-fiber diet than emphysema mice (**Fig. 5f**).

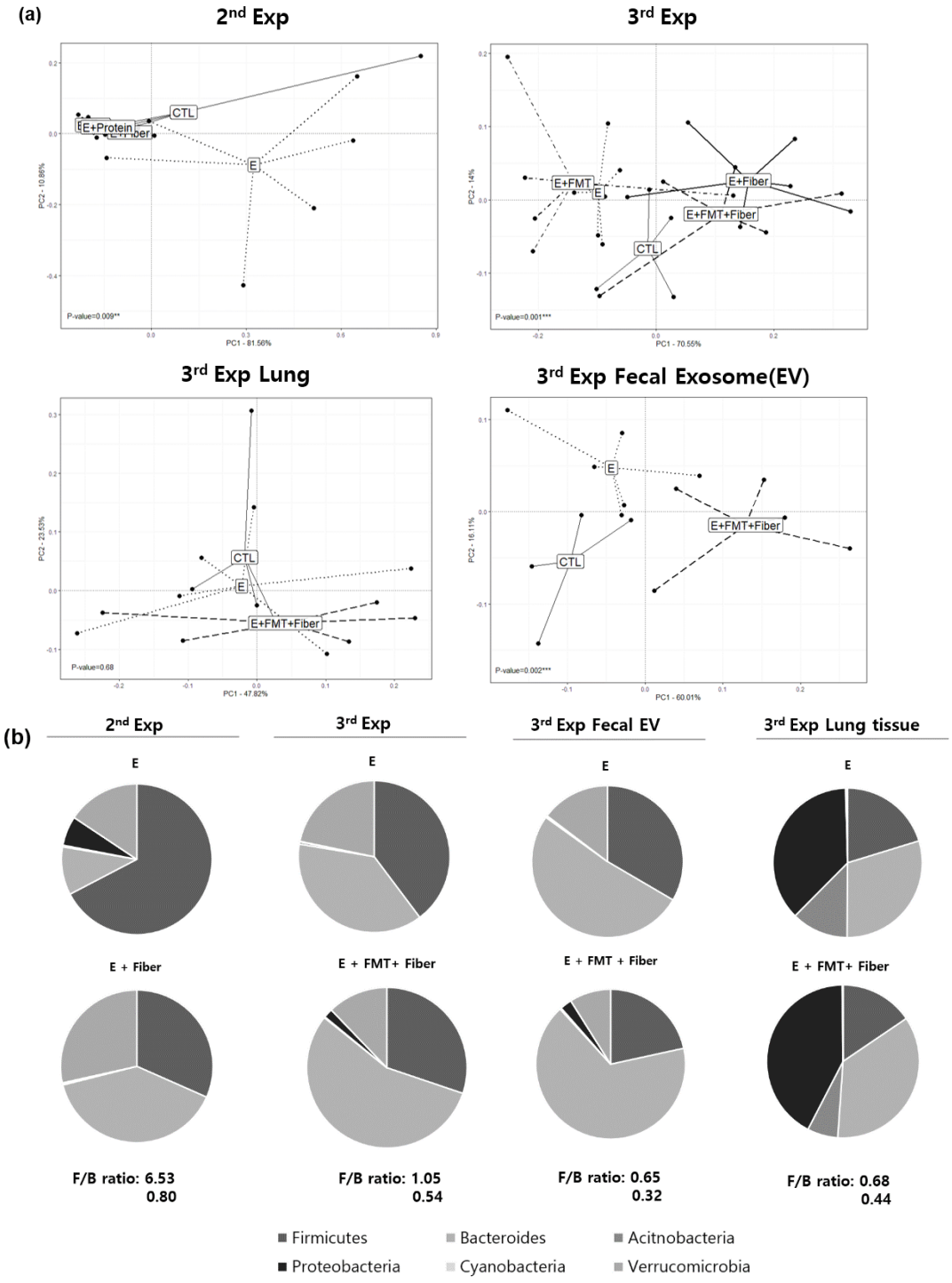

Figure 5. FMT and dietary modification alters the gut microbiota and local SCFA concentration. **(a)** Principal component analysis (PCA) of fecal samples, fecal exosome, or lung tissues from different experiments. **(b)** The proportions of Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, Cyanobacteria, and Verrucomicrobia phyla in emphysema mice compared to mice with diet modifications. *Continued*

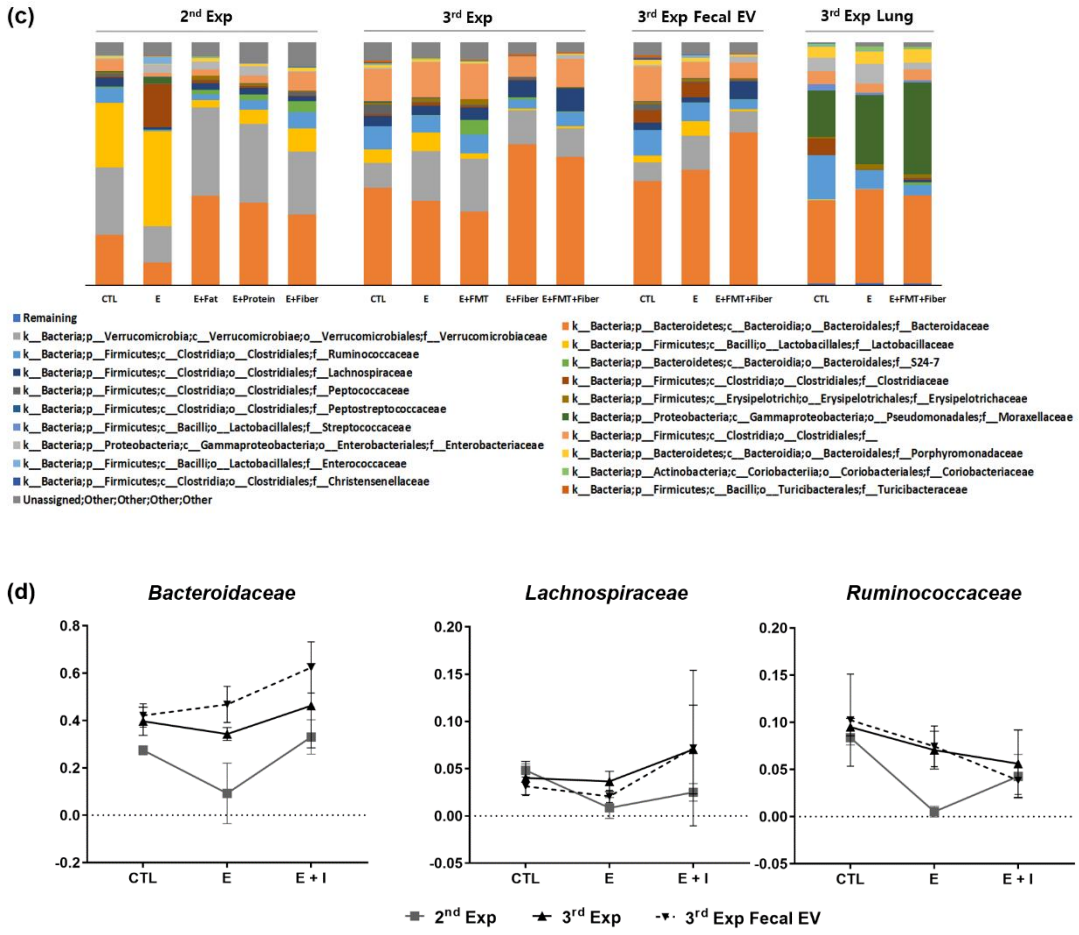

Figure 5. FMT and dietary modification alters the gut microbiota and local SCFA concentration. **(c)** Family level pyrosequencing analysis of the microbial composition of feces, fecal exosome, or lung tissues from mice treated with FMT, dietary modification, or both. **(d)** Bacteria-specific relative composition changes throughout all experiments at the family level. Intervention (I) means below; 2nd Exp = diet modification including fat, protein and fiber diets (n = 18); 3rd Exp = FMT, high-fiber diet, or both (n = 17); 3rd Exp Fecal EV = FMT and high-fiber diet (n=5). *Continued*

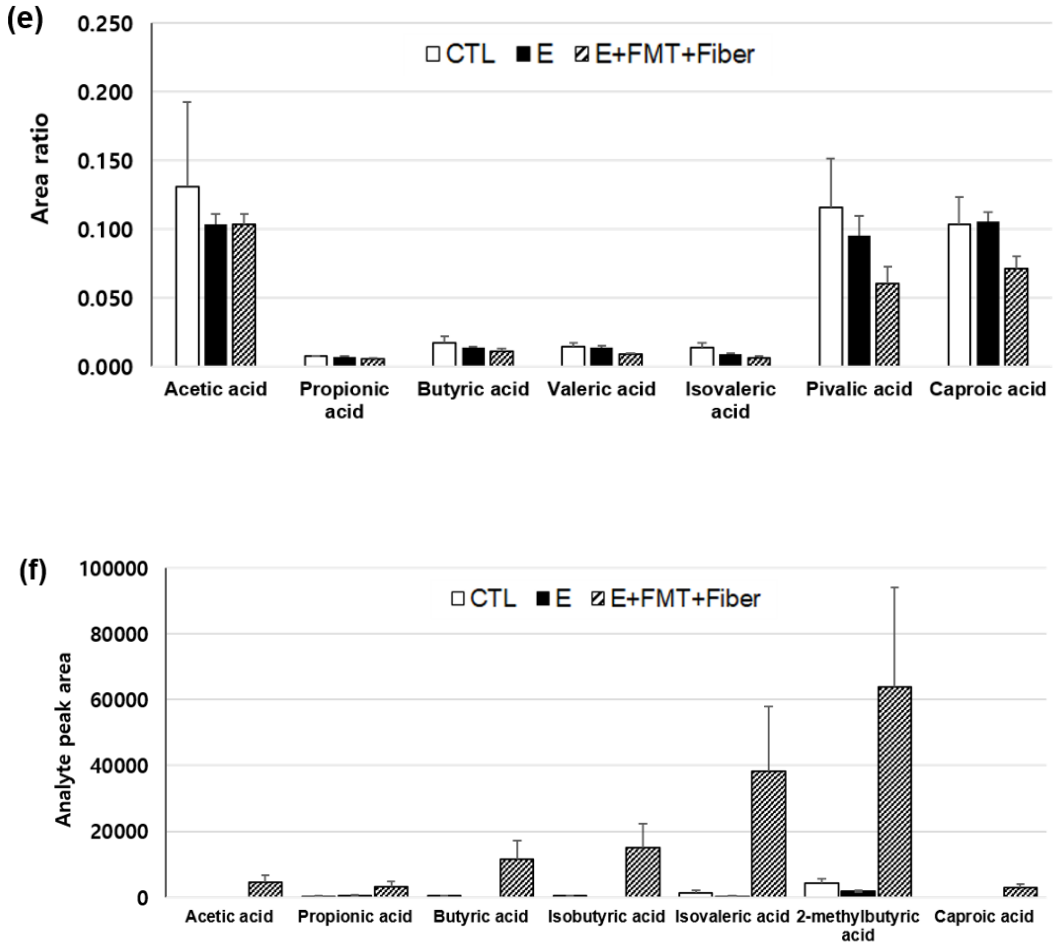

Figure 5. FMT and dietary modification alters the gut microbiota and local SCFA concentration. **(e, f)** SCFA analysis in the serum **(e)** and feces **(f)**. (n = 4 control mice, n = 6 emphysema mice, n = 6 emphysema mice with both FMT and high-fiber diet). CTL, control; E, Emphysema; I = Intervention.

5. Effect of oral SCFA administration on emphysema

Since dietary modification influenced the local SCFA concentration, we directly administered SCFA. We expected that SCFA itself might decrease the local and systemic inflammatory response and attenuate emphysema. The mixture of SCFAs including acetate, propionate, and butyrate was administered to emphysema mice via drinking water for the latter three weeks of the experimental period.

The relative body weight of all groups increased from baseline at day 20 (**Fig. 6a**). Dietary intake was generally lower in the CS exposure group than the control group (**Fig. 6b**). The decrease in dietary intake was prominent during the initial 1-2 weeks of CS exposure. The dietary intake was similar between the emphysema mice and the emphysema mice with SCFA for the third and fourth weeks of the study period.

The emphysema mice showed severe emphysema, but SCFA administration resulted in decreased alveolar destruction (**Fig. 6c**). Emphysema mice receiving SCFAs had lower MLI compared to emphysema mice (**Fig. 6d**). IL-6 and IFN-γ cytokine levels in the BALF and serum decreased in emphysema mice receiving SCFAs compared to emphysema mice (**Fig. 6e, 6f**).

The relative mRNA levels of TNF-α, IFN-γ, IL-8, IL-18, IRF-5, and TGF-β increased in the emphysema mice. Thus, the expression of factors associated with tissue destruction and healing, including MMP-12, MMP-9, and Cathepsin S, also increased in the emphysema mice. In contrast, emphysema mice with SCFAs showed reduced expression of the noted mRNAs compared to emphysema mice (**Fig. 6g**). Among them, IL-18 and Cathepsin S expression levels were significantly altered in response to SCFA administration. We observed that postbiotic SCFA administration attenuated emphysema development, but SCFAs were less effective than a prebiotic high-fiber diet. These results indicate that a prebiotic high-fiber diet has more potent preventive effects and is associated with better lung function related to emphysema development.

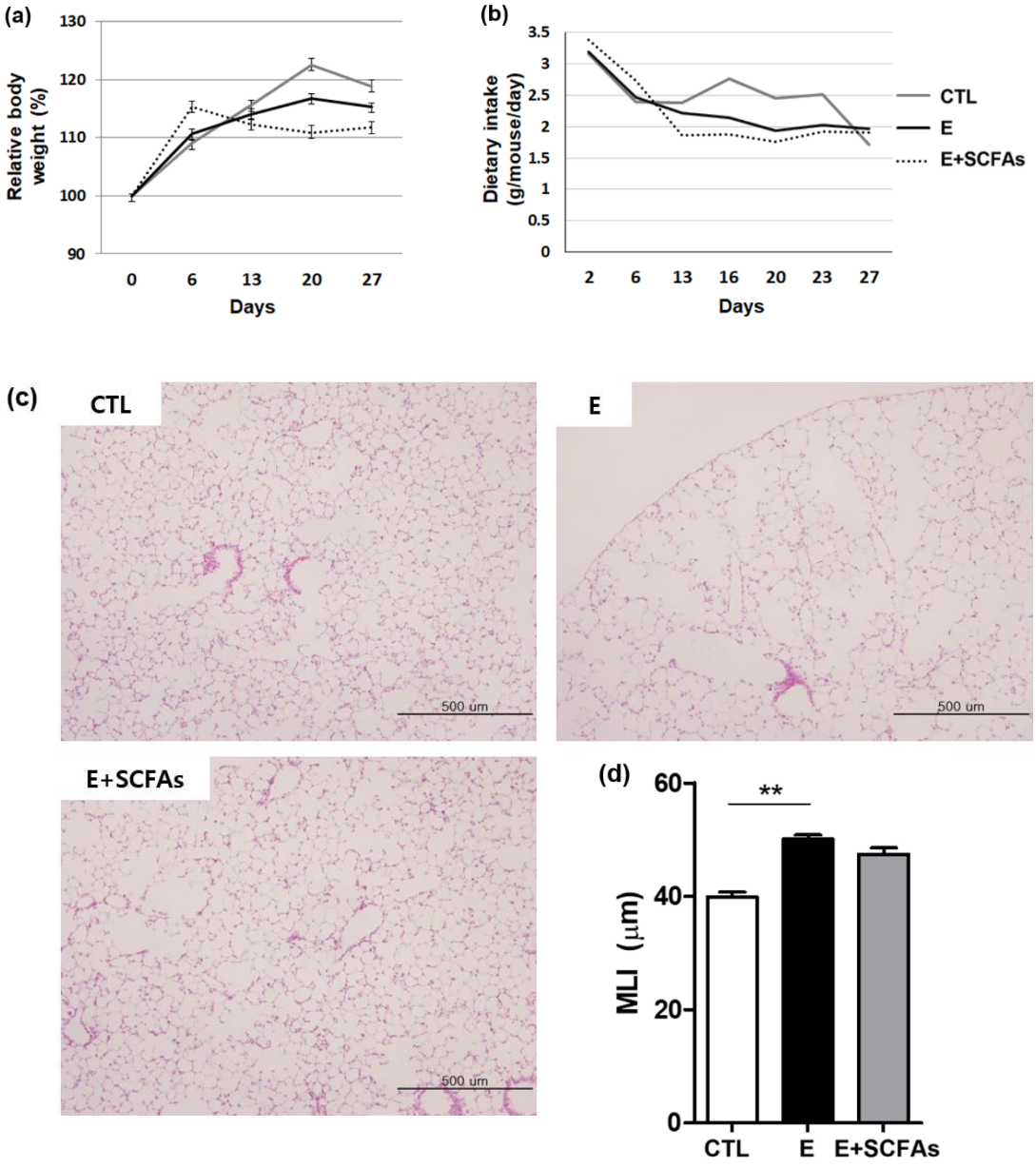

Figure 6. SCFA supplementation reduced the inflammatory response and emphysema (Experiment 4). (a) Relative body weight change during 4 weeks of experimental period. **(b)** Dietary intake changes during 4 weeks of experimental period. **(c)** Representative H&E-stained lung tissues from mice in the control, emphysema, and emphysema with SCFAs supplementation groups (magnification: 100x). **(d)** MLI of lung tissues from each group. *Continued*

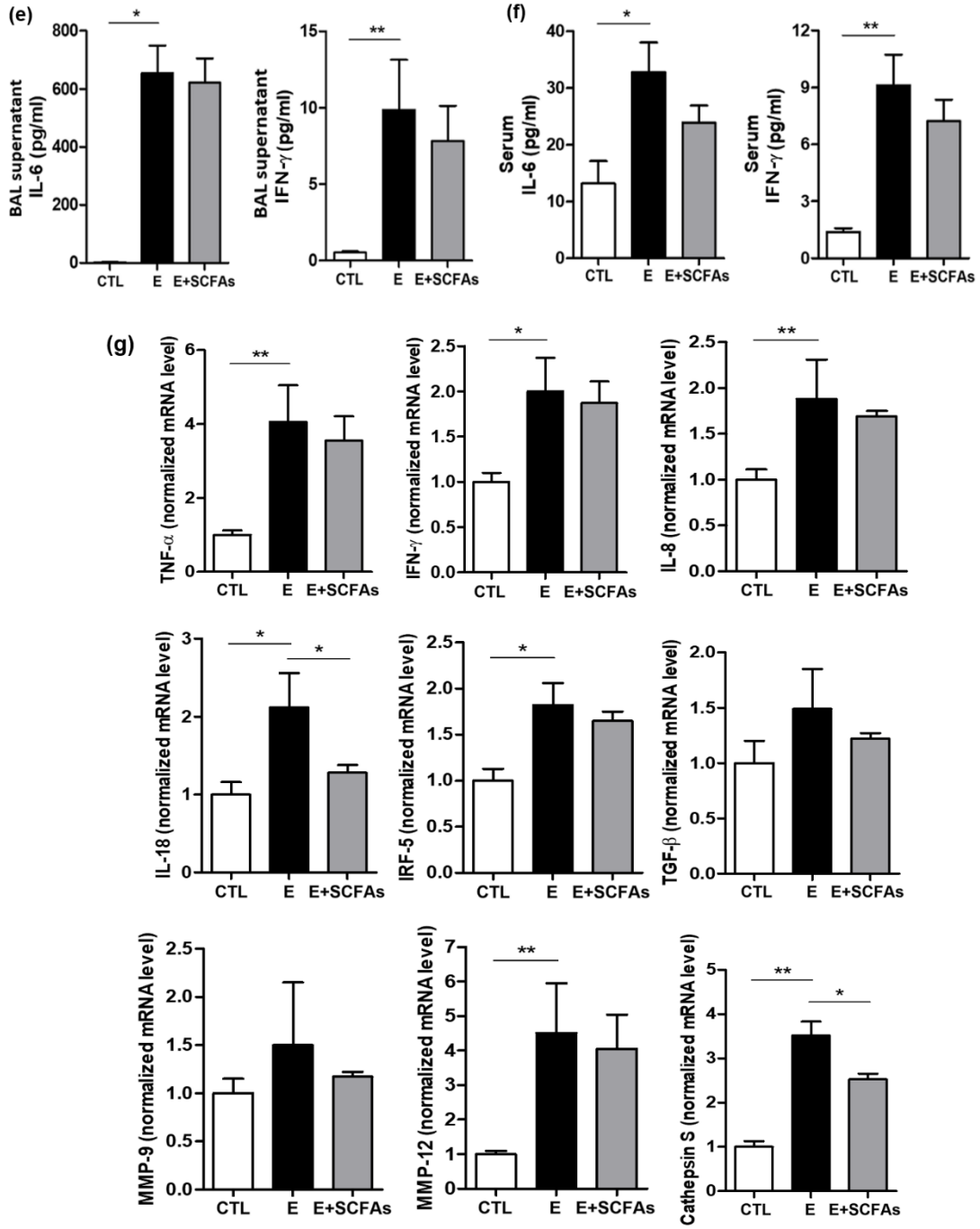

Figure 6. SCFA supplementation reduced the inflammatory response and emphysema (Experiment 4). (e, f) The levels of the cytokines IL-6 and IFN-γ in the BALF **(e)** and serum **(f)** measured by ELISA. **(g)** The relative mRNA levels of TNF-α, IFN-γ, IL-8, IL-18, IRF-5, TGF-β, MMP-9, MMP-12, and Cathepsin S in the lung tissue (n = 5 mice per group; n = 4 control mice for IL-6 analysis in BAL). Values are expressed as the mean ± SE. *P < 0.05 and **P < 0.01. CTL, control; E, Emphysema.

Discussion

Here, we demonstrated the therapeutic potential of FMT and a high fiber diet in emphysema treatment. The modulation of gut microbiota with prebiotics and FMT changed gut microbiota composition, consequently attenuating smoking-induced emphysema. Prebiotics and a high-fiber diet showed the most prominent benefit among various diets. Microbiota transplantation with feces from high-fiber diet further improved emphysema. The therapeutic implication with gut microbiota has been mainly studied in gastrointestinal diseases, and it has not been tried in pulmonary diseases except for a few allergic diseases. This study suggested the meaningful possibility of a new emphysema treatment, offering hope to treat a chronic incurable disease.

Extensive studies have revealed that the gut microbiota is crucial in maintaining of host homeostasis and health via immune system interactions[6, 33]. The gut microbiota has a systemic impact beyond intestine via various metabolites[34, 35]. The lungs are not the exception: accumulating data support a link between gut microbial dysbiosis and chronic respiratory disease[8-10, 15, 29]. Low gut microbial diversity is associated with increased risk of allergic disease development during childhood[36]. Children with cystic fibrosis also display low gut microbial diversity with distinct changes in microbial composition[37]. Although a number of studies demonstrated dysbiotic airway microbiota in COPD patients, including overall increases in Firmicutes and Proteobacteria and a decrease in Bacteroidetes[8], the role of intestinal microbiota in COPD development and progression is less clear.

Although the gut microbiota is influenced by several factors, diet is considered as a key factor that regulates the microbial composition and its metabolic function[28, 30, 38]. Therefore, it is natural to expect that a healthy diet or direct transfer of good microbiota would have a beneficial effect on lung, especially under pathological conditions. Among various diets, a high-fiber diet has been extensively studied for its beneficial effect in “diet-microbiota-immunity” link[15, 29, 30].

Here, we focused on a high-fiber diet. It was significantly associated with attenuation of both local and systemic inflammation, alveolar destruction, and cellular apoptosis. Dietary modification with a high -fiber diet led to a distinct change in the gut microbial structure, including a decrease in the F/B ratio, and an increase in local SCFA concentrations. The

combination of FMT and high-fiber diet additionally counterbalanced the harmful effects of CS exposure. The beneficial effects of the high-fiber diet in decreasing systemic inflammation were previously reported[39-41]. Inverse associations were noted between dietary fiber intake and the activity of serum C-reactive protein (CRP), a marker of acute inflammation[39, 40]. A small randomized crossover trial demonstrated that a fiber diet decreases CRP levels by 20-30% from the baseline[31]. The relationship between dietary fiber intake and lung function was evaluated in prospective cohort studies in the UK and USA[42-44]. In those studies, fiber-rich diets were positively associated with improved lung function and negatively associated with the risk of COPD development. In large epidemiologic studies[45, 46], a high-fiber diet was also significantly associated with reduced respiratory-related deaths.

The underlying mechanism through which a high-fiber diet plays a beneficial role in lung health with "diet-microbiota-immunity" link has been researched extensively[15, 47]. SCFAs are considered one of the key microbial metabolites in this link. In the allergic airway model, the fiber-rich diet changes gut microbiota and also affects the lung immune response by increasing SCFA[11, 48, 49]. Dietary-fiber derived SCFAs also demonstrated a protective role in tissue damage in the influenza-infected mice model[50]. SCFAs dampen the harmful innate immune response by activating G-protein receptors, inhibiting histone deacetylase, and serving as energy substrate for many immune regulating cells[15, 29]. Here, the high-fiber diet successfully increased the local SCFA concentration and improved inflammation and alveolar destruction. Meanwhile, the oral intake of SCFAs had a smaller effect than the high-fiber diet. Since gut microbiota produces other metabolites like amino acid derivatives and polyamines, the beneficial effects of the high-fiber diet more likely came from the combination with other metabolites rather than from SCFA alone. In this context, the result of the recent metabolomic study that suggested the association between several metabolites and clinical outcomes in COPD patients is not surprising[51]. Further studies with metabolomics may help to disclose potential diagnostic and therapeutic candidates in emphysema.

COPD is primarily considered a respiratory disease, but it also includes systemic manifestations like weight loss or being underweight, which are poor prognostic factors[52, 53]. Therefore, it seems reasonable to hypothesize that COPD prognosis can be improved by managing systemic manifestations like weight loss[54]. Here, mice with FMT were protected

from weight loss during emphysema development. The composition of Bacteroides, known to be associated with obesity[55], increased when emphysema improved. The proportion of Bacteroidetes (specifically, Prevotella spp.) is also known to be significantly decreased in COPD[56]. The intestine is the most densely bacteria colonized surface of the human body, and the lower respiratory tract which is one of the least populated. Several bacteria appear in the intestine before being detected in the respiratory tract[57]. This points toward a contribution of gut microbes and their therapeutic application for respiratory diseases.

Here, we demonstrated the potential role of gut microbiota modulation in emphysema, and we identified certain families of microbiota presenting consistent changes throughout the experiments. The beneficial effects of probiotics in chronic lung disease were addressed in preclinical studies or small scaled clinical trials. Administration of various probiotic bacteria attenuated the allergic response in a murine model[8,29]. In cystic fibrosis patients, administration of *Lactobacillus rhamnosus* GG was associated with restored intestinal microbiota and decreased local inflammation[37]. There are no reports on the role of probiotics in COPD progression. Understanding the complex relationship between prebiotics, specific microbiota, and metabolites in emphysema will provide future therapeutic applications that prevent COPD progression.

Conclusion

In conclusion, we demonstrate that FMT and high-fiber diet modulate gut microbiota and attenuate the degree of emphysema in a murine model. FMT and high-fiber diet decreased local and systemic inflammation and protected against alveolar destruction and cellular apoptosis. These findings highlight the importance of dietary fiber in COPD patients and provide new interventional insights for preventing or delaying COPD progression.

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국문요약

연구 배경: 만성 폐쇄성 폐질환(COPD)는 높은 사망률과 이환율을 보이는 난치성 질환이다. 최근 장-폐 축의 개념이 대두되면서 세균총의 불균형과 만성 호흡기 질환의 연관성이 제시되고 있다. 그러나 아직 COPD 에서 장내 세균총의 역할과 치료적 적응을 위한 연구는 제한적이다. 본 연구는 장내 세균총 조절이 폐기종의 진행을 완화시킬 수 있는지 확인하고자 하였다.

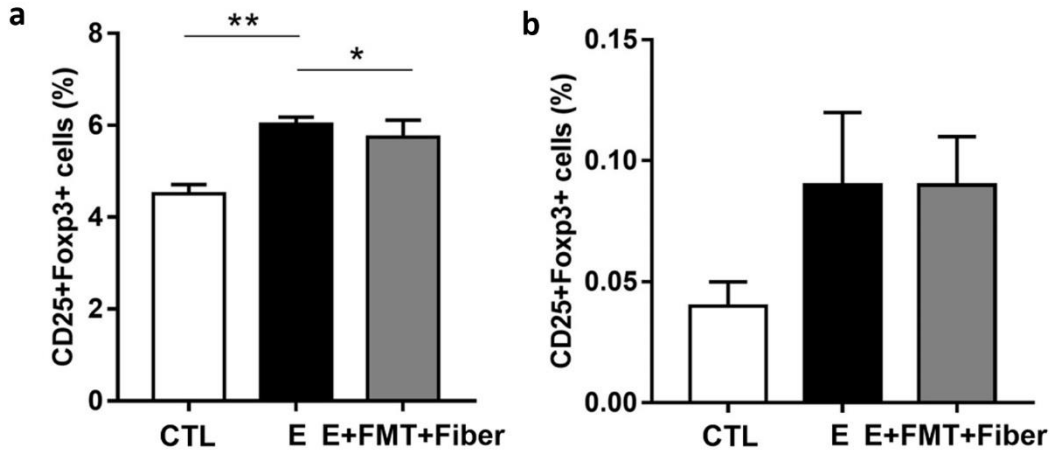
연구 방법: 흡연과 poly I:C 를 통해 쥐 폐기종 모델을 확립하였고, 장내 세균총을 조절하기 위해 분변 이식 및 구성을 변화시킨 사료를 활용하였다. 폐기종 심각도는 평균 선형 절편 (mean linear intercept; MLI)으로 평가하였고, TUNEL assay 로 세포 자멸 정도를 평가하였다. 분변 및 미세 소포(extracellular vesicle) 검체에서 미생물 분석을 진행하였다.

연구 결과: 흡연 이후 폐기종 쥐에서 MLI 는 유의미하게 증가하였으나 분변 이식 및 고섬유 식이에서는 이러한 변화 정도가 감쇄되었다. 분변 이식 시 폐기종 쥐에서 관찰되었던 체중 감소가 관찰되지 않았다. 폐기종 쥐와 비교하였을 때 고섬유 식이는 폐포 세척액의 대식 세포 및 림프구 수를 유의미하게 감소시켰고, 폐포 세척액과 혈장의 IL-6 와 IFN-γ 를 감소시켰다. TUNEL 점수는 분변이식과 고섬유 식이를 시행한 쥐에서 유의미하게 낮게 관찰되어 세포 자멸이 적게 발생하였음을 시사하였다. 미생물 분석에서 단쇄지방산(short chain fatty acids; SCFAs)을 생성하는 것으로 알려진 *Bacteroidaceae* 와 *Lachnospiraceae* 과(family)에 속하는 세균이 분변 이식군 및 고섬유 식이군에서 증가하였다. 실제로 분변 이식과 고섬유 식이를 시행한 군에서 분변의 SCFAs 농도가 증가되었다.

연구 결론: 분변 이식과 고섬유 식이는 국소 및 전신 염증 반응을 억제하고, 장내 세균의 구성을 변화시켜 폐기종의 진행을 감쇄하였다. 본 연구는 장내 세균총 조절이 새로운 폐기종의 치료 전략으로 활용될 수 있는 가능성을 제시하였다는 데 의의가 있다.

중심 단어: 불균형(dysbiosis), 폐기종(emphysema), 세균총(microbiota), 프리바이오틱스(prebiotics), 프로바이오틱스(probiotics).

Supplementary Materials



Supplementary Figure 1. Fluorescence-assisted Cell Sorting (FACS) analysis (a) Percentage of CD25+Foxp3+ T cells retrieved from spleens. **(b)** Percentage of CD25+Foxp3+ T cells retrieved from lung tissues. (n = 6 mice per group; n = 4 control mice. Values are expressed as the mean ± SE. **P* < 0.05 and ***P* < 0.01. CTL = control; E = Emphysema; FMT = Fecal microbial transplantation.

Supplementary Table 1. Design of animal studies

	Study 1	Study 2	Study 3	Study 4
Groups	1. CTL 2. E only 3. E with FMT	1. CTL 2. E only 3. E with high-fat diet 4. E with high-protein diet 5. E with high-fiber diet	1. CTL 2. E only 3. E with FMT 4. E with high-fiber diet 5. E with FMT and high-fiber diet	1. CTL 2. E only 3. E with SCFAs
Dietary fiber composition		20% cellulose	10% cellulose + 10% pectin	
FMT source	CTL mice		High fiber-diet mice	
Duration	4 weeks	4 weeks	4 weeks	4 weeks

CTL, control; E, emphysema;

Supplementary Table 2. **Primer sequences for quantitative PCR**

Gene	Forward/reverse	Primer sequence 5′-3′
Actin	Forward	AAGAGCTATGAGCTGCCTGA
	Reverse	CACAGGATTCCATACCCAAG
IFN-γ	Forward	AAGCGTCATTGAATCACACCTG
	Reverse	TGACCTCAAACTTGGCAATACTC
IL-1β	Forward	CCAAGCAACGACAAAATACC
	Reverse	GTTGAAGACAAACCGTTTTTCC
TGF-β	Forward	CTGCTGACCCCCACTGATAC
	Reverse	GTGAGCGCTGAATCGAAAGC
TNF-α	Forward	GACAGTGACCTGGACTGTGG
	Reverse	TGAGACAGAGGCAACCTGAC
IL-6	Forward	TGTGCAATGGCAATTCTGAT
	Reverse	GGTACTCCAGAAGACCAGAGGA
IL-8	Forward	TTGGTGATGCTGGTCATCTT
	Reverse	TTTAGATGCAGCCCAGACAG
IL-18	Forward	GCTGTGACCCTCTCTGTGAA
	Reverse	GGCAAGCAAGAAAGTGTCCT
MMP-9	Forward	CTGGACAGCCAGACACTAAAG
	Reverse	CTCGCGGCAAGTCTTCAGAG
MMP-12	Forward	GGCCATTCCTTGGGGCTGCA
	Reverse	GGGGGTTTCACTGGGGCTCC
IRF-5	Forward	GCTGGCTACAGGGTTCTGAG
	Reverse	CTGCTGGCTTCATTTCTTCC
Cathepsin S	Forward	GCCAGCCATTCCTCCTTCTT
	Reverse	AGCCAACCACAAGAACACCA

IFN-γ, interferon-γ; IL, interleukin; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α; MMP matrix metalloprotein ase; IRF-5, interferon regulatory factor-5