Am J Transl Res 2018;10(10):3171-3185
www.ajtr.org /ISSN:1943-8141/AJTR0077798

Original Article

Alterations of fecal bacterial communities in patients with lung cancer

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Received April 12, 2018; Accepted September 7, 2018; Epub October 15, 2018; Published October 30, 2018

Abstract: Emerging evidence suggests the microbiome may affect a number of diseases, including lung cancer. However, the direct relationship between gut bacteria and lung cancer remains uncharacterized. In this study, we directly sequenced the hypervariable V1-V2 regions of the 16S rRNA gene in fecal samples from patients with lung cancer and healthy volunteers. Unweighted principal coordinate analysis (PCoA) revealed a clear difference in the bacterial community membership between the lung cancer group and the healthy control group. The lung cancer group had remarkably higher levels of *Bacteroidetes*, *Fusobacteria*, *Cyanobacteria*, *Spirochaetes*, and *Lentisphaerae* but dramatically lower levels of *Firmicutes* and *Verrucomicrobia* than the healthy control group ($P < 0.05$). Despite significant interindividual variation, eight predominant genera were significantly different between the two groups. The lung cancer group had higher levels of *Bacteroides*, *Veillonella*, and *Fusobacterium* but lower levels of *Escherichia-Shigella*, *Kluyvera*, *Fecalibacterium*, *Enterobacter*, and *Dialister* than the healthy control group ($P < 0.05$). Most notably, correlations between certain specific bacteria and serum inflammatory biomarkers were identified. Our findings demonstrated an altered bacterial community in patients with lung cancer, providing a significant step in understanding the relationship between gut bacteria and lung cancer. To our knowledge, this is the first study to evaluate the correlations between certain specific bacteria and inflammatory indicators. To better understand this relationship, further studies should investigate the underlying mechanisms of gut bacteria in lung cancer animal models.

Keywords: Lung cancer, gut bacteria, carcinogenesis, microbial dysbiosis, 16S rRNA gene sequencing

Introduction

Lung cancer, a disease of global geographic reach, is the most common type of cancer and the leading cause of cancer-related death among men [1]. According to the annual report on the status of cancer in China, the number of new lung cancer diagnoses increased by 34.8% from 2005 to 2011 [2]. Currently, limited clinical approaches exist for the prevention and treatment of lung cancer, resulting in an 11% five-year survival rate for patients [3]. Smoking accounts for approximately 80% of the global lung cancer burden in men and 50% in women [4]. Tobacco smoke includes carcinogens that are attributable to increased mutation and misreplication of DNA damage [5]. However, studies also demonstrate that the progression of lung cancer is associated with the systemic immune and inflammatory response [6, 7], which may be modulated by human gut microbiota.

More than 1,000 different species, totaling 10^{14} bacterial cells in the human gastrointestinal tract, play a vital role in the maintenance of normal physiological function of the human intestine [8]. Accumulating evidence shows that gut bacteria, including *Fusobacterium nucleatum*, *Escherichia coli*, *Bacteroides fragilis*, and *Proteobacteria*, are associated with carcinogenesis [7, 9]. Previous studies also have revealed the possible effects of gut bacteria on carcinogenesis, which primarily involves modulating the immune response, activating Toll-like receptors, producing carcinogenic toxins, and

Table 1. Descriptive data of included adults in the study

Parameter	Lung cancer (N = 41)	Healthy controls (n = 41)	*P*-Value
Age (years; mean ± SD) (range)*	57.97 (7.68) (41-71)	59.05 (6.78) (48-74)	0.50
Females/Males, No. (%)#	11/30 (26.83%/73.17%)	15/26 (36.59%/63.41%)	0.48
BMI (mean, SD) (range)*	24.05 (1.91) (20.08-27.16)	23.38 (2.09) (19.13-28.08)	0.13
Smoking status (%)#			0.12
Never smoker	15 (36.59%)	23 (56.10%)	
Ever smoker	26 (63.41%)	18 (43.90%)	
Alcohol consumption status (%)#			0.89
Never drinker	25 (60.98%)	23 (56.10%)	
< 1 standard drink per day	6 (14.63%)	11 (26.83%)	
≥ 1 standard drink per day	10 (24.39%)	7 (17.07%)	
Final diagnosis		NA	
Non-small cell cancer	34 (82.93%)		
Adenocarcinoma	22 (53.66%)		
Squamous cell cancer	12 (29.27%)		
Stage I/II/III/IV	11/12/18/0 (26.83%/29.27%/43.90%/0)		
Small cell cancer	7 (17.07%)		
NLR, mean (SD)	2.60 (1.25)	NA	
PNI, mean (SD)	49.43 (5.04)	NA	
PLR, mean (SD)	146.15 (53.58)	NA	
PLT, mean (SD)	241.17 (80.07)	NA	
IL-6 (pg/ml), mean (SD)*	5.75 (4.23)	3.90 (2.53)	0.020
IL-12 (pg/ml), mean (SD)*	20.39 (6.58)	23.08 (7.54)	0.089
IL-17 (pg/ml), mean (SD)*	33.72 (1.63)	25.20 (1.19)	0.008
sCTLA-4 (ng/ml), mean (SD)*	9.53 (6.25)	21.51 (2.78)	0.009

*Student's *t* test; #Chi-square test; Abbreviations: BMI, body mass index; NLR, neutrophil-lymphocyte ratio; PNI, prognostic nutritional index; PLR, platelet-lymphocyte ratio; PLT, platelet; IL-6, interleukin-6; IL-12, interleukin-12; IL-17, interleukin-17; s-CTLA-4, soluble cytotoxic T lymphocyte associated antigen-4; NA, not available.

inducing chronic inflammation [7, 10]. Further evidence has demonstrated that alterations of gut bacteria play an important role in the development of extraintestinal cancers, such as hepatocellular carcinoma and breast cancer [11, 12]. A series of studies have shown that an altered bacteria community in lung tissue, sputum, bronchoalveolar lavage fluid, or saliva samples is prospectively associated with an increased risk of lung cancer [13-18], and one study reported that *Lactobacillus* shows antitumor effects in the intestinal tract of a Lewis lung cancer mouse model [19]. Additionally, gut bacteria affect the efficacy of tumor therapy against epithelial tumors [20-22], raising the possibility that the quality of existing therapeutic approaches may be improved in combination with treatment of certain specific bacteria. Studies have also revealed that gut bacteria affect immune and inflammatory responses not only locally at the mucosal level but also systemically, including the pulmonary organs through the gut-lung axis [12, 23-27]. Furthermore, certain bacteria have been recognized as potential biomarkers for cancer detection and classification [18, 28]. However, the exact composition of gut bacteria in patients with lung cancer remains poorly understood. Moreover, the correlations between gut bacteria and certain prognostic indexes have not yet been observed.

To better understand the relationship between gut bacteria and lung cancer, we directly sequenced the 16S rRNA gene in fecal samples from patients with lung cancer and healthy volunteers using next-generation sequencing technology. We compared the unique compositions of bacteria in lung cancer patients with those in healthy volunteers. Additionally, for the first time, we evaluated the correlations between certain specific bacteria and clinical

inflammatory indicators. The identification of specific genera correlated with lung cancer may provide a broader understanding of gut bacteria and pave the way for further inspiring exploration in this research area.

Materials and methods

Patients and samples

This study was approved by the Research Ethics Board of the Second Hospital of Shandong University (Jinan, China) and Shandong Provincial Chest Hospital (Jinan, China) and complied according to the ethical guidelines outlined in the 1975 Declaration of Helsinki. Prior to inclusion in this study, written informed consent was obtained from all participants.

The study was conducted in 2016 from May 1 to October 30. Forty-one patients with lung cancer (aged 41-71 years) were recruited from the Department of Thoracic Surgery at the Second Hospital of Shandong University and Shandong Provincial Chest Hospital. All patients enrolled in our study had a definite postoperative pathological diagnosis. No patients received chemotherapy, radiation therapy, or surgery for lung cancer before sample collection. Forty-one age-, gender-, and BMI-matched healthy volunteers were recruited from the Physical Examination Center of the Second Hospital of Shandong University. The healthy status of these volunteers was self-reported. All study participants were Han Chinese residents who had lived in the Jinan district for at least 5 years prior to the date of sample collection. For participant recruitment, the following exclusion criteria were strictly applied: (i) use of antibiotic, probiotics, or steroids within the past year; (ii) acute or chronic infection within the preceding 3 months; (iii) clinically diagnosed with psychiatric disorders; (iv) previous diagnosis of hypertension, diabetes, gastrointestinal tract disease, autoimmune disease, or any metabolic diseases; or (v) a history of gastrointestinal surgery. Additionally, the clinical characteristics of all participants are listed in **Table 1**.

Detection of serum cytokines

Matched blood samples were collected from participants immediately after recruitment, transferred to the laboratory in an icebox, and stored at -80°C within 15 min after preparation for further analysis. Serum levels of interleukin-6, IL-12, IL-17, and soluble cytotoxic T-lymphocyte associated antigen 4 (sCTLA-4) were determined using enzyme-linked immunosorbent assay (ELISA) kits. Human IL-6 (#KE00007), IL-12 (#KE00019) and IL-17 (#KE00015) ELISA kits were purchased from Proteintech Group (Wuhan, China); sCTLA-4 (#437407) ELISA kit was obtained from Biolegend (San Diego, CA, USA).

Fecal sample collection and DNA extraction

The fecal samples from all participants were freshly collected into sterile plastic cups and were placed on ice immediately for transfer to -80°C freezer. All samples were stored at -80°C until they were further processed. Fecal bacterial DNA was extracted with the QIAamp DNA stool mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The quality of the DNA was evaluated by a NanoDrop ND-1000 spectrophotometer (Thermo, USA), and the concentration and purity were monitored on 2% agarose gels. All DNA was stored at -20°C until further analysis.

16S rRNA gene PCR amplification and sequencing

The hypervariable V1-V2 region of the bacterial 16S rRNA gene was amplified with specific barcoded primers targeting 27F and 355R (27F: 5'-AGAGTTTGATCMTGGCTCAG-3'; 355R: 5'-GCTGCCTCCCGTAGGAGT-3'). All PCR reactions were conducted with Phusion® High-Fidelity PCR Master Mix (New England Biolabs). Then, the PCR products were purified with a gel extraction kit (Qiagen, Germany) and quantified by a NanoDrop ND-1000 spectrophotometer. Sequencing libraries were generated using TruSeq® DNA PCR-free sample preparation kit (Illumina, USA) and sequenced on an Illumina MiSeq platform at Novogene Bioinformatics Technology Co., Ltd. (Beijing, China). The blank controls, carried out with an empty sterile tube, were processed for DNA extraction, amplified, and sequenced with the same procedures and reagents for the fecal samples. No detectable amplification was found in the negative controls by qPCR.

Bioinformatics and statistical analysis

The 16S sequence paired-end reads were merged using the FLASH method (version 1.2.7, http://ccb.jhu.edu/software/FLASH/) [29]. A

Table 2. Comparison of phylotype coverage and diversity estimation of the 16S rRNA gene libraries at 97% similarity from the sequencing analysis

Group	No. of reads	No. of OTUs[1]	Good's[2] (%)	PD-whole tree	Richness estimator				Diversity index	
					ACE	95% CI	Chao 1	95% CI	Shannon	Simpson
Lung cancer	3,240,700	30,680	99.10%	47.77	774.81	674.63-874.98	755.29	655.44-855.14	5.02	0.88
Healthy controls	1,749,961	29,279	99.18%	49.11	721.87	674.86-768.88	699.81	649.94-749.67	5.13	0.90

[1]The operational taxonomic units (OTUs) were defined with 97% similarity level. [2]The coverage percentage (Good's) and richness estimators (ACE and Chao 1), and diversity indices (Shannon and Simpson) were calculated using the R software. No alpha diversity index between the lung cancer group and healthy controls reached the statistically different ($P < 0.05$, Student *t* test).

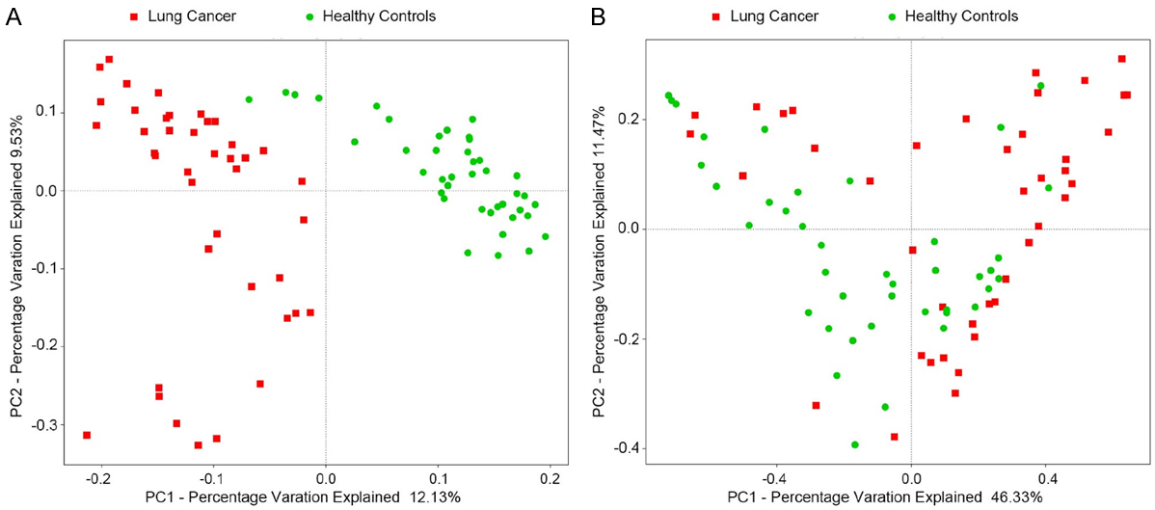

Figure 1. Beta diversity comparisons by principal coordinate analysis (PCoA) plots based on (un)weighted UniFrac distances. The percentage of explained variability of each PC is indicated on the axis. Each point represents a sample and is colored by sample types (red squares, lung cancer; green circles, healthy controls). A. PCoA based on the unweighted UniFrac distances. B. PCoA based on the weighted UniFrac distances.

quality-controlled process was performed according to the Quantitative Insights into Microbial Ecology (QIIME, version 1.7.0, http://qiime.org/index.html) pipeline [30, 31] to obtain high-quality clean tags. These tags were compared with the reference database (Gold database, http://drive5.com/uchime/uchime_download.html) using the UCHIME algorithm (UCHIME algorithm, http://www.drive5.com/usearch/manual/uchime_algo.html) [32, 33], and then the effective tags were obtained. Sequence analyses were performed using Uparse software (Uparse version 7.0.1001, http://drive5.com/uparse/) [34]. Sequences with ≥ 97% similarity were assigned to the same operational taxonomic units (OTUs). For each representative sequence, the GreenGene Database(http://greengenes.lbl.gov/cgi-bin/nph-index.cgi) [35] was used based on the ribosomal database project classifier (version 2.2, http://sourceforge.net/projects/rdp-classifier/) [36] algorithm to annotate taxonomic information. Alpha diversity indices in our samples were calculated with QIIME and displayed with R software (version 2.15.3, http://www.R-project.org). Beta diversity was determined by principal coordinate analysis (PCoA) using R software, and unweighted pair-group method with arithmetic means clustering was conducted using QIIME software.

Metastats analysis was performed on the relative abundance to determine which taxa were statistically different between the two groups. Only taxa with an average abundance greater than 1%, a *P* value less than 0.05, and a low *Q* value (false -discovery rate) were considered significant [37]. The linear discriminant analysis (LDA) effect size method (http://huttenhower.sph.harvard.edu/lefse/), which emphasizes both the statistical significance and biological relevance [38], was used to identify bacterial biomarkers that were enriched in the fecal samples of the lung cancer group, with a significance alpha of 0.05 and an LDA score greater than 4.0.

Intergroup comparisons of ELISA data were performed using Student's t-test. A Spearman's

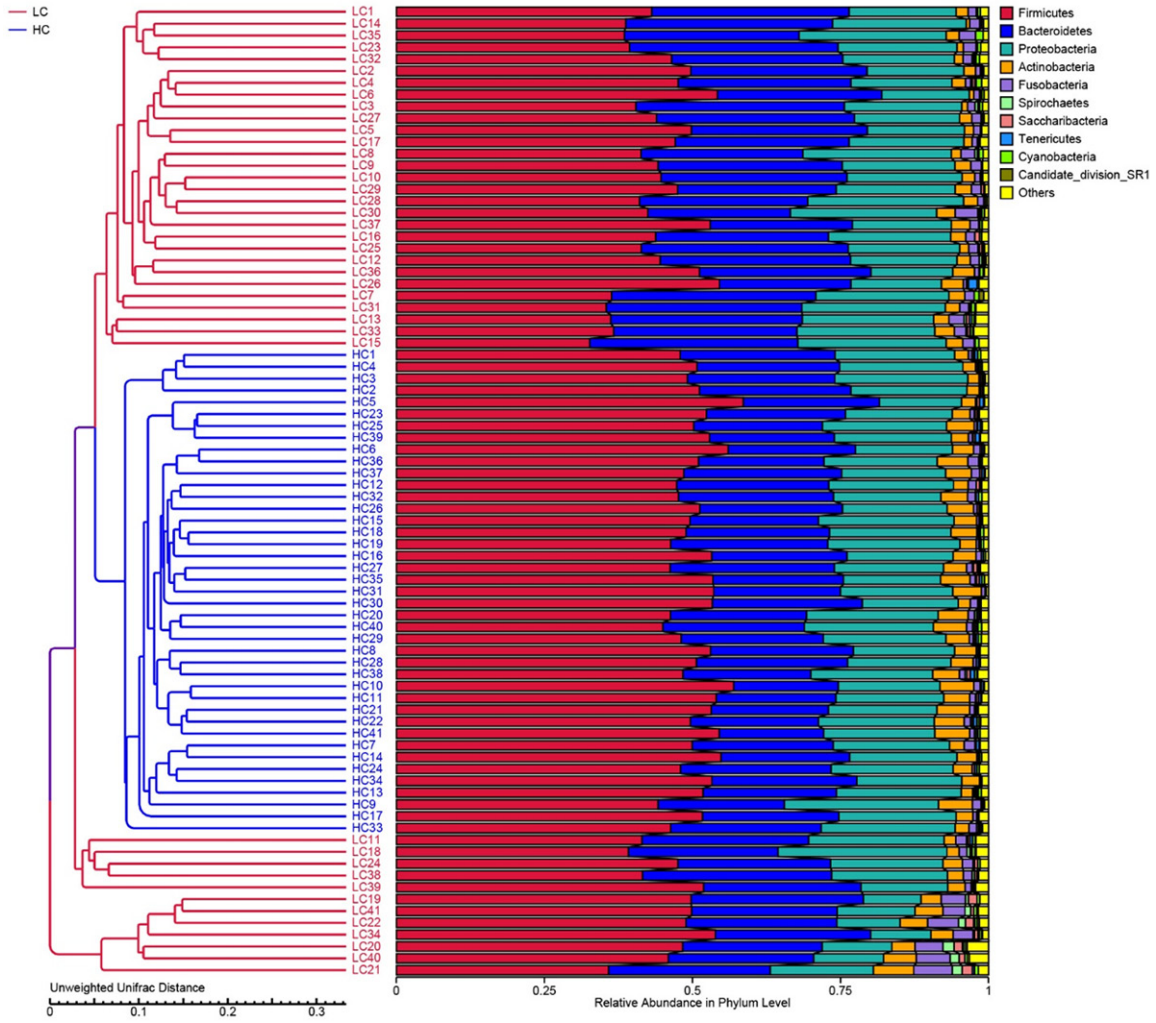

Figure 2. UPGMA tree based on unweighted UniFrac distance matrix. The community differentiation was measured by the unweighted UniFrac algorithm, and the scale bar indicates the distance between the clusters in UniFrac units. The red bars represent patients with lung cancer, and the blue bars indicate healthy volunteers.

rank-correlation analysis was used for correlation analyses between the altered bacteria and inflammation indicators. A *P* value greater than 0.05 was considered to indicate statistical significance. Statistical analyses were performed using SPSS software version 17.0 for Windows (IBM, SPSS Inc., Chicago, IL, USA).

Availability of data and materials

The sequence data from this study have been submitted to NCBI Sequence Read Archive (accession number: SRA096351, http://www.ncbi.nlm.nih.gov/sra).

Results

Differences in fecal bacterium diversity

Fecal bacterium diversity profiles were generated from a total of 41 patients with lung cancer and 41 healthy volunteers. No difference in the age, gender, BMI, smoking status, and alcohol consumption was observed between the two groups ($P < 0.05$). The clinical characteristics are listed in **Table 1**.

Barcoded 16S rRNA amplicon sequencing using Illumina MiSeq yielded a total of 5,755,467 effective reads. From these, 4,990,760 taxon reads were selected, with a mean of 60,863 reads per barcoded sample (range, 20,514-152,145). A total of 59,959 OTUs were obtained, based on the conventional criterion of 97% sequence similarity, with 30,680 OTUs in the lung cancer group and 29,279 OTUs in the healthy control group. The values of Good's coverage in our study were nearly 99% for all samples, indicating that the reads obtained from both groups represented most of the bacteria

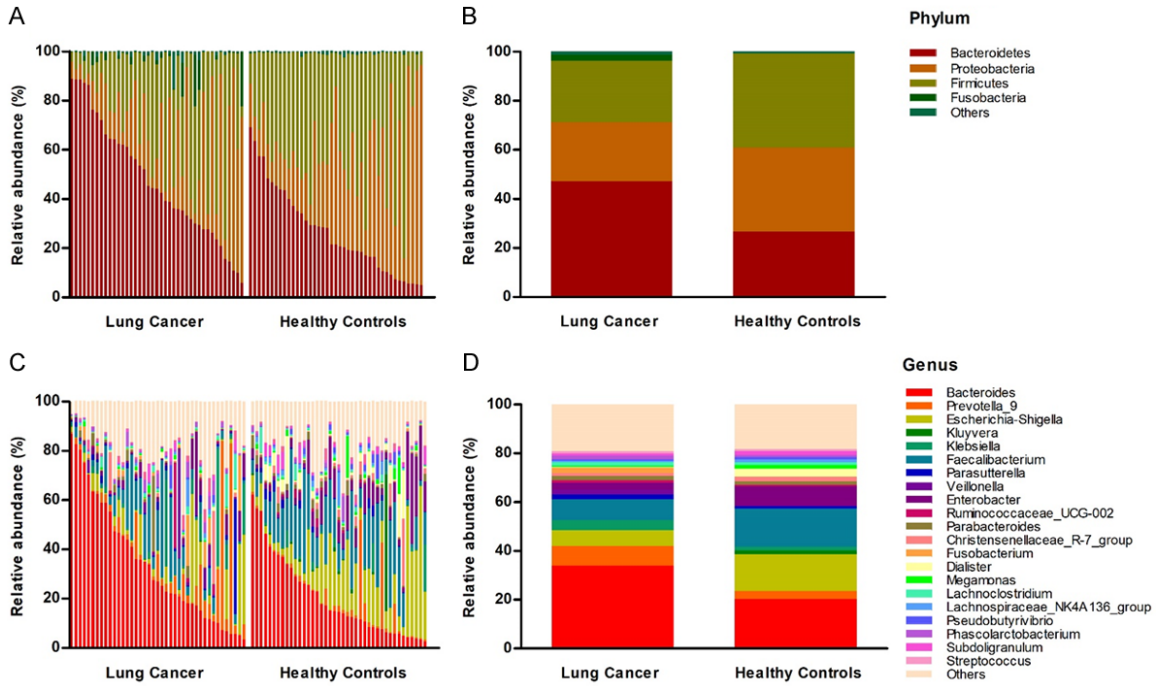

Figure 3. Relative abundances of the predominant taxa in fecal microbiota from patients with lung cancer and healthy volunteers. A, C. Abundances of fecal microbiota in each sample at phylum level and genus level, respectively. B, D. Abundances of fecal microbiota in lung cancer and healthy control groups at the phylum level and genus level, respectively.

presented in the samples of this study. Alpha diversity was determined to analyze the complexity of species diversity in each sample. No differences were found in the alpha diversity indices between the healthy control and lung cancer groups ($P < 0.05$) (**Table 2**). A summary of these results is presented in **Table 2**. Taken together, they suggest a high similarity in compositional complexity of gut bacteria between lung cancer patients and healthy volunteers.

To characterize the dysbiosis in the gut microbial communities of lung cancer patients, the beta diversity of the microbiota was used by evaluating the overall structure features. As is expected, PCoA based on unweighted UniFrac distances at the OUT level revealed a statistically significant separation of the two groups (**Figure 1A**). However, there was no significant difference in the PCoA based on the weighted UniFrac distance (**Figure 1B**), indicating that the primary difference lies in a less abundant taxon. Next, we performed an analysis of similarities. The results indicated that the structure of the gut bacteria in patients with lung cancer was significantly different from that in healthy volunteers (Adonis, $R = 0.270$, $P = 0.001$). Additionally, the cluster tree analysis based on the relative abundance of the OTUs in each sample indicated that the bacterial communities were divided into clusters between lung cancer patients and healthy volunteers, and the microbiota composition from the same community was more similar (**Figure 2**).

Differences in bacterial communities

A taxon-dependent analysis using the ribosomal database project classifier was conducted to explore lung cancer-associated differences in the fecal microbiota. Nineteen phyla and two candidate divisions (SR1 and WCHB1-60) were revealed from the study samples of the two groups. The analysis showed that *Bacteroidetes*, *Firmicutes*, and *Proteobacteria* were the most common phyla identified in the two groups, contributing 96.26% and 99.18% of the gut bacteria in the lung cancer group and healthy control group, respectively. However, the lung cancer group had a conspicuously lower abundance in *Firmicutes* and *Proteobacteria* than the healthy control group (**Figure 3C**, **3D**). Additionally, the Metastats analysis showed that three predominant phyla-*Bacteroidetes*,

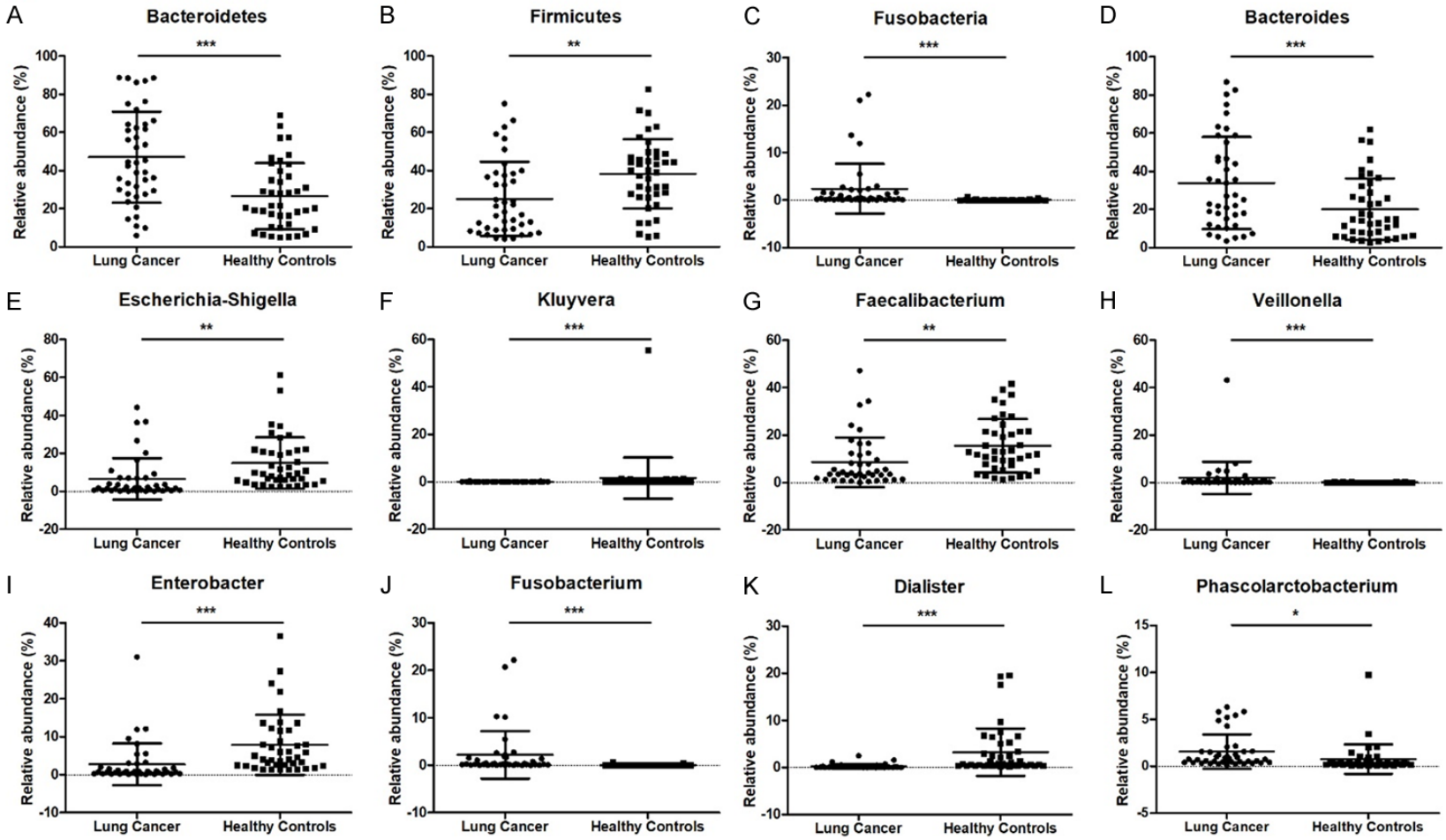

Figure 4. Significant differences of the predominant taxa in fecal microbiota from lung cancer patients and healthy volunteers. A-C. Phylum level. D-L. Genus level. Data are presented as the means ± SEM; horizontal bars indicate means. Statistical analysis was performed by Metastats analysis. *P < 0.05, **P < 0.01, ***P < 0.001.

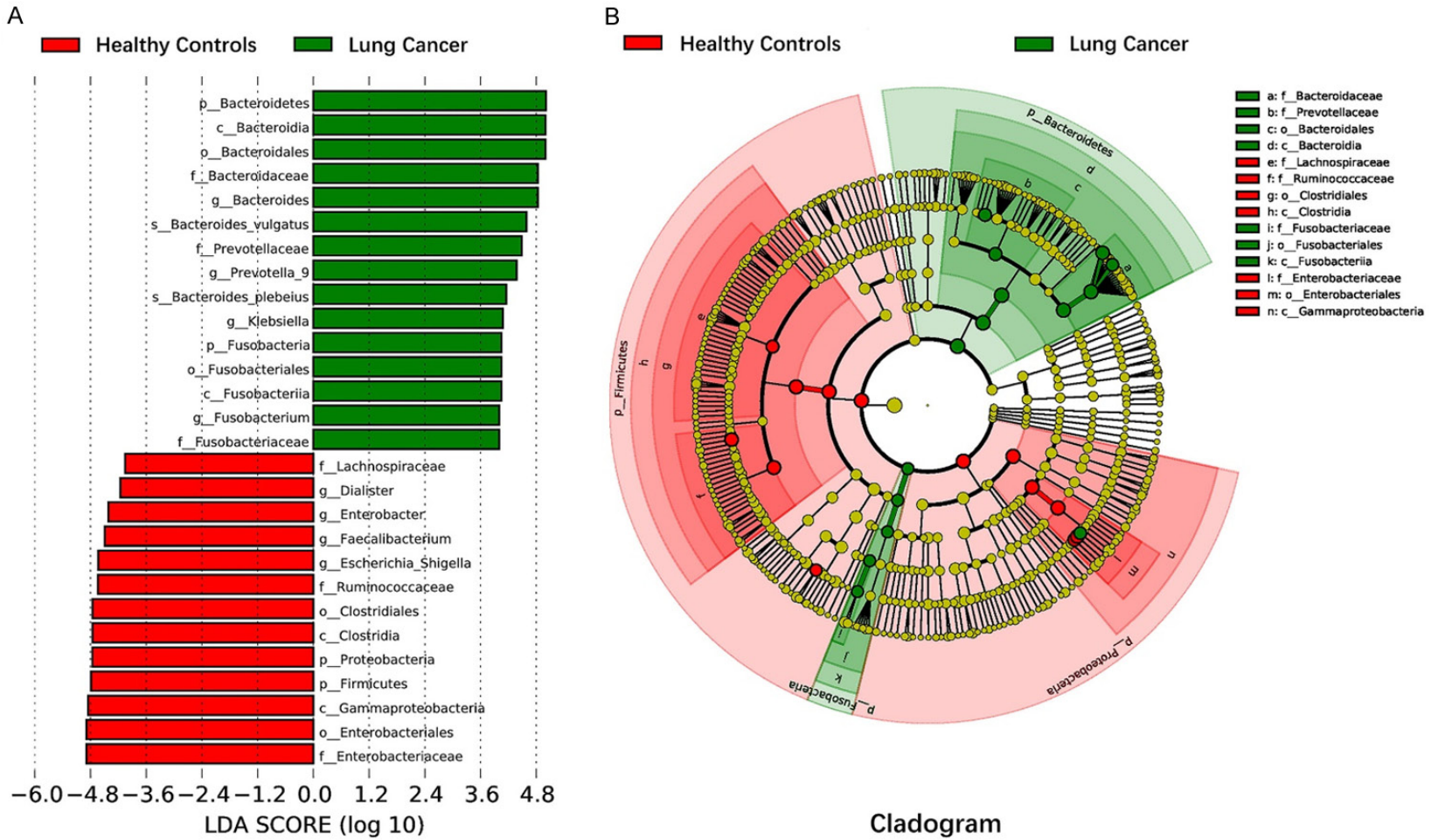

Figure 5. Different structures of fecal microbiota from patients with lung cancer and healthy volunteers. A. Histogram of the linear discriminant analysis (LDA) scores for differentially abundant taxon (relative abundant ≥ 1%). The enriched taxa in lung cancer group are indicated with a positive LDA score (green), and taxa enriched in healthy volunteers have a negative score (red). Only taxa that met an LDA significant threshold > 4 are shown. B. Taxonomic representation of statistically and biologically consistent differences between patients with lung cancer and healthy volunteers. Differences are represented by the color of the most abundant taxon (green, lung cancer; yellow, non-significant; red, healthy). The diameter of each dot is proportional to the taxon's abundance.

Table 3. Summary of the significant correlations between systemic inflammatory indicators and certain specific genera

Correlating pair of the variables	Correlation Coefficient (R)	*P* value
NLR-Escherichia-Shigella	0.453	0.003
NLR-Enterobacter	0.439	0.004
NLR-Dialister	-0.323	0.040
PLR-Dialister	-0.397	0.010
IL12-Veillonella	0.449	< 0.001
IL12-Dialister	-0.259	0.019
IL12-Fusobacterium	0.278	0.011
IL17-Veillonella	-0.224	0.043
IL17-Fusobacterium	-0.278	0.012
sCTLA-4-Escherichia	0.307	0.005
sCTLA-4-Veillonella	-0.300	0.006
sCTLA-4-Fusobacterium	-0.305	0.005
sCTLA-4-Enterobacter	0.318	0.004
sCTLA-4-Dialister	0.293	0.008

The spearman rank correlation (R) and probability (*P*) were used to evaluate statistical importance, and the correlation was filtered by P < 0.05.

Firmicutes, and *Fusobacteria*-were significantly different between the lung cancer and healthy control group (**Figure 4A-C**).

At the genus level, 398 genera were classified from the fecal bacteria, with 335 genera in the lung cancer group and the same number of genera in the healthy control group. The predominant genera were defined as comprising greater than 1% of the total gut bacteria. Among the total genera identified in the gut bacteria, 15 predominant genera were detected in the lung cancer group, and 16 were detected in the healthy control group, with 10 predominant genera found in both groups. These predominant genera accounted for 78.22% and 79.39% of the total sequences from the lung cancer group and the healthy control group, respectively; and the most predominant genera in the two groups were *Bacteroides* (**Figure 3A**, **3B**). The Metastats analysis showed that 169 genera differed significantly between the two groups, including eight predominant and 161 less predominant genera. Among the predominant differential genera, three genera-*Bacteroides*, *Veillonella*, *Fusobacterium*-were higher and five genera-*Escherichia-Shigella*, *Kluyvera*, *Fecalibacterium*, *Enterobacter*, and *Dialister*-were lower in the lung cancer group than in the healthy control group (**Figure 4D-L**).

Additionally, the metagenomic biomarker discovery approach was used to identify the greatest different phylotypes responsible for the difference in gut bacteria between the two groups. We found that the lung cancer group was associated with significantly higher *Bacteroidetes* and *Fusobacteria*. *Bacteroides* was the prominent genus level biomarker for lung cancer group, and thus, the lung cancer group might be designated by the *Bacteroides* dominant cluster. Other biomarkers for lung cancer group included *Prevotella-9*, *Klebsiella*, and *Fusobacterium*. For healthy controls, *Proteobacteria* and *Firmicutes* were the top two abundant phyla, and *Dialister*, *Enterobacter*, *Faecalibacterium*, and *Escherichia-Shigella* were the most prominent genus level biomarker. Thus, the healthy control group might be designated as the *Prevotella* and *Prevotella_7* dominant cluster. The other biomarkers for healthy controls included *Porphyromonadaceae*, *Prevotellaceae*, *Bacteroidia*, and *Bacteroidales*. These dominant phylotypes contributed to this difference between the lung cancer and healthy control groups (**Figure 5**).

Differences in expressed cytokines

To investigate the associations between fecal bacteria and clinical indicators, we detected the serum levels of IL-6, IL-12, IL-17, and sCTLA-4 in the lung cancer and the healthy control groups. No significant difference was observed in serum IL-12 level between the two groups. However, the serum levels of IL-6, IL-17, and sCTLA-4 in the lung cancer group were significantly higher than those in the healthy control group (**Table 1**) ($P < 0.05$).

We also retrospectively reviewed medical records of the patients with lung cancer for systemic inflammation-related markers, including neutrophil-to-lymphocyte ratio (NLR), platelet-to-lymphocyte ratio (PLR), lymphocyte-to-monocyte ratio (LMR), and prognostic nutritional index (PNI), which are potentially independent prognostic factors in survival of lung cancer (**Table 1**). Moreover, we evaluated the correlations between these systemic inflammation-related markers and the relative abundance of the bacterial genera with significant differences. Because of significant interindividual variation, two identified genera, *Escherichia-Shigella* and *Enterobacter*, were positively correlated with serum NLR level, and *Dialister* was nega-

tively correlated with serum levels of NLR and PLR. Furthermore, correlations were found between *Dialister* and serum levels of IL-12 and CTLA-4. Significant correlations between certain bacteria and inflammatory cytokines are presented in **Table 3**.

Discussion

In the present study, we characterized the unique composition of gut bacteria in 41 patients with lung cancer and healthy volunteers. Our study found an altered gut bacterial community in patients with lung cancer. Furthermore, we evaluated the correlations between certain specific bacteria and clinical indicators. For the first time, correlations were identified between certain specific bacteria and inflammatory status in patients with lung cancer. These findings provided a broader understanding of gut bacteria in lung cancer patients, paving the way for further investigation in this research area.

Altered gut bacteria composition has been identified in patients with intestinal or extraintestinal cancers [39, 40]. Up to now, studies on microbiota were primarily focused on bronchoalveolar lavage fluid, sputum and salivary samples, airway microbiome, and lung tumor tissues [10, 13, 15, 18, 41, 42] in patients with lung cancer. These studies provided insights into the microbiota communities of these patients and their potential link to lung cancer. Previous studies also documented that gut bacteria played an important role in the carcinogenesis of gastrointestinal cancer and other cancers via metabolism, inflammation, and immune response [7, 10-12]. These findings highlighted the reciprocal relationship between gut bacteria and lung cancer.

Decreased alpha diversity of bacteria from tumor lung tissues [15], bronchoalveolar lavage fluid, and sputum samples from cancer patients [14, 43] is commonly reported to be associated with cancer states. However, in the present study, no significant difference in alpha diversity was found between the two groups. This result does not exist in isolation as a similar result was also reported in buccal samples from patients with lung cancer [43]. Inflammation and immune status may be two of the major factors that affect gut bacterial alpha diversity [9, 44]. Diet, lifestyle, age, and other related factors also cannot be ignored [45]. Given the limited residence and diet style of our study participants, our results merely represent the patients commonly seen in clinical practice in the Jinan district. Therefore, large well-conducted studies are needed to further elucidate the lack of difference in alpha diversity.

In the present study, we provided evidence that patients with lung cancer had lower abundances in *Firmicutes* and *Proteobacteria*, along with relatively higher levels of *Bacteroidetes* and *Fusobacteria*, indicating the potential links between gut bacteria and lung cancer. In general, the dysbiosis of gastrointestinal tract metabolism has been repeatedly associated with a reduced *Firmicutes/Bacteroidetes* ratio [46, 47]. Our findings agree with this feature, as the lung cancer group exhibited a low *Firmicutes/Bacteroidetes* ratio. This low ratio results in a low concentration of circulating short-chain fatty acids, which are important influencing elements for host systemic immunity and systemic inflammation [48, 49]. Moreover, butyrate, one of the most crucial of these fatty acids, is associated with trophic and anti-inflammatory activities, and induces differentiation of regulatory T cells, cellular proliferation, and apoptosis through the activation of signaling pathways (such as NF-κB) [50, 51]. Additionally, all butyrate-producing bacteria belong to the *Firmicutes* phylum in human bacterial communities. Increased opportunistic pathogens, such as *Proteobacteria*, constitute a major structural imbalance of gut microbiota in patients with cancer [52, 53]. These data provided initial insights into the dysbiosis in gut microbiota associated with lung cancer. However, we cannot rule out that the altered bacteria diversity may be a passive byproduct of tumor progression. Therefore, additional studies of the longitudinal changes of the gut bacteria are warranted.

Our findings indicated that several other bacteria abundant in the intestines had a potential function in lung cancer (**Figure 5**). Fusobacterium, which is reportedly correlated with the development of several types of malignant tumors [54], was found to be significantly higher in patients with lung cancer than in healthy volunteers. Overgrowth of *Fusobacterium*, a potential inducer of T regulatory cells or carcinogens [40, 55, 56], promotes autophagy activation with poor outcomes in

colon cancers [21]. Additionally, a higher abundance of this bacteria is found in the bronchoalveolar lavage fluid samples from "healthy" smokers [57]. Thus, it is not a stretch to infer that *Fusobacterium* may contribute to the progression of lung cancer. An elevated abundance of *Veillonella* in patients with lung cancer was identified as a potential diagnostic biomarker for lung cancer in saliva samples [18], indicating a potential link between this type of gut bacteria and lung cancer. However, further mechanism studies are required to investigate this hypothesis. *Faecalibacterium*, the major type of butyrate-producing bacteria, and *Bacteroides* belonging to the *Bacteroidetes* phylum have been reported to enrich anti-cytotoxic T-lymphocyte-associated protein (CTLA) 4 plus anti-programmed cell death protein (PD) 1 receptors in epithelial tumors, indicating the potential synergistic antitumor effect on immunotherapy in patients with cancer [22, 58]. Furthermore, *Kluyvera* and *Dialister*, which were significantly lower in patients with lung cancer, were barely reported in relation to carcinogenesis until now. These results further supported the hypothesis that gut bacteria is linked with lung cancer. However, because the complex roles of those bacteria are unreaveled, further research, preferably with longitudinal studies, should be conducted to clarify the underlying mechanisms at work.

Interestingly, our study revealed that gut bacteria are correlated with inflammation indicators. A previous study showed that gut microbiota could regulate the lifespan of neutrophils and inflammatory monocytes [59, 60]. Elevated NLR and PLR were associated with the poor prognosis in patients with lung cancer [61, 62]. Moreover, combination immune checkpoint-targeted therapies such as CTLA-4 and PD-1/PD-L1 and bacterial treatments for cancer patients exerted great promise in antitumor responses [22, 63]. These evidences mentioned above make us infer that there might have been a direct relationship between the gut bacteria and inflammatory indicators in lung cancer patients. Our findings demonstrated the correlations between certain specific bacteria and inflammation indicators and, furthermore, provided the associations between bacterial markers and lung cancer. While these findings have sparked much new interest, the current data is unlike to yield any firm conclusion on whether the observed associations are a consequence of the disease or a causative mechanism. Future studies would benefit from a precise longitudinal study design to evaluate the causal relationships between inflammatory indicators and gut bacteria.

We acknowledge that our study contained some limitations. First, the sample size of patients was small. We could not comprehensively and systematically profile the bacterial communities of lung cancer patients. Thus, additional larger number of subjects is needed to verify our observations. Second, our experiments did not monitor the bacterial community structure dynamically in the process of lung cancer, which may contribute to a better understanding of the altered gut bacteria associated with lung cancer. Third, the effect of depression on gut bacteria in patients with lung cancer was not evaluated in this study. Nevertheless, further research on this topic is required, preferably studies with a longitudinal study design using a lung cancer animal model to investigate the underlying mechanisms of the relationship between gut bacteria and lung cancer.

In conclusion, we presented a detailed description of the altered fecal bacteria in patients with lung cancer, providing a significant first step in understanding the relationship between fecal bacteria and lung cancer. Moreover, our work is the first to evaluate the correlations between certain specific bacteria and inflammatory indicators. Our work not only extends this observation to patients with lung cancer, but also might facilitate clinical therapeutic strategies for monitoring and altering gut bacteria dysbiosis in lung cancer patients.

Acknowledgements

We acknowledge the patients and volunteers for their collaboration and collection of the fecal and serum samples. This study was supported by the Natural Science Foundation of Shandong Province (ZR2015HM054), the Key Research and Development Program of Shandong Province (2016ZDJS07A15 and 2017G006028), and the Science and Technology Department Public Welfare Project of Shandong Province (2014kjhm0107).

Disclosure of conflict of interest

None.

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