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Characterization of lung and oral microbiomes in lung cancer patients using culturomics and 16S rDNA sequencing

yifan sun, Yujing Bi, hongwei zhang, yuejiao liu, jianjie li, Tongtong An, bo jia, minglei zhuo, menglei ma, Ziping Wang, yafang tan, Ruifu Yang, and zhiyuan pan

Corresponding Authors: Ziping Wang, Peking University Cancer Hospital, and Ruifu Yang and Yujing Bi, State Key Laboratory of Pathogen and Biosecurity

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February 11, 2023

Dr. Yujing Bi
Beijing Institute of Microbiology and Epidemiology
Beijing
China

Re: Spectrum00314-23 (Characterization of lung and oral microbiome in lung cancer patients by cultruomics and 16S rDNA sequencing)

Dear Dr. Yujing Bi:

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2. Notch box plot should be used instead of box whisker plot.
3. The no of clinical samples may be increased.
4. The authors should also comment on the keystone species and species diversity of each mirobiome and their changes.

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Diyan Li

Editor, Microbiology Spectrum

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American Society for Microbiology
1752 N St., NW
Washington, DC 20036
E-mail: spectrum@asmusa.org

Reviewer comments:

Reviewer #10 (Comments for the Author):

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authors to consider. I believe the manuscript will be much improved if the authors incorporate my suggestions.
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Reviewer #12 (Comments for the Author):

The objective of this research aimed to analyze the lung and oral microbiome of patients with lung cancer.

There is a lack of information concerning the patients (oral pathologies?). The conclusions must be accompanied by several reservations concerning the size of the sample, the natural biodiversity of the microbiome between patients. Comparing the microbiomes (oral and pulmonary) of the same patient from a cancerous lung area with another non-cancerous area of the same patient cannot be 100% reliable.
In the study, a significant segregation was found at the level of the genus, but without having sought the equivalent level at the species. The taxonomic levels of the genus are not the relevant biological measurement units for some authors (BIK, ME et al. Bacterial diversity in the oral cavity of 10 healthy individuals) 1nternational society for microbial ecology. (2010). However in the mouth, the level of ecological interest chosen to label is the genus rather than species. There also reservations concerning the

conclusions of the article are to be made.

See the other remarks in the attached PDF. Corrections on the form and the bottom are necessary. Several reservations may call into question the validity of the result.

Add Bibliography.

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Zhang WQ. Et al 2018. Alterations of fecal bacterial communities in patients with lung cancer.
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Mortensen MS. The developing hypopharyngeal microbiota in early life. Microbiome 2016.

Das S. et al . A prevalent and culturable microbiota links ecological balance to clinical stability of the human lung after transplantation. Nat Commun 2021.

Reviewer #20 (Comments for the Author):

This is an interesting study aimed at characterizing and associating the oral and pulmonary microbiome of lung cancer patients. However, the study has not considered that the oral microbiome depends mainly on the oral condition. It has been widely demonstrated that high bacterial richness in the salivary microbiota is significantly associated with poor oral health, as indicated by decayed teeth, periodontitis, and poor oral hygiene. Therefore, it becomes crucial to understand oral microbial diversity and how it fluctuates under conditions of disease/disturbance. Advances in metagenomics and next-generation sequencing techniques generate rapid sequences and provide extensive information on the microorganisms inhabiting a niche. Therefore, the information retrieved can be used to develop microbiome-based biomarkers for use in the early diagnosis of oral and associated diseases. However, a homogenization of the oral clinical conditions of the samples must be considered for the results to be robust.

Reviewer #5 (Comments for the Author):

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Staff Comments:

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Characterization of lung and oral microbiome in lung cancer patients by cultruomics and 16S rDNA sequencing

Yifan Sun[1,#], Yuejiao Liu[1,#,] Jianjie Li[2], Yafang Tan[1], Tongtong An[2], Minglei Zhuo[2], Zhiyuan Pan[1], Menglei Ma[2], Bo Jia[2], Hongwei Zhang[2], Ziping Wang[2,*], Ruifu Yang[1,*], Yujing Bi[1,*]

1. State Key Laboratory of Pathogen and Biosecurity, Beijing Institute of Microbiology and Epidemiology, Beijing 100071, China;
2. Department of thoracic oncology, Peking University Cancer Hospital, Beijing 100142, China

#These authors contributed equally to this work.

* Corresponding authors: Ziping Wang (wangzp2007@126.com)
Ruifu Yang (13801034560@163.com)
Yujing Bi (byj7801@sina.com);

Abstract:

Microbiota dysbiosis in lung cancer has attracted more attention recently. However, most relevant studies on lung microbes are based on sequencing, making the potentially functional bacteria with extremely low abundance uncovered. Here, we employed both cultruomics and 16S rDNA sequencing to characterize and compare microbiota in lung and oral cavity. From BALF samples, 198 bacteria at species level were isolated and Firmicutes predominated (40%). 20 bacterial species isolated from BLAF samples occurred in at least half of patients, which were also present in a high proportion of oral samples. *Streptococcus* and *Veillonella* were the highly dominant group among all isolated strains. We showed that the abundance of genus *Prevotella* and *Veillonella* displayed a decreased trend from oral site to lung, whereas *Pseudomonas* increased. LEfSE analysis demonstrated that *Prevotella* increased in "Healthy" side (H) than Cancerous side (C), which was consistent with the species *Prevotella orails* only isolated from H group by cultruomics. Moreover, *Gemella sanguinis*, and *Streptococcus intermedius* were only isolated from non-small cell lung cancer (NSCLC) group while the 16S rDNA amplicon sequencing also showed a tendency that they are higher in the NSCLC than small cell lung cancer (SCLC) group. Furthermore, *Bacillus* and *Castellaniella* were enriched in lung adenocarcinoma (ADC), but *Brucella* in lung squamous cell carcinoma (SCC). Overall, we founded that the microbial community changed in lung cancer patients, which diversity might be site- and pathological-dependent. Combined with cultruomics and 16S rDNA amplicon sequencing provides deeper insights about the pulmonary and oral microbiota changes of lung cancer patients.

Keywords: Microbiota, Lung cancer, BALF, Oral bacteria, Cultruomics, 16S rDNA

Introduction

Lung cancer is the most common cancer worldwide, and is closely associated with chronic inflammation [1]. Inflammation caused by microbial infection may contribute to cancer development and progression [2]. Polymorphic microbiomes have been recently added to one of the four new "Hallmarks" of cancer [3]. Evidence is mounting that the lung microbiome may play a role in cancer pathogenesis.

Healthy lungs that were traditionally thought to be sterile are now known to harbor a diverse microbiota [4]. To date, a substantial number of studies applying culture-independent analysis have reported that microbial population diversity was associated with lung cancer [5, 6]. Richness of lung microbiota was reduced in lung cancer patients but the composition of the bacterial flora of patients in phylum Bacteroidetes was significantly higher compared with control subjects [7, 8]. A study found that genus *Streptococcus* was more abundant in BALF samples of lung cancer patients than healthy controls [9]. Few studies showed links between lung bacteria and histological subtypes of lung cancers; the genera *Veillonella, Megasphaera, Enterobacter, Morganella*, and *Klebsiella* were significantly higher in lung adenocarcinoma than lung squamous cell carcinoma [10, 11]. Oral cavity is the entry point for the respiratory tract, and the oral microbiome may contribute to lung cancer risk [12]. Tsay et al. reported that lower airway dysbiosis induced by microaspiration of oral commensals affects lung tumorigenesis by promoting an IL-17-driven inflammatory phenotype [13]. A study showed that the oral microbiome of bacterial genera *Sphingomonas* and *Blastomonas* were relatively higher in lung cancer patients [14]. However, the possible variations of oral and lung microbiota in lung cancer patients and the difference in microbial diversity in samples from the saliva and BALF has yet to be defined.

While numerous DNA sequencing-based investigations have been performed to explore the relationship between the lung microbiota and cancer, it has several inherent drawbacks, such as depth bias and a high detection threshold [15]. Culture-dependent approaches are indispensable for further studies of lung microbial function. Cultruomics, which uses multiple culture conditions combined with rapid identification, is developed to provide new perspectives on host-bacteria relationships [16]. However, cultruomics has rarely been reported for the culture and identification of bacteria in BALF.

In this work, we propose to apply a comprehensive approach combining cultruomics and 16S rDNA amplicon sequencing to saliva and BLAF samples from 25 lung patients with unilateral lobar masses. We reported the bacteria diversity and richness of oral and BALF microbiota from lung cancer patients and applied cultruomics for the first time to culture and identify them.

Materials and methods

1 Patient recruitment and samples collection

This study was approved by the Institutional Review Board of the Peking University School of Oncology, China, and informed consent was obtained from all subjects. BALF sample collection was performed as previously described [8]. Before the patients were selected for bronchoscopy examination, saliva samples were collected. All the participants were instructed to not eat and drink for 1 h prior to saliva sample collection. 25 patients with unilateral lobar masses were enrolled from patients who consented to bronchoscopy examination at Peking University Cancer Hospital. All patients underwent transbronchoendoscope, which avoided contamination of the upper respiratory tract or oral microbiota, and paired BALF samples (one from the cancerous side (C), the other from the contralateral healthy lung (H)) were collected before the operation. Before the bronchoscopy, total 21 patient oral samples were collected. The sample was divided into two parts: one part (including 45 samples from 15 lung cancer patients) was used for culturing bacteria, and the other part (all of the 71 samples) was used for 16S rDNA amplicon sequencing. The fresh samples were collected in a sterile tube, placed on ice, and transported to the laboratory within 1 h. Cultruomics was carried out on the day of collection in the laboratory immediately, while aliquots were then stored at −80°C before high-throughput sequencing.

2 Cultruomics

2.1 Process of cultruomics

Cultruomics is a high-throughput method that multiplies culture conditions to detect higher bacterial diversity and pure bacterial cultures. This cultruomics study was pre-cultured under different conditions: aerobically supplemented with 5% sheep blood; aerobically supplemented with 5% rumen fluid; anaerobically with 5% sheep blood;

anaerobically with 5% rumen fluid. Sample dilution, strain isolation, and identification were performed as previously described [17]. On days 1, 3, 6, 9, 15, and 30, samples of enriched cultures were extracted from the bottles by syringe, and 100ul doubling dilutions were spread onto Columbia agar supplemented with 5% sheep for culture at 37°C aerobic conditions for 24 h or anaerobic conditions for 72 h. Colonies were picked and identified using MALDI-TOF MS systems (Autof MS1000). Colonies that were not identified by MALDI-TOF MS systems of database V1.1.12 (score < 9) were subjected to 16S rDNA gene sequencing with primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). Sequencing results were analyzed by the NCBI BLAST algorithm4 for homologous sequence searches with type strains. If 16S rDNA is <98.65% similar to the closest type strain, the isolate could be a new species [18].

2.2 Classification of cultivated species

Classify all isolates into four categories: oral/respiratory(this study and [19]), gut [20], urine [21, 22], and vagina [23, 24]. We also conducted literature searches on PubMed to compare against published papers and confirm the classification.

Sequences of the 16S rDNA of isolated strains were aligned using the CLUSTALX program. A phylogenetic tree was constructed using neighbor-joining with MEGA version X with 1000 replicate bootstrap values. The classical tree visualization is supported online tool (https://itol.embl.de) [25].

3 DNA Extraction, 16S rDNA Amplification and Sequencing

DNA was extracted from each sample using Hipure Bacterial DNA kit (Mageon, China) according on the manufacturer's recommendation. The V3-V4 region of 16S rDNA gene was amplified using specific primers (341F: 5'-CCTAYGGGRBGCASCAG-3'; 806R: 5'-GGACTACNNGGGTATCTAAT-3'). Amplicons were sequenced using the Miseq platform. Sequencing was performed on an Illumina Novaseq6000 sequencing platform (Illumina, San Diego, CA, United States), and 250 bp paired-end reads were generated.

4 Sequence data analysis

Raw reads were filtered to remove adaptors and low-quality and ambiguous bases,

clean data were extracted from Raw data using USEARCH 8.0. Operational taxonomic units (OTUs) were classified based on 97% similarity after chimeric sequences removed using UPARSE (version 7.0.1001 http://drive5.com/uparse/) and the representative sequence from each OTU cluster was obtained. Alpha diversity was assessed on the basis of the nonparametric Shannon index and Simpson index. The β diversity was calculated using Bray-Curtis distances in QIIME and visualized by principal coordinate analysis (PCoA). The linear discriminant analysis (LDA) effect size (LEfSE) was used to detect taxa with differential abundance among groups. The Metastats analysis was employed to detect the differences in microbiota composition among groups at the genus levels.

5 Statistical analysis

Statistical analysis was performed by R software (v3.4.10) and SPSS 20.0. For demographic and clinical data, independent t test and chi-square test were used. Fischer Exact test with FDR correction were used for higher isolated species between the two groups. Sample diversity metrics were assessed on the basis of the nonparametric Shannon–Wiener diversity index and Chao1 diversity index. To visualize separation of samples based on pairwise distances, principal coordinate analysis (PCoA) plots were generated, and anosim was performed to test statistically whether there is a significant difference in PCoA analysis. All statistical analyses were performed using R software (Version 2.15.3). P value < 0.05 was considered statistically significant.

RESULTS

1、Clinical information

A total of 25 patients with unilateral lobar masses were recruited between January 2021 and May 2022 from Beijing Peking University Cancer Hospital. Among all the patients, 23 patients were newly diagnosed with lung cancer by histological confirmation, but 2 patients were not. There were 16 non-small cell lung cancer (NSCLC) including 8 adenocarcinoma (ADC), 7 squamous cell carcinoma (SCC), and

one with non-specified NSCLC, and 7 small cell lung cancer (SCLC). The lung cancer patients were not previously received any anticancer therapy nor were treated with any antibiotics. In 23 lung cancer patients, there were14 males and 9 females, and totally 18 smokers. There were 6 cases without distant metastasis and 17 with distant metastasis (Table 1).

2、Characteristics of bacteria isolated from lung and oral cavity by cultruoims

The cultruomics workflow is shown in Figure 1. Briefly, total 45 samples from 15 lung cancer patients were collected, and 4 culture conditions were tested for each sample. From BALF samples (including both cancerous lung (C) and the contralateral "healthy" lung (H)), a total of 12379 colonies were obtained, and 198 bacteria at species level were identified by MALDI-TOF or 16S rDNA gene sequencing. The identified species belonged to 6 phyla, including Firmicutes (40%), Proteobacteria (28%), Actinobacteria (19%), Bacteroidetes (10%), Fusobacteria (2%), and Synergistetes (2%) (Figure 2A and B). By comparing the previously established repertoire of isolated microorganisms from the human gut, urine, vagina, and oral/respiratory tract, about 1/4 of the isolated species in this study were previously isolated from 4 multiple sites in the human body (47/198, 23.7%) (Figure 2C).

From oral samples, a total of 5671 colonies were observed, and 156 bacteria at species level were identified (Fig. S1). At the phylum level, the bacterial diversity of the oral sample was similar to BALF, Firmicutes and Proteobacteria were predominated (> 66%).

In addition to previously known bacteria, 15 potentially new species were isolated from this cultruomics study. (Table S1)

3、Comparison of microbiota in lung and oral cavity

We compared the microbiota composition between cancerous-side lung (C), "healthy"-side lung (H), and oral cavity (O) by 16S rDNA amplicon sequencing. There was obvious difference between lung and oral cavity, but C and H seemed moderate difference at phylum level (Figure 3A). Three dominant phyla were Proteobacteria, Firmicutes, and Bacteroidetes. At genus level, *Pseudomonas*

(Proteobacteria), *Streptococcus* (Firmicutes), *Veillonella* (Firmicutes), and *Prevotella*_7 (Bacteroidetes) were the most common in the BALF samples. In contrast, *Prevotella*_7 (Bacteroidetes), *Neisseria* (Proteobacteria), *Streptococcus* (Firmicutes), *Veillonella* (Firmicutes), and *Haemophilus* (Proteobacteria) were the most common in oral samples (Figure S2A). In the BALF group, cancer lung was not significantly from "healthy" lung in richness and diversity of microbial community (α-diversity), as measured by the Shannon (P=0.527) and Chao1 diversity index (P=0.428), or overall microbiota (β-diversity), as measured by Bray-Curtis distances (P=0.390) (Figure S3). Nevertheless, the oral sample was significantly different from both the cancerous side and the "healthy" side of the lung in α and β-diversity (Figure 3B, 3C). Metastat analysis based on genus level further revealed unique anatomy-related microbial features, such as more abundant *Prevotella* in oral samples (Figure 3D). *Pseudomonas* was the only genus concentrated in BALF samples (Figure 3D, S2B).

We also compared the bacteria isolated by cultruomics between different groups. Different with sequence data, there was no obvious difference for bacterial proportion at phylum level between cancerous-side (C), "healthy"-side (H), and oral cavity (O). Firmicutes predominated in all three sites (Figure 3E). More than half of the species (89) were isolated in all three sites, and there was 34, 32 and 39 special species isolated in C, H and O group respectively (Figure 3F). Further, we analyzed the prevalence bacteria in lung and oral cavity. For cultruomics results, we defined the strains isolated from more than 50% of patients as prevalent strains. For BALF samples, 20 species were identified as prevalent strains, which belonged to 12 genus (Table 2). *Streptococcus* was the major genus, and *Streptococcus oralis*, *Veillonella atypica*, *Parvimonas micra*, and *Actinomyces odontolyticus* were found in nearly all BALF samples. Similar, these 20 species were also cultured at a high frequency in oral samples, which indicated the pulmonic microbiota maybe come from oral cavity.

4、Difference in microbiota compositions between cancerous and "healthy" lung

To compare the relative contribution of different taxa, we used the LEfSE to detect taxa with differential abundance among the two groups. A total of 14 different taxa at

various levels with significant abundances across two groups were identified, of which four differentially abundant taxa at the genus level were noted. *Prevotella* and *Prevotella_7* increased in H group, whereas *Carnobacterium* and *Brucella* increased in C group (Figure 4A).

At the same time, we also compared the differences between H and C groups of bacteria obtained by cultruomics. We drew a heat map of the proportion of each bacterium in the total sample. *Streptococcus orails*, *Veillonella ayptica*, and *Parvimonas micra* were both cultured at a high frequency without significant group differences. *Prevotella orails* was found with a significantly higher frequency in H group (P=0.019) (Figure 4B). This was coincident with sequencing result, which also showed the prevalent of *Prevotella* in H group.

5、Different pathological types shaped different pulmonary microbiomes

We then sought to disclose differences in lung microbiota of NSCLC and SCLC. Basic information included age, sex, smoking status and distant metastasis were comparable between 2 groups (Table S2). Bacteria with abundance greater than 4% were considered as dominant. Across the 32 NSCLC lung samples, the dominant genera were *Pseudomonas* (32%), *Streptococcus* (5%), and *Bacillus* (4%). While in SCLC, the dominant genus was *Pseudomonas* (31%), *Veillonella* (7%), and *Prevotella_7* (6%) (Figure 5A). NSCLC was not significantly different from SCLC in α-diversity, as measured by Shannon (P=0.931) and Chao1 diversity index (P=0.720), or β-diversity as measured by Bray-Curtis distances (P=0.489) (Figure S4 A, B). By cultruomics study, we found that 77 species were only isolated from NSCLC lung samples and 37 species were only isolated from SCLC lung samples (Figure 5B). Only the bacterial species isolated at least half of the patients in the group were selected to the next step of comparison. Then we analyzed bacteria, which only isolated from one group with isolation rate ≥50% (The bacterial species isolated from at least half of the patients in the group). *Gemella sanguinis*, *Pseudoramibacter alactolyticus*, *Bifidobacterium dentium*, and *Streptococcus intermedius* were the four species with a relatively high frequency only isolated from the NSCLC group. Of all the species isolated from SCLC lung samples, *Prevotella pallens* was the species which had a relatively high frequency. Then, we compared the relative abundance of

above five bacteria at genus level with 16S rDNA amplicon sequencing. Though there is no significant difference between the two groups, the abundance showed a higher tendency in the only isolated site (Figure 5C).

In subtypes analysis of NSCLC patients, among the 16 ADC samples, the dominant genera were *Pseudomonas* (26%) and *Bacillus* (8%). While in 14 SCC samples, the dominant genera were *Pseudomonas* (38%) and *Streptococcus* (8%) (Figure 5D). Shannon (P=0.270) and Chao1 diversity index (P=0.402) were not significantly different in ADC and SCC groups, while the β-diversity showed that microbiota constitution in ADC lung samples was clearly different than the SCC samples (P=0.021) (Figure S4 C, D). In the LEfSE analysis of ADC and SCC, 11 various taxa were detected to display contrasting correlations between NSCLC subtypes. A differential abundance analysis at the genus level between ADC and SCC showed an enrichment of *Bacillus* and *Castellaniella* in ADC patients, whereas SCC had a higher abundance of *Brucella* (Figure 5E).

Discussion

To date, criteria for the normal composition of lung microbiota have not yet been established, but the available data indicate that their composition in cancer patients differs considerably from that of healthy individuals [26]. An association between the lung microbiome and histologic classification of lung cancer was also observed. *Pseudomonas* showed a correlation with ADC [27]. Interestingly, the bacteria found in BALF can originate in the mouth [28]. With more oral microbes entering to the lungs being associated with increased lung proinflammatory cytokines [29]. The microbiome has been implicated in lung cancer in a variety of specific ways, but the role of lung microbiota in carcinogenic processes has not yet been elucidated. One of the possible reasons for this is the current studies are mostly based on the sequencing levels and resulting in a lack of strains materials. In the current study, both cultruomics and 16S rDNA amplicon sequencing were employed to evaluate and compare the structure and diversity characteristics of oral and pulmonary microbiota associated with lung cancer.

Cultruomics can identify bacteria to strain level, and successfully isolating living bacteria is crucial for carrying out subsequent experimental work [15, 30, 31]. A study summarized a list of microbes isolated from the human lower respiratory tract showed

that the lung microbiome was dominated by the phyla *Pseudomonadota*, *Firmicutes*, *Bacteroidota*, and *Actinomycetota*, which was consistent with our study [19]. In this study, we have cultured 198 identified bacterial species from the human BALF and 156 from oral of lung cancer patients, and 15 potentially new taxa. The related article about novel bacteria new.4 and new.10 which belonged to a novel genus we named '*Curtanaerobium*' was submitted to IJSEM (under review). The present study enabled to expand of the human respiratory and oral repertoire. We also identified 20 species as prevalent strains in both BLAF and oral samples of lung cancer patients, which indicated the pulmonic microbiota maybe come from oral cavity. Cultruomics could reduce the number of these unclassed or no-rank OTUs by increasing the number of pure cultured microorganism species. In our study, *Pseudoramibacter alactolyticus* was recovered with a relatively high frequency only isolated from the NSCLC group but was not detected in 16S rDNA amplicon sequencing. *Parvimonas micra* was reported to reveal a high abundance in colorectal cancer patients, and Yu et al. reported that *P. micra* promoted colorectal tumorigenesis by inducing colonocyte proliferation and altering Th17 immune response [32, 33]. Still, there was no report of the correlation with lung cancer. In this study, *P. micra* was cultured at a high frequency in lung cancer patients, and the roles of these strains provided materials for further study.

Microbiota composition analysis at the genus level through taxonomic assignment were performed, and the results showed that representative flora differed by sampling site. We found that *Streptococcus*, *Veillonella*, and *Prevotella* were enriched in the oral samples, while *Pseudomonas* was enriched in BLAF samples. *Streptococcus* and *Veillonella* which were considered as oral commensals were reported to increase in lower airways of lung cancer patients [34]. *Pseudomonas* species are commonly found in the respiratory tract; and are involved in the pathogenesis of lung diseases, such as chronic obstructive pulmonary disease and cystic fibrosis [35, 36]. One study reported that *Pseudomonas aeruginosa* was significantly more abundant in brain metastasis of NSCLC patients [37]. Likewise, the majority of patients in our study was with distant metastasis, and *Pseudomonas* was the most genus in BALF samples. These findings, including ours, seem to suggest that *Pseudomonas* colonize more frequently in the respiratory tract of lung cancer patients and induce a promotion of distant metastasis.

Gemella sanguinis, *Pseudoramibacter alactolyticus*, and *Streptococcus intermedius* are involved in human inflammatory diseases, such as endocarditis [38], periodontal infectious [39], and brain abscess [40]. Germ-free mice or mice treated with antibiotics had a significantly lower incidence of lung cancer than pathogen-free mice [41, 42]. Our results showed that the three species were only isolated from lung microbiota of NSCLC, not isolated from SCLC patients, and the tendency was confirmed by 16S rDNA amplicon sequencing, which indicated that the specific bacterial might mediate the development of NSCLC by inducing chronic inflammation.

When stratifying by pathological subtypes of lung cancer, a significant enrichment in *Bacillus* and *Castellaniella* was observed in the BALF samples of ADC patients, and an enrichment of *Brucella* was observed in the SCC group. Another study reported the enrichment of *Acinetobacter* in BALF samples of ADC, while *Bacillus* and *Brucella* showed no significant differences between ADC and SCC groups [11]. These conflicting associations of microbiome with different pathological subtypes could be attributable to the significant variation across different individuals and sampling methods.

In conclusion, we examined pneumonic and oral microbiota in lung cancer patients using cultruomics and 16S rDNA sequencing, and found that the microbial community changed in lung cancer patients, which diversity might be site- and pathological-dependent. We found that *Streptococcus* and *Veillonella* were the highly dominant bacteria both in both pneumonic and oral samples of lung cancer patients by cultruomics, which suggested possible deleterious effects of airway microbial dysbiosis originating from oral cavity. We showed that *Prevotella orails* was only isolated from H group and *Gemella sanguinis* was only isolated from NSCLC group, which was consistent with the 16S rDNA amplicon sequencing. This study provides basic data on the microbiota diversity in pneumonic and oral samples from lung cancer patients. These features may be potential bacterial biomarkers and new targets for lung cancer diagnosis and treatment and the isolated strains provide materials for exploring the causative relationships.

AUTHOR CONTRIBUTIONS

Ethics approval and consent to participate

All Procedures performed in studies involving human participants or animals were approved by the Institutional Review Board of the Peking University School of Oncology (No. 2018KT89). All patients gave their written consent before inclusion in the study.

Consent for publication

Not applicable.

Availability of data and materials

All the data generated or analyzed in this study are included in this published article (or its Supplementary Information files). The read sequences obtained from Illumina NovaSeq were submitted to the NCBI Sequence Read Archive (SRA) under accession number PRJNA904049 (BioProject ID) (http://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA904049).

Competing Interest

The authors declare no competing interests.

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Authors' contributions

Y.J.B. designed research and project outline. R.F.Y and Z.P.W. directed the research. Y.F.S, J.J.L., Y.J.L., A.T.T, Z.M.L, M.M.L, J.B, and Z.H.W. performed isolation, deposition and identification. Y.F.T. and Z.Y.P. performed genome analysis. Y.F.S. and Y.J.B. drafted the manuscript. All authors read and approved the final manuscript.

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501

Fig 1. Summary of culturomics methods and workflow.

Fig 2. The bacteria identified from the BALF samples. **A** Phylogenetic tree of the isolated bacterial species from BALF samples. **B** Proportion of bacterial species isolated from the BALF samples listed according to their phylum. **C** Upsetplot showing the shared cultured species between human oral/respiratory, gut, urine and vagine.

Fig 3. The microbial composition varied in different anatomy sites. **A** Taxonomic composition at phylum level in BALF and oral samples based on 16S rDNA amplicon sequencing (Top 30). **B** The Shannon diversity index of BALF samples and oral. *P* values were calculated with Wilcoxon test. **P* < 0.05, ***P* < 0.01 **C** PCoA analysis of three different anatomy sites samples in lung cancer patients. Anosim was performed to test statistically whether there is a significant difference. ***P* < 0.01 **D** Box-and-whisker plots illustrating the differences of the significantly 2 genus relative abundances in three different anatomy sites. * *P* < 0.05 **E** Proportion of bacterial species isolated from C, H and O samples listed according to their phylum. **F** Veen diagram of culturable bacterial species in three different anatomy sites. C: samples from the cancerous site of lung cancer patients, H: samples from the contralateral "healthy" controls of lung cancer patients. O: samples from oral site of lung cancer patients.

Fig 4. Differentially abundant taxonomy between cancer and paired "healthy" lung. **A** The LEfSE was used to identify the bacterial microbiota that significantly differed between cancer and lung. Only taxa meeting a significant LDA threshold value of **>** 3.5 and *P* **<** 0.05 are shown. **B** Heatmap analysis between C and H of lung microbiota was done based on the culturomics of the cancer patients (n=15). Each row represents an individual species with the higher isolated rate in the two groups. The top 25 bacterial species recovered from either group. From blue to red represents the frequency of culturomics recovery from 0 to 1. Statistical comparisons were made using the Fischer Exact test with FDR correction. **P*< 0.05

Fig 5. Characterization of the lung microbiota in the NSCLC and SCLC and the differences in ADC and SCC. **A** Taxonomic composition at genera level in NSCLC and SCLC lung samples. **B** Veen diagram of culturable bacterial species only isolated from NSCLC and only isolated from SCLC lung samples. **C** Difference of relative the genus abundance corresponding to species isolated from NSCLC and SCLC lung. To identify differential microbial taxa, paired t-test was performed and P-values were adjusted for multiple comparison by the FDR. **D** Taxonomic composition at genera level in ADC and SCC lung samples. **E** The LEfSE was used to identify the bacterial microbiota that significantly differed between NSCLC and SCLC. Only taxa meeting a significant LDA threshold value of **>** 4 and *P* **<** 0.05 are shown.

546

547 **Table 1.** Clinical characteristics of patients.

Variable	NSCLC	SCLC	Non
N	16	7	2
Age-mean (SD)	65 (7.0)	67 (12.8)	69 (8.2)
Gender			
Male, n (%)	9 (56%)	5 (72%)	2
Female, n (%)	7 (44%)	2 (14%)	0
Smoking			
Current or former Smoker, n (%)	12(75%)	6(86%)	1
Never smoker, n (%)	6(25%)	1(14%)	1
Pathological diagnosis			
Adenocarcinoma, n (%)	8(50%)	——	——
Squamous cell carcinoma, n (%)	7(44%)	——	——
Unidentified	1(6%)	——	——
Distant			
MO	4(25%)	2(29%)	——
M1	12(75%)	5(71%)	——

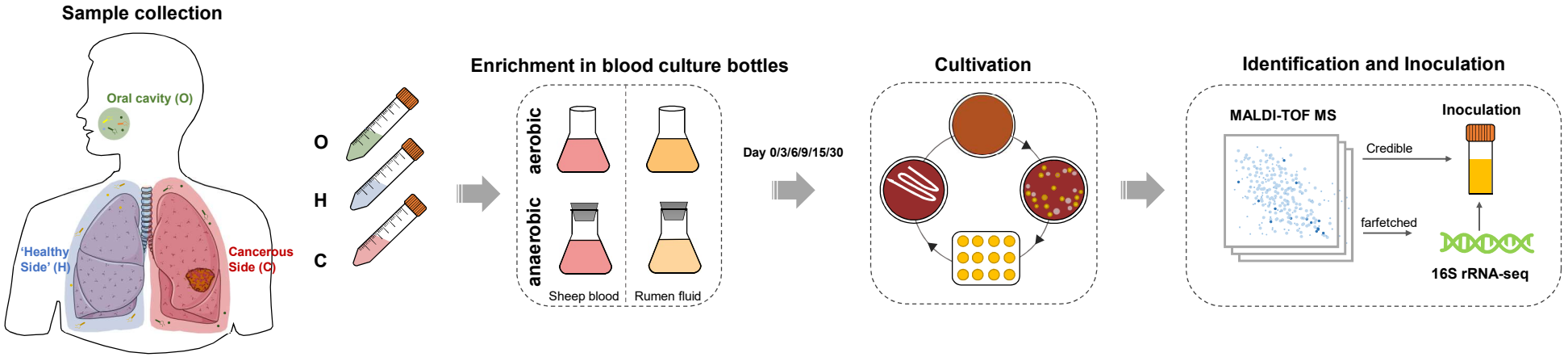
548

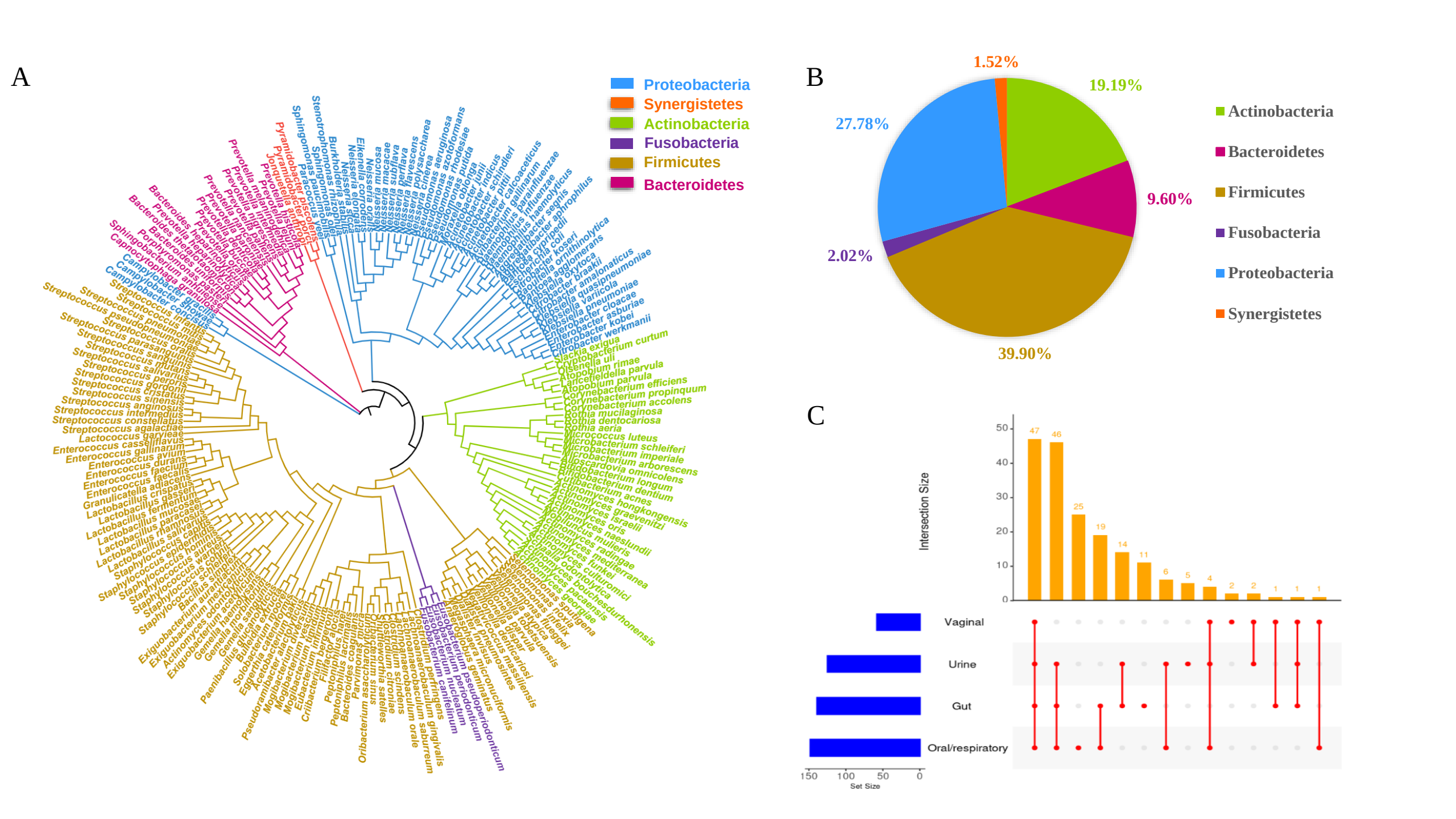
549

Table 2. Summary of the proportion of the TOP20 bacterium in BALF samples and the related proportion in oral cavity.

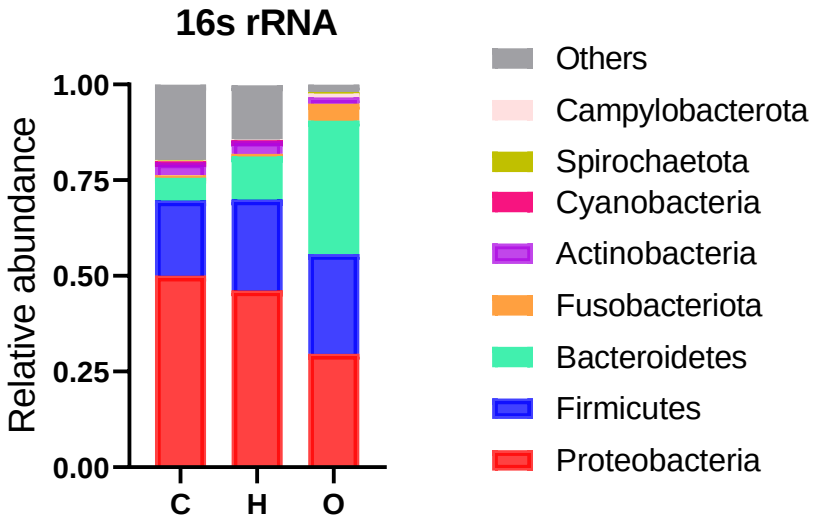
Genera	Species	C	H	O
Streptococcus	***Streptococcus salivarius***	60.00%	60.00%	73.33%
	Streptococcus pseudopneumoniae	53.33%	40.00%	33.33%
	Streptococcus parasanguinis	93.33%	86.67%	100.00%
	Streptococcus oralis	93.33%	93.33%	100.00%
	Streptococcus mitis	93.33%	86.67%	100.00%
	Streptococcus gordonii	53.33%	53.33%	80.00%
	Streptococcus constellatus	66.67%	73.33%	93.33%
	Streptococcus anginosus	66.67%	66.67%	100.00%
Veillonella	***Veillonella parvula***	60.00%	60.00%	73.33%
	Veillonella atypica	66.67%	80.00%	66.67%
Solobacterium	***Solobacterium moorei***	66.67%	60.00%	73.33%
Slackia	***Slackia exigua***	60.00%	73.33%	60.00%
Parvimonas	***Parvimonas micra***	80.00%	80.00%	93.33%
Mogibacterium	***Mogibacterium diversum***	53.33%	46.67%	53.33%
Granulicatella	***Granulicatella adiacens***	66.67%	53.33%	46.67%
Gemella	***Gemella morbillorum***	66.67%	53.33%	66.67%
Fusobacterium	***Fusobacterium nucleatum***	40.00%	66.67%	80.00%
Dialister	***Dialister invisus***	73.33%	53.33%	73.33%
Anaeroglobus	***Anaeroglobus geminatus***	53.33%	60.00%	73.33%
Actinomyces	***Actinomyces odontolyticus***	80.00%	80.00%	66.67%

C: samples from the cancerous site of lung cancer patients, **H**: samples from the contralateral "healthy" controls of lung cancer patients. **O**: samples from oral site of lung cancer patients.

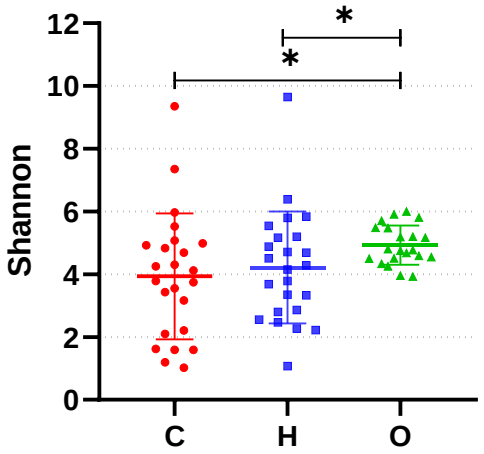

Sample collection
Oral cavity (O)
'Healthy Side' (H)
Cancerous Side (C)
O
H
C
Enrichment in blood culture bottles
aerobic
anaerobic
Sheep blood
Rumen fluid
Day 0/3/6/9/15/30
Cultivation
Identification and Inoculation
MALDI-TOF MS
Credible
farfetched
Inoculation
16S rRNA-seq


A
Proteobacteria
Synergistetes
Actinobacteria
Fusobacteria
Firmicutes
Bacteroidetes
B
1.52%
19.19%
27.78%
9.60%
2.02%
39.90%
Actinobacteria
Bacteroidetes
Firmicutes
Fusobacteria
Proteobacteria
Synergistetes
C
Intersection Size
50
40
30
20
10
0
47
46
25
19
14
11
6
5
4
2
2
1
1
1
Vaginal
Urine
Gut
Oral/respiratory
150
100
50
0
Set Size

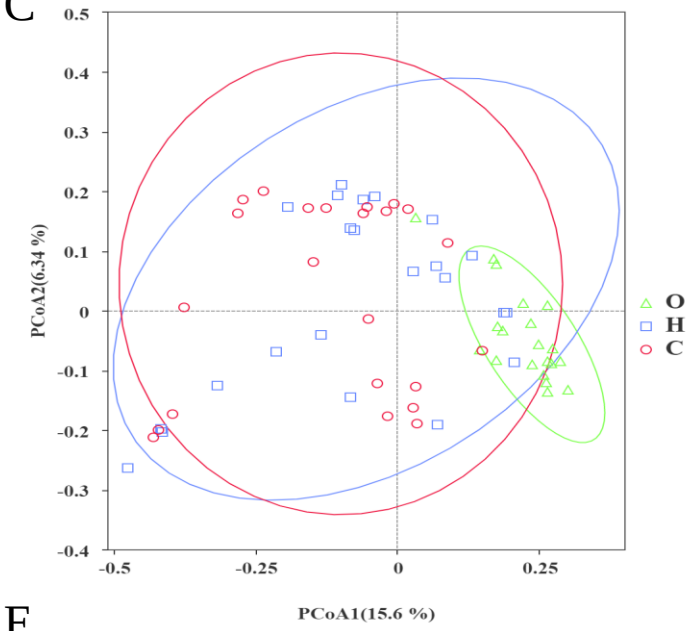
A


16s rRNA
Relative abundance
1.00
0.75
0.50
0.25
0.00
C
H
O
Others
Campylobacterota
Spirochaetota
Cyanobacteria
Actinobacteria
Fusobacteriota
Bacteroidetes
Firmicutes
Proteobacteria

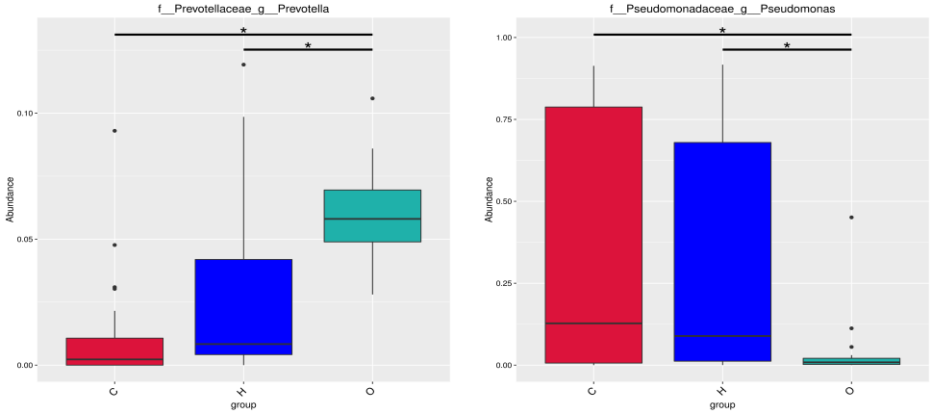
B


Shannon
12
10
8
6
4
2
0
*
*
C
H
O

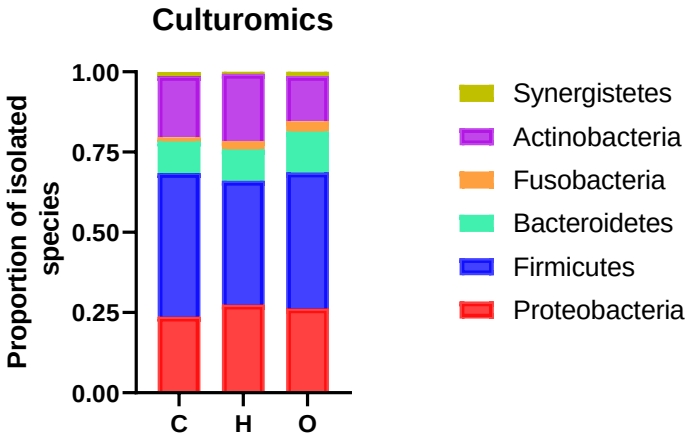
C


PCoA2(6.34 %)
PCoA1(15.6 %)
0.5
0.4
0.3
0.2
0.1
0
-0.1
-0.2
-0.3
-0.4
-0.5
-0.25
0.25
O
H
C

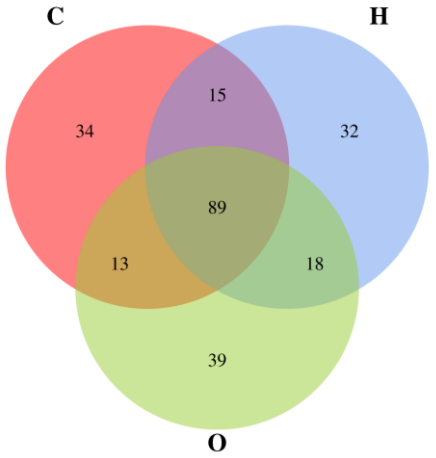
D


f__Prevotellaceae_g__Prevotella
f__Pseudomonadaceae_g__Pseudomonas
Abundance
group
C
H
O

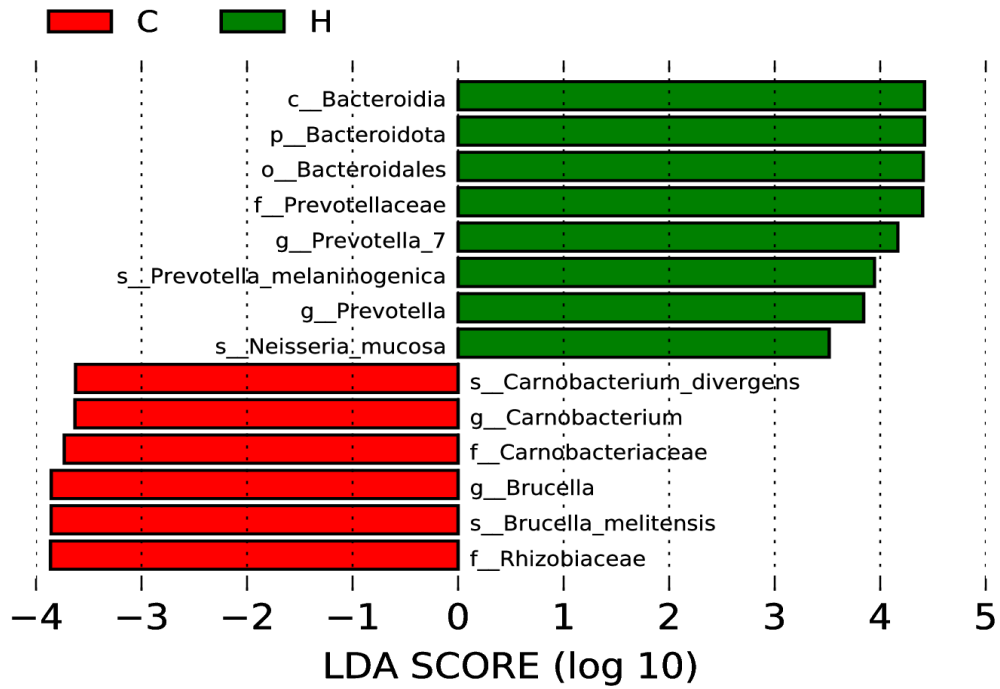
E


Culturomics
Proportion of isolated species
1.00
0.75
0.50
0.25
0.00
C
H
O
Synergistetes
Actinobacteria
Fusobacteria
Bacteroidetes
Firmicutes
Proteobacteria

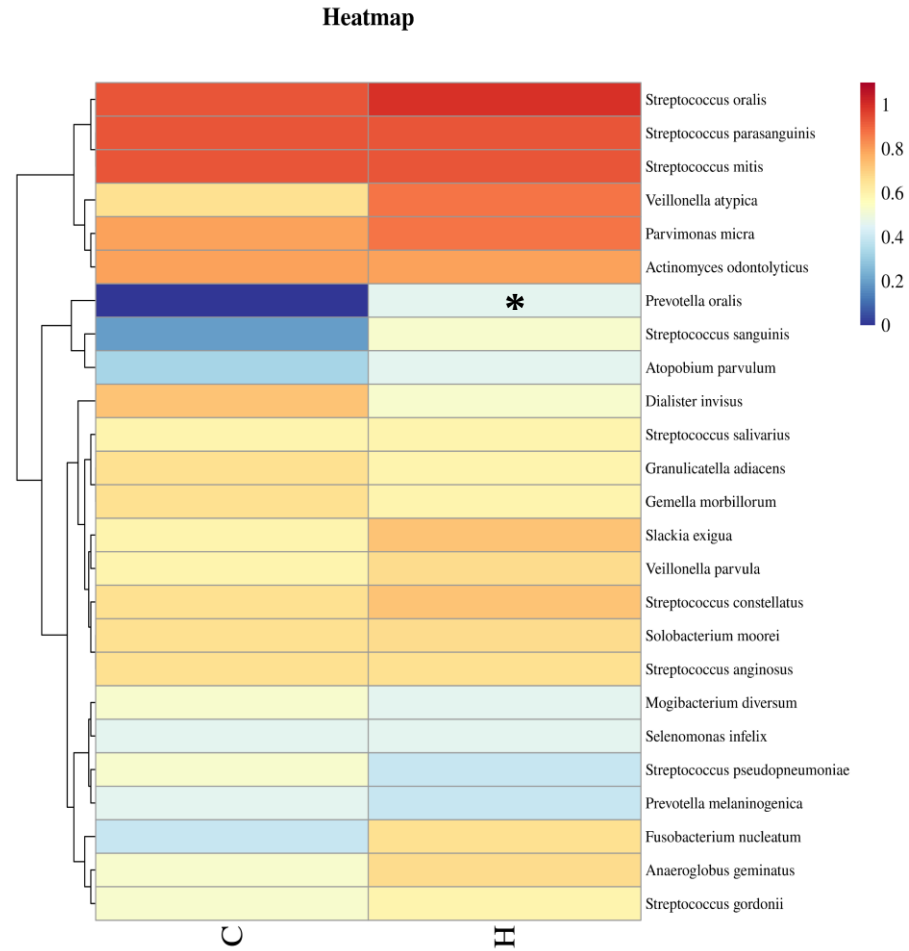
F


C
H
O
34
15
32
89
13
18
39

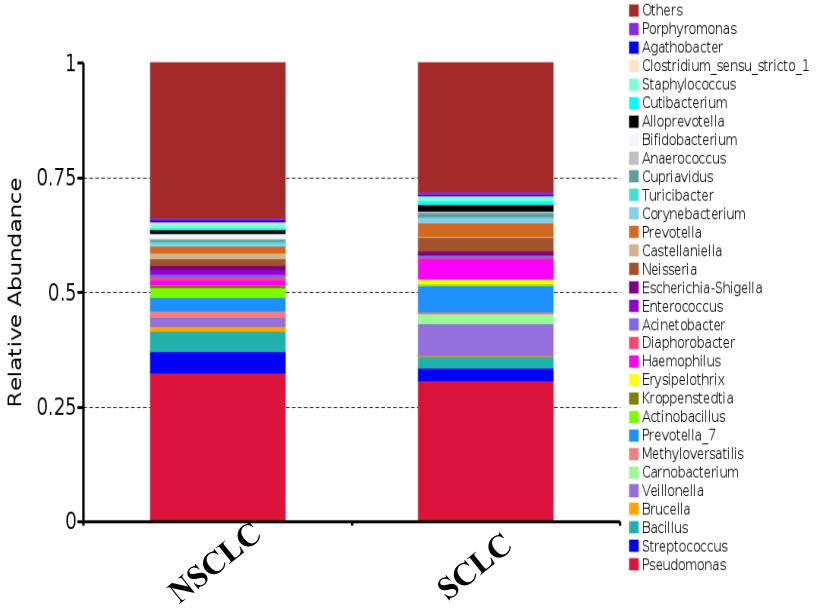
A


C
H
c__Bacteroidia
p__Bacteroidota
o__Bacteroidales
f__Prevotellaceae
g__Prevotella_7
s__Prevotella_melaninogenica
g__Prevotella
s__Neisseria_mucosa
s__Carnobacterium_divergens
g__Carnobacterium
f__Carnobacteriaceae
g__Brucella
s__Brucella_melitensis
f__Rhizobiaceae
−4
−3
−2
−1
0
1
2
3
4
5
LDA SCORE (log 10)

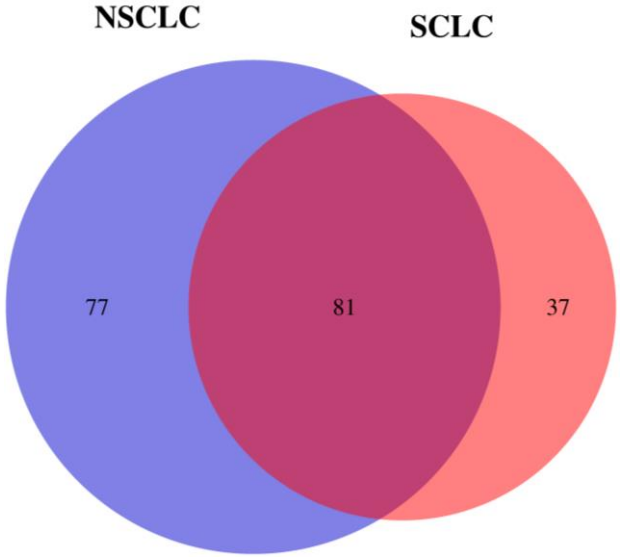
B


Heatmap
Streptococcus oralis
Streptococcus parasanguinis
Streptococcus mitis
Veillonella atypica
Parvimonas micra
Actinomyces odontolyticus
Prevotella oralis
Streptococcus sanguinis
Atopobium parvulum
Dialister invisus
Streptococcus salivarius
Granulicatella adiacens
Gemella morbillorum
Slackia exigua
Veillonella parvula
Streptococcus constellatus
Solobacterium moorei
Streptococcus anginosus
Mogibacterium diversum
Selenomonas infelix
Streptococcus pseudopneumoniae
Prevotella melaninogenica
Fusobacterium nucleatum
Anaeroglobus geminatus
Streptococcus gordonii
*
1
0.8
0.6
0.4
0.2
0
C
H

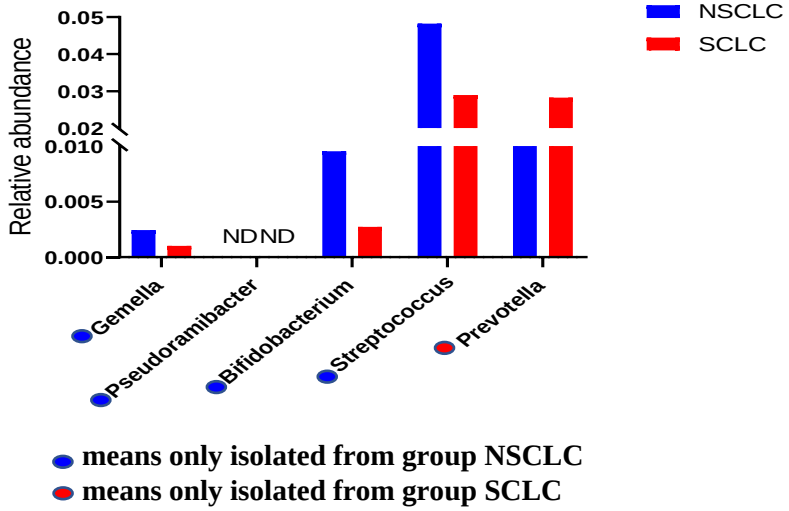
A


Relative Abundance
1
0.75
0.5
0.25
0
NSCLC
SCLC
Others
Porphyromonas
Agathobacter
Clostridium_sensu_stricto_1
Staphylococcus
Cutibacterium
Alloprevotella
Bifidobacterium
Anaerococcus
Cupriavidus
Turicibacter
Corynebacterium
Prevotella
Castellaniella
Neisseria
Escherichia-Shigella
Enterococcus
Acinetobacter
Diaphorobacter
Haemophilus
Erysipelothrix
Kroppenstedtia
Actinobacillus
Prevotella_7
Methyloversatilis
Carnobacterium
Veillonella
Brucella
Bacillus
Streptococcus
Pseudomonas

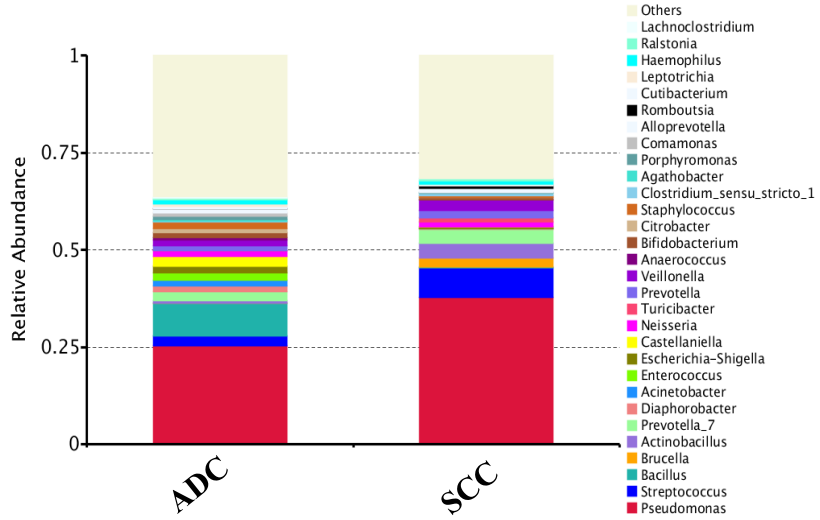
B


NSCLC
SCLC
77
81
37

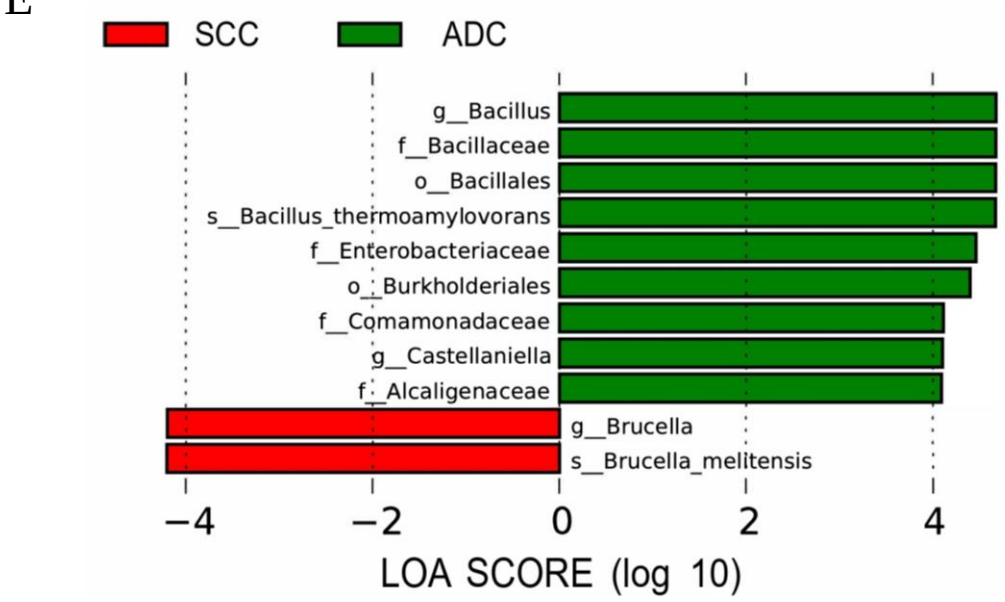
C


Relative abundance
0.05
0.04
0.03
0.02
0.010
0.005
0.000
ND ND
Gemella
Pseudoramibacter
Bifidobacterium
Streptococcus
Prevotella
NSCLC
SCLC
means only isolated from group NSCLC
means only isolated from group SCLC

D


Relative Abundance
1
0.75
0.5
0.25
0
ADC
SCC
Others
Lachnoclostridium
Ralstonia
Haemophilus
Leptotrichia
Cutibacterium
Romboutsia
Alloprevotella
Comamonas
Porphyromonas
Agathobacter
Clostridium_sensu_stricto_1
Staphylococcus
Citrobacter
Bifidobacterium
Anaerococcus
Veillonella
Prevotella
Turicibacter
Neisseria
Castellaniella
Escherichia-Shigella
Enterococcus
Acinetobacter
Diaphorobacter
Prevotella_7
Actinobacillus
Brucella
Bacillus
Streptococcus
Pseudomonas

E


SCC
ADC
g__Bacillus
f__Bacillaceae
o__Bacillales
s__Bacillus_thermoamylovorans
f__Enterobacteriaceae
o__Burkholderiales
f__Comamonadaceae
g__Castellaniella
f__Alcaligenaceae
g__Brucella
s__Brucella_melitensis
−4
−2
0
2
4
LOA SCORE (log 10)

Response to Reviewer comments

Reviewer #10 (Comments for the Author):

The manuscript titled, "Characterization of lung and oral microbiome in lung cancer patients by cultruomics and 16S rDNA sequencing" by Sun et al provides valuable insight about the bacterial populations in the lung and oral bacteriome in lung cancer patients. Overall, I firmly believe the study provides important information which can be translated to future animal models, and also pave the way for studies that may be use the identified bacterial species as biomarkers. The methods are also well explained and the bioinformatic tools were utilized well in the study. However, I have some major and minor comments for the authors to consider. I believe the manuscript will be much improved if the authors incorporate my suggestions.

Major comments

1. The authors use the word "cultruomics" throughout the Text, but I believe the word is "culturomics". Is it a typo throughout the manuscript? I think the authors must address this point, because the word is a part of the manuscript title.

Response: We apologize for the mistake. We agree with the reviewer's comment, and we have thoroughly checked and revised the manuscript.

2. I firmly believe the manuscript requires extensive English editing. I have come across numerous grammatical errors and typos. I have tried to highlight most of them in the minor comments, but still, I have the feeling that the text requires further editing.

Response: We apologize for the poor language of our manuscript. We have now worked on the language and have also involved native English speakers for language corrections.

3. In the discussion section, I think the authors should add some information about the bacterial species that were identified in the cancer patients. For instance, why Pseudomonas is such a potential threat in lung cancer, is it due to certain pathogenicity factors that are encoded by its genome? Similarly, I think adding some information about the other identified species will provide more rigor to the Discussion section.

Response: Thanks for the suggestion. We have added the information about the differential bacteria at species level in the discussion section, including *Pseudomonas aeruginosa* and *Parvimonas micra*, and *Brucella melitensis*. And we think combined the discussion of these species and our findings will provide a deeper insight for understanding the links between the species and the lung cancer.

4. Minor comments

• Line 25, mention the full name of BALF not just the abbreviation
• Line 26, "Twenty" not 20.
• Line 27, "BALF" instead of "BLAF".
• Line 31, mention the full name of "LEfSE".
• Line 33, "Prevotella Oralis" not "Prevotella orails"
• Line 39, "found" not "founded".
• Line 41, add "this study" before "provides"

• Line 56-58, should be rephrased.
• Line 67, add "an" instead of "the"
• Line 68 has some spacing issues.
• Line 81 again "BLAF".
• Line 87, add a full stop after 1
• Line 93, "Twenty five" instead of "25"
• Line 93, I think "from patients" is a mistake.
• Line 95, the word should be "transbronchoendoscopy"
• Line 98-99, replace ", total 21 patients' oral samples were collected" with "oral samples from 21 patients were collected".
• Line 113, "100 μL" not "100ul"
• Line 116, mention the full name of "MALDI-TOF MS".
• Line 118 has a strange spacing kindly check.
• Line 141 "raw" instead of "Raw".
• Line 143 add "were" after "sequences"
• Line 171, replace "were not" with "did not".
• Line 176, check if the word "cultruoims" is correct?
• Line 182, I think the exact values should be mentioned that are given in the Figure 2A.
• Line 188, you mention that 156 bacterial species were identified, but there is not mention of this in the Supplementary information.
• Line 204-205, its hard to understand the meaning, kindly rephrase.
• Line 220, add "of" after "prevalence"
• Line 240, "results" instead of "result"
• Line 241, "prevalence" not "prevalent"
• Line 242 , "Different pathological types shaped different pulmonary microbiomes" the name should be changed to "Different pathological strains reshape different pulmonary microbiomes"
• Line 318, again "BLAF"
• Line 325, add "prevalent" before "genus".
• Line 331, there is a double space before "Germ-free".
• Line 336, "bacteria" not "bacterial".
• Line 363, "procedures" not "Procedures".

Response: We are grateful to the reviewer for his remarks, and we have corrected all these errors in the revised manuscript.

Reviewer #12 (Comments for the Author):

The objective of this research aimed to analyze the lung and oral microbiome of patients with lung cancer.

1. There is a lack of information concerning the patients (oral pathologies?).
Response: Thanks for the suggestion. As our study focused on the microbiomes (both oral and pulmonary) of the lung cancer patients, we took into account the oral condition when setting the criteria for patient recruitment. The inclusion criteria had been added in the revised manuscript.

2. The conclusions must be accompanied by several reservations concerning the size of the sample, the natural biodiversity of the microbiome between patients.
Response: We thank the reviewer for his suggestion. We agree that the presence of natural biodiversity of the microbiome across individuals. We have added the reservations concerning the size of the sample in the discussion section as suggested . And we will call for larger and dynamic longitudinal studies in the future to verify the association between microbiome and lung cancer.

3. Comparing the microbiomes (oral and pulmonary) of the same patient from a cancerous lung area with another non-cancerous area of the same patient cannot be 100% reliable.
Response: We thank the reviewer for his relevant comment. We admit that spatial variation of microbiota within an individual is significantly less than variation across individuals in healthy lungs. However, the local tumor microenvironment of cancerous lung may be changed. We assume that the microbial differences between the cancerous lung and "healthy lung" may be associated with the tumor initiation and development. To date, two studies used high-throughput sequencing for characterizing the difference in microbiota compositions between cancerous and "healthy" lung. Zhuo et al found that the relative abundance of family Spiroplasmataceae, and its genus *Spiroplasma* was significantly increased in cancerous lung (Zhuo, M., et al., *Characterization of Microbiota in Cancerous Lung and the Contralateral Non-Cancerous Lung Within Lung Cancer Patients.* Frontiers In Oncology, 2020. 10: p. 1584)**. Liu et al found that the lower airway microbial communities of lung cancer were distinguishable from that of "healthy" controls** (Liu, H.-X., et al., *Difference of lower airway microbiome in bilateral protected specimen brush between lung cancer patients with unilateral lobar masses and control subjects.* International Journal of Cancer, 2018. 142(4): p. 769-778).

4 In the study, a significant segregation was found at the level of the genus, but without having sought the equivalent level at the species. The taxonomic levels of the genus are not the relevant biological measurement units for some authors (BIK, ME et al. Bacterial diversity in the oral cavity of 10 healthy individuals) 1nternational society for microbial ecology. (2010). However, in the mouth, the level of ecological interest chosen to label is the genus rather than species. There also reservations concerning the conclusions of the article are to be made.

Response: We are grateful to the reviewer for his suggestion. As the reviewer appointed, the 16S rDNA sequencing gave the bacterial identification at genus level, which is relatively accurate, as we compared to the results of culturomics, which gave the identification at species level. We simply compared them at genus level, indicating that only equivalent genus abundance corresponding to species obtained by the culturomics was considered. We have discussed this issue in the discussion section, according to the reviewer's suggestion, pointing out the limitation of this research.

4. Add Bibliography.

Ramirez-Labrada, AG et al. The influence of microbiota on lung carcinogenesis immunity and immunotherapy. Trends Cancer 2020. 6, 86-97.

Yagi, K.; Huffnagle, G.B.; Lukacs, N.W.; Asai, N. The Lung Microbiome during Health and Disease. Int. J. Mol. Sci. 2021, 22, 10872. https://doi.org/10.3390/ ijms221910872

Dickson, R.P.; Erb-Downward, J.R.; Martinez, F.J.; Huffnagle, G.B. The Microbiome and the Respiratory Tract. Ann. Rev. Physiol. 2016, 78, 481-504. [CrossRef]

Pizzo F. et al. Role of the microbiota in lung cancer insights on prevention and treatment.Int Mol Sci. 2022 23. (11) 6138.

Q. et al. Alterations of fecal bacterial communities in patients with lung cancer. Am J Transl 2018. 10 3171 -3185.

Georgiou K et al . Gut microbiota and lung cancer. Where Do we stand? Int J mol Sci 2021.

Goubet AG. Et al The impact of the intestinal microbiota in therapeuthic responses against cancer. Comptes Rendus Biol 2018. 341 284-289.

Segal LN et al. enrichment of the lung microbiome with oral taxa is associated with lung inflammation of Th17 phenotype. Nat Microbiol 2016.

Mortensen MS. The developing hypopharyngeal microbiota in early life. Microbiome 2016.

Das S. et al . A prevalent and culturable microbiota links ecological balance to clinical stability of the human lung after transplantation. Nat Commun 2021.

Response: Thanks for the suggestion. We have carefully read the bibliographies and added them in the manuscript.

Reviewer #20 (Comments for the Author):

This is an interesting study aimed at characterizing and associating the oral and pulmonary microbiome of lung cancer patients. However, the study has not considered that the oral microbiome depends mainly on the oral condition. It has been widely that high bacterial richness in the salivary microbiota is significantly associated with poor oral health, as indicated by decayed teeth, periodontitis, and poor oral hygiene. Therefore, it becomes crucial to understand oral microbial diversity and how it fluctuates under conditions of disease/disturbance. Advances in metagenomics and next-generation sequencing techniques generate rapid sequences and provide extensive information on the microorganisms inhabiting a niche. Therefore, the information retrieved can be used to develop microbiome-based biomarkers for use in the early diagnosis of oral and associated diseases. However, a homogenization of the oral clinical conditions of the samples must be considered for the results to be robust.

Response: Thanks for the suggestion. As our study focusing on the microbiomes (oral and pulmonary) of the lung cancer patients, we took into account the oral condition when setting the criteria for patient recruitment. All enrolled patients had not recently suffered from oral disease. We have added the inclusion criteria in the patient recruitment and samples collection section.

Reviewer #5 (Comments for the Author):

1. p should be represented properly in the manuscript as it is a statistical symbol.
Response: We are grateful to the reviewer for his remark. We have corrected in the manuscript.

2. Notch box plot should be used instead of box whisker plot.
Response: We appreciate the reviewer's suggestion. We have redesigned the figures as suggested.

3. The no of clinical samples may be increased.
Response: Thanks for the suggestion. We have added the reservations concerning the size of the sample in the discussion section as suggested. And we will call for larger and dynamic longitudinal studies in the future to verify the association between microbiome and lung cancer.

4. The authors should also comment on the keystone species and species diversity of each mirobiome and their changes.
Response: Thanks for the suggestion. We have added the information and changes of the keystone species in the discussion section, including *Pseudomonas aeruginosa*, *Parvimonas micra*, and *Brucella melitensis*.

April 3, 2023

Dr. Yujing Bi
Academy of Military Medical Sciences State Key Laboratory of Pathogen and Biosecurity
Beijing
China

Re: Spectrum00314-23R1 (Characterization of lung and oral microbiomes in lung cancer patients using culturomics and 16S rDNA sequencing)

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Characterization of lung and oral microbiomes in lung cancer patients using culturomics and 16S rDNA sequencing

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1. State Key Laboratory of Pathogen and Biosecurity, Beijing Institute of Microbiology and Epidemiology, Beijing 100071, China;
2. Department of thoracic oncology, Peking University Cancer Hospital, Beijing 100142, China

#These authors contributed equally to this work.

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Ruifu Yang (13801034560@163.com)
Yujing Bi (byj7801@sina.com);

Abstract:

Recently, microbiota dysbiosis in lung cancer has attracted immense attention. Studies on lung microbes are mostly based on sequencing, which has left the potentially functional bacteria with extremely low abundance uncovered. In this study, we characterized and compared the lung and oral cavity microbiota using culturomics and 16S rDNA sequencing. Of the 198 bacteria identified at the species level from bronchoalveolar lavage fluid (BALF) samples, Firmicutes was predominant (39.90%). Twenty bacterial species isolated from BALF samples were present in at least half of the patients and were also highly abundant in oral samples. Of all isolated strains, *Streptococcus* and *Veillonella* were highly dominant. The abundance of *Prevotella* and *Veillonella* decreased from the oral cavity to the lung, whereas that of *Pseudomonas* increased. Linear discriminant analysis effect size demonstrated that *Prevotella* was more abundant in the healthy samples than in the cancerous ones, which is in accordance with the isolation of *Prevotella oralis* only from the healthy group using culturomics. Moreover, *Gemella sanguinis* and *Streptococcus intermedius* were isolated only from the non-small-cell lung cancer (NSCLC) group, and 16S rDNA sequencing showed that they were higher in the NSCLC than in the small-cell lung cancer group. Furthermore, while *Bacillus* and *Castellaniella* were enriched in lung adenocarcinoma, *Brucella* was enriched in lung squamous cell carcinoma. Overall, alterations were observed in the microbial community of patients with lung cancer, whose diversity might be site and pathology dependent. Using culturomics and 16S rDNA amplicon sequencing, this study has provided insights into pulmonary and oral microbiota alterations in patients with lung cancer.

Importance: The relationship between lung microbiota and cancer have been explored based on DNA sequencing, however, culture-dependent approaches are indispensable for further studies on lung microbiota. In this study, we applied a comprehensive approach combining culturomics and 16S rDNA amplicon sequencing to detect microbiota from saliva and BALF samples of patients with unilateral lobar masses. We found alterations in the microbial community of patients with lung cancer, whose diversity might be site and pathology dependent. These features may be potential bacterial biomarkers and new targets for lung cancer diagnosis and treatment. In addition, a lung and oral microbial biobank from lung cancer patients

was established, which represents a useful resource for studies of host-microbe interactions.

Keywords：Microbiota, Lung cancer, BALF, Oral bacteria, Culturomics, 16S rDNA

Introduction

Lung cancer, the most common type of cancer worldwide, is closely associated with chronic inflammation [1]. Inflammation caused by microbial infections might contribute to cancer development and progression [2]. Polymorphic microbiomes have recently been added as one of the four new “hallmarks” of cancer [3]. Increasing evidence shows that the lung microbiome is involved in cancer pathogenesis.

Healthy lungs, conventionally considered to be sterile, are now known to harbor diverse microbiota [4]. To date, several culture-independent analyses have reported the association between microbial population diversity and lung cancer [5, 6]. Lung tissue samples of lung cancer patients demonstrated an increase in α-diversity and phylum Bacteroidetes compared with lung tissue samples from emphysema patients [7]. A study found that the genus *Streptococcus* was more abundant in the bronchoalveolar lavage fluid (BALF) samples of patients with lung cancer than in those of healthy controls [8]. Few studies have shown the association between lung bacteria and the histological subtypes of lung cancers; the genera *Veillonella, Megasphaera, Enterobacter, Morganella*, and *Klebsiella* were significantly higher in lung adenocarcinoma (ADC) than in lung squamous cell carcinoma (SCC) [9, 10]. As the oral cavity is the entry point to the respiratory tract, its microbiome may contribute to lung cancer [11]. Tsay et al. reported that lower airway dysbiosis induced by microaspiration of oral commensals promotes an interleukin-17-driven inflammatory phenotype, which exacerbates lung tumorigenesis [12]. *Sphingomonas* and *Blastomonas* were shown to be relatively more abundant in the oral microbiome of lung cancer patients [13]. However, the possible variations in the oral and lung microbiota of lung cancer patients and the differences in the microbial diversity of their saliva and BALF samples remain unclear.

While numerous DNA sequencing-based investigations have explored the relationship between lung microbiota and cancer, it has several inherent drawbacks, such as depth bias and a high detection threshold [14]. Therefore, culture-dependent approaches are indispensable for further studies on lung microbiota. Culturomics, in which multiple culture conditions are combined for rapid identification, provides new perspectives on host–bacteria relationships [15]. However, the use of culturomics for the culture and identification of BALF bacteria has rarely been reported.

In this study, we applied a comprehensive approach combining culturomics and

16S rDNA amplicon sequencing to the saliva and BALF samples from 25 patients with unilateral lobar masses. To the best of our knowledge, this is the first study reporting the bacterial diversity and richness in the oral and BALF microbiota of patients with lung cancer, using culturomics and 16S rDNA sequencing.

Materials and methods

1. Patient recruitment and sample collection

This study was approved by the Institutional Review Board of Peking University School of Oncology, China, and informed consent was obtained from all participants. The BALF samples were collected as previously described [16]. The saliva samples were collected before the patients underwent bronchoscopy examination. All the participants were instructed to not eat and drink for 1 h prior to saliva sample collection. Twenty-five patients with unilateral lobar masses who consented to undergo bronchoscopic examination at Peking University Cancer Hospital were enrolled. All patients underwent transbronchoendoscopy to avoid contaminating the upper respiratory tract or oral microbiota, and paired BALF samples (one each from the cancerous site (C) and the contralateral healthy lung (H)) were collected before the operation. None of the participants had recently suffered from oral disease. Before the bronchoscopy, oral samples from 21 patients were collected. The sample was divided into two parts: one part (including 45 samples from 15 lung cancer patients) was used for culturing bacteria, and the other part (all of the 71 samples) was used for 16S rDNA amplicon sequencing. Fresh samples were collected in a sterile tube, placed on ice, and transported to the laboratory within 1 h. Culturomics was performed immediately after sample collection in the laboratory, and the aliquots were stored at −80°C before high-throughput sequencing.

2. Culturomics

2.1. The process of culturomics

Culturomics is a high-throughput method that multiplies culture conditions to detect high bacterial diversity and pure bacterial cultures. This analysis involved preculturing under different conditions: aerobically supplemented with 5% sheep

blood; aerobically supplemented with 5% rumen fluid; anaerobically with 5% sheep blood; anaerobically with 5% rumen fluid. Sample dilution, strain isolation, and identification were performed as previously described [17]. On days 1, 3, 6, 9, 15, and 30, the enriched culture samples were extracted from the bottles using a syringe. Subsequently, 100 μL doubling dilutions were spread onto Columbia agar supplemented with 5% sheep blood at 37℃ under aerobic conditions for 24 h or anaerobic conditions for 72 h. Colonies were picked and identified using matrix-assisted laser desorption ionization-time-of-flight mass spectroscopy (MALDI-TOF MS) systems (Autof MS1000). Colonies that could not be identified using the MALDI-TOF MS database V1.1.12 (score < 9) were subjected to 16S rDNA gene sequencing with primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-GGTTACCTTGTTACGACTT-3′). The sequencing results were analyzed using the NCBI BLAST algorithm for homologous sequence searches with type strains. If the 16S rDNA sequence is <98.65% similar to the closest type strain, the isolate might be a new species [18].

2.2. Classification of cultivated species

We classified all isolates into four categories: oral/respiratory (this study and [19]), gut [20], urine [21, 22], and vagina [23, 24]. We also performed literature searches on PubMed to compare our results with the published data to confirm the classification.

3. DNA extraction, 16S rDNA amplification, and sequencing

DNA was extracted from each sample using a Hipure Bacterial DNA kit (Mageon, China) according to the manufacturer's instructions. The V3–V4 region of the 16S rDNA gene was amplified using specific primers (341F: 5′-CCTAYGGGRBGCASCAG-3′; 806R: 5′-GGACTACNNGGGTATCTAAT-3′). Sequencing libraries were generated usingTruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, USA) following manufacturer's recommendations and index codes were added. The library quality was assessed on the Qubit@ 2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system. The sequencing was sequenced on an Illumina Novaseq6000 sequencing platform (Illumina, San Diego, CA, United States) and 250 bp paired-end reads were generated.

4. Sequence data analysis

Raw reads were filtered to remove adaptors, low-quality reads, and ambiguous bases. Clean data was extracted from the raw data using USEARCH 8.0. The operational taxonomic units (OTUs) were classified based on 97% similarity after chimeric sequences were removed using UPARSE (version 7.0.1001 http://drive5.com/uparse/), and the representative sequence from each OTU cluster was obtained. α diversity was assessed using the nonparametric Shannon and Simpson indices. β diversity was calculated using Bray–Curtis distances in QIIME and visualized by principal coordinate analysis (PCoA). Linear discriminant analysis effect size (LEfSe) was used to detect taxa with differential abundance among groups. The Metastats analysis was employed to detect the differences in the microbiota composition at the genus level.

5. Statistical analyses

Statistical analyses were performed using the R software (v3.4.10) and SPSS 20.0. Independent t- and chi-square tests were used to analyze the demographic and clinical data. Fischer's exact test with false discovery rate (FDR) correction was used to compare frequency of isolated species between groups. Sample diversity metrics were assessed based on the nonparametric Shannon index and Chao1 index. PCoA plots were generated to visualize the separation of samples based on pairwise distances, and ANOSIM was performed to evaluate the statistically significant difference in PCoA analysis. p-value < 0.05 was considered statistically significant.

RESULTS

1. Clinical information

We recruited 25 patients with unilateral lobar masses from Beijing Peking University Cancer Hospital between January 2021 and May 2022, of which 23 were newly diagnosed with lung cancer via histological confirmation but 2 were not. There were 16 non-small-cell lung cancer (NSCLC) patients, including 8 ADC, 7 SCC, and one with non-specified NSCLC, and 7 small-cell lung cancer (SCLC) patients. None previously had received any anticancer therapy, radiation therapy or any antibiotics treatment. Of the 23 patients with lung cancer, 14 were men and 9 were women.

Furthermore, 18 were smokers. There were 17 and 6 patients with and without distant metastasis, respectively (Table 1).

2. Characteristics of bacteria isolated from the lung and oral cavity via culturomics

Figure 1 shows the culturomics workflow. Briefly, total 45 samples were collected from 15 lung cancer patients, and four culture conditions were tested for each sample. We obtained 12379 colonies from the BALF samples (both C and H samples), and 198 bacteria were identified at the species level using MALDI-TOF or 16S rDNA gene sequencing. The identified species belonged to six phyla, including Firmicutes (39.90%), Proteobacteria (27.78%), Actinobacteria (19.19%), Bacteroidetes (9.60%), Fusobacteria (2.02%), and Synergistetes (1.52%) (Figures 2A and B). Comparison with the previously established repertoire of microorganisms isolated from the human gut, urine, vagina, and oral/respiratory tract revealed that approximately 1/4th of the species isolated in this study were previously isolated from these four sites (47/198, 23.7%) (Figure 2C).

We obtained 5671 colonies from the oral samples and identified 156 bacterial species (Fig. S1). At the phylum level, the bacterial diversity of the oral sample was similar to that of BALF, consisting predominantly of Firmicutes and Proteobacteria (>66%). In addition to the previously known bacteria, we isolated 15 potentially new species (Table S1).

3. Comparison of microbiota in lung and oral cavity

We compared the microbiota composition between the C and H lung sites and the oral cavity (O) using 16S rDNA amplicon sequencing. There was a noticeable difference in the microbiota composition between the lung and oral cavity. In the lung samples, we observed a moderate difference between the C and H samples at the phylum level (Figure 3A). The three dominant phyla were Proteobacteria, Firmicutes, and Bacteroidetes. At the genus level, *Pseudomonas* (Proteobacteria), *Streptococcus* (Firmicutes), *Veillonella* (Firmicutes), and *Prevotella*_7 (Bacteroidetes) were the most common ones in the BALF samples. Contrastingly, *Prevotella*_7 (Bacteroidetes), *Neisseria* (Proteobacteria), *Streptococcus* (Firmicutes), *Veillonella* (Firmicutes), and *Haemophilus* (Proteobacteria) were the most common ones in the oral samples

(Figure S2A). We did not observe any significant difference in the richness or diversity of the microbial community (α diversity) between the BALF samples from C and H sites, as measured using the Shannon index (p = 0.527) and Chao1 index (p = 0.428). Moreover, there was no difference in the overall microbiota (β diversity) between the C and H groups, as measured using the Bray–Curtis distances (p = 0.39) (Figure S3). Nevertheless, the oral sample was significantly different from both the C and H lung samples in α and β diversity (Figures 3B, 3C). Metastats analysis at the genus level further revealed unique anatomy-related microbial features, such as higher abundance of *Prevotella* in oral samples (Figure 3D). *Pseudomonas* was the only genus concentrated in BALF samples (Figure 3D, S2B).

Furthermore, we compared the bacteria isolated via culturomics among different groups. Unlike the sequencing data, there was no obvious difference in the bacterial proportion at the phylum level among C, H, and O samples, with Firmicutes being dominant in all three sites (Figure 3E). More than half of the species (89) were isolated in all three sites, and 34, 32, and 39 unique species were isolated from the C, H, and O groups, respectively (Figure 3F). In addition, we analyzed the prevalence of bacteria in the lungs and oral cavity. Based on the culturomics results, we defined the strains isolated from over 50% of the patients as prevalent strains. Twenty prevalent strains were identified from the BALF samples, which belonged to 12 genera (Table 2). *Streptococcus* was the major genus, and *Streptococcus oralis*, *Veillonella atypica*, *Parvimonas micra,* and *Actinomyces odontolyticus* were found in almost all BALF samples. These 20 species were also cultured at a high frequency from oral samples, which indicated that the pulmonary microbiota might originate from the oral cavity.

4. Differences in the microbiota composition between cancerous and healthy lungs

To compare the relative contribution of different taxa, we used the LEfSe to detect taxa with differential abundance between the two groups We identified 14 different taxa at various levels with significant abundances across the two groups, of which four were differentially abundant at the genus level. *Prevotella* and *Prevotella_7* were more abundant in the H group, whereas *Carnobacterium* and *Brucella* were higher in the C group (Figure 4A).

Moreover, we compared the differences between the H and C groups of bacteria

obtained via culturomics. We drew a heat map of the proportion of each bacterium in the total sample. *Streptococcus oralis*, *Veillonella ayptica*, and *Parvimonas micra* were cultured at a high frequency without significant differences. A significantly higher frequency of *Prevotella oralis* was found in the H group (p = 0.019) (Figure 4B), which agreed with the sequencing results.

5. Different pathological strains reshape different pulmonary microbiomes

We examined the differences in the lung microbiota of NSCLC and SCLC groups with comparable basic information, including age, sex, smoking status, and distant metastasis (Table S2). Bacteria with an abundance of >4% were considered dominant. *Pseudomonas* (32%), *Streptococcus* (5%), and *Bacillus* (4%) were the dominant genera in the 32 NSCLC lung samples. While in SCLC, the dominant genera were *Pseudomonas* (31%), *Veillonella* (7%), and *Prevotella_7* (6%) (Figure 5A). The α diversity of NSCLC was not significantly different from that of SCLC, as measured using the Shannon index (p = 0.931) and Chao1 index (p = 0.72), or β diversity, as measured using the Bray–Curtis distances (p = 0.489) (Figures S4 A, B). The culturomics study revealed that 77 and 33 species were isolated only from NSCLC and SCLC lung samples, respectively (Figure 5B). Only those bacterial species isolated from at least half of the patients in the group were compared. Later, we analyzed the bacteria isolated from only one group with an isolation rate of ≥50%. *Gemella sanguinis*, *Pseudoramibacter alactolyticus*, *Bifidobacterium dentium*, and *Streptococcus intermedius* were the four species isolated with a relatively high frequency only from the NSCLC group. *Prevotella pallens* was the only species isolated at a relatively high frequency from the SCLC lung samples. Subsequently, we compared the relative abundance of these five bacteria at the genus level using 16S rDNA amplicon sequencing. Although there was no significant difference between the two groups, their abundance tended to be higher in the site from which they were isolated (Figure 5C).

In the subtype analysis of patients with NSCLC, *Pseudomonas* (26%) and *Bacillus* (8%) were dominant in the 16 ADC samples. Contrastingly, in the 14 SCC samples, the dominant genera were *Pseudomonas* (38%) and *Streptococcus* (8%)

(Figure 5D). Shannon index (p = 0.27) and Chao1 index (p = 0.402) were not significantly different between the ADC and SCC groups. However, β diversity showed that the microbiota constitution of the ADC lung samples was significantly different from that of the SCC samples (p = 0.021) (Figures S4 C, D). In the LEfSe analysis of ADC and SCC, 11 taxa displayed contrasting correlations between the NSCLC subtypes. A differential abundance analysis at the genus level between ADC and SCC showed enrichment of *Bacillus* and *Castellaniella* in patients with ADC, whereas those with SCC had a higher abundance of *Brucella* (Figure 5E).

Discussion

The relationship between microbiota and cancer is being extensively investigated [25]. Accumulating evidence indicates that gut microbiota contributes to carcinogenesis and response to immunotherapy [25, 26]. While many studies have focused on analyzing the influence of gut microbiota, its composition differs substantially from that of the lung microbiota [27, 28]. Although microaspiration commonly occurs in healthy subjects, it is more frequent in those with chronic inflammatory airway diseases [29, 30]. The entry of more oral microbes into the lungs is associated with the increased release of lung proinflammatory cytokines and a proinflammatory phenotype characterized by elevated Th-17 lymphocytes [31, 32]. Lung microbiota dysbiosis might modulate the risk of malignancy at multiple levels, including chronic inflammation and activation of oncogenes [33, 34]. Although the lung microbiome has been implicated in lung cancer in several specific ways, its exact role in carcinogenesis has not yet been elucidated. One of the possible reasons for this is the current studies are mostly based on the sequencing levels, which results in a lack of strains materials for further study. Here, we utilized both culturomics and 16S rDNA amplicon sequencing to evaluate and compare the structure and diversity characteristics of oral and pulmonary microbiota associated with lung cancer.

Culturomics can identify bacteria at strain level, and successfully isolating living bacteria is crucial for carrying out subsequent experimental work [14, 35]. A previous study summarizing a list of microbes isolated from the human lower respiratory tract showed that the lung microbiome was dominated by the phyla Pseudomonadota, Firmicutes, Bacteroidota, and Actinomycetota, which was consistent with our study [19]. Here, we cultured 198 identified bacterial species from human BALF and 156

from oral samples of lung cancer patients, including 15 potentially new taxa. We have submitted the article describing the novel bacteria, new.4 and new.10, belonging to a novel genus, which we named "*Curtanaerobium*," to *International Journal of Systematic and Evolutionary Microbiology* (under review). The present study has enabled us to expand the human respiratory and oral repertoire. We also found 20 prevalent strains in both BALF and oral samples of patients with lung cancer, which indicates that the pulmonary microbiota might originate from the oral cavity. Culturomics might reduce the number of these unclassed or no-rank OTUs by increasing the number of pure-cultured microbial species. In our study, *Pseudoramibacter alactolyticus* was recovered with a relatively high frequency only from the NSCLC group but was not detected in 16S rDNA amplicon sequencing.

Parvimonas micra is an opportunistic pathogen that is frequently associated with several human infections and also causes purulent infections in multiple organs [36, 37]. *P. micra* is highly abundant in patients with colorectal cancer and promotes colorectal tumorigenesis by inducing colonocyte proliferation and altering the Th17 immune response [38, 39]. Our previous results showed that *P. micra* promoted colorectal cancer progression by upregulating miR-218-5p expression and ultimately activating the Ras/ERK/c-Fos signaling pathway [40]. However, its correlation with lung cancer has not yet been reported. In this study, *P. micra* was cultured at a high frequency in patients with lung cancer, which provides valuable information for further studies.

We analyzed the microbiota composition at the genus level via taxonomic analysis, and the results showed that the representative flora differed based on the sampling site. We found that *Streptococcus*, *Veillonella*, and *Prevotella* were enriched in the oral samples, whereas *Pseudomonas* was enriched in the BALF samples. S*treptococcus* and *Veillonella* which were considered as oral commensals were reported to increase in lower airways of lung cancer patients [41]. *Pseudomonas* species, commonly found in the respiratory tract, are involved in the pathogenesis of lung diseases, such as chronic obstructive pulmonary disease and cystic fibrosis [42, 43]. A study demonstrated that transplanted lungs are susceptible to the growth of multiple *Pseudomonas* species [44]. *Pseudomonas aeruginosa* possesses numerous virulence factors that attack the respiratory epithelial cells, such as the type III secretion system [45]. *P. aeruginosa* possesses pili and flagella that are necessary for motility and respiratory infection as they enable attachment to the respiratory

epithelium via respiratory mucins and the glycolipid asialoGM1 [46]. A study reported that *P. aeruginosa* was significantly more abundant in NSCLC patients with brain metastasis than in those without metastasis [47]. Likewise, most of the patients in our study had distant metastasis, and *Pseudomonas* was the most prevalent genus in the BALF samples. These findings, including ours, suggest that *Pseudomonas* frequently colonizes the respiratory tract of patients with lung cancer and might promote distant metastasis.

Based on 16S rDNA sequencing, we found that *Brucella melitensis* was more enriched in the cancerous lung than in the healthy lung. The *Brucella* genus includes several significant human and veterinary pathogens, which predominantly maintain an intracellular lifestyle in their mammalian hosts. These pathogens exhibit numerous pathogen-associated molecular patterns that can be recognized by innate receptors in airway epithelial cells [48]. Their type IV secretion system secretes effector molecules into the host cell cytoplasm, which direct the intracellular trafficking of the brucellae and modulate host immune responses [49]. Thus, we speculated that *Brucella melitensis* is a possible lung cancer biomarker.

Gemella sanguinis, *Pseudoramibacter alactolyticus*, and *Streptococcus intermedius* are involved in human inflammatory diseases, such as endocarditis [50], periodontal infections [51], and brain abscess [52]. Germ-free mice or mice treated with antibiotics had significantly lower incidence of lung cancer than specific pathogen-free mice [53, 54]. Our results showed that these three species were isolated only from the lung microbiota of patients with NSCLC and not from those with SCLC. This observation was consistent with the results of 16S rDNA amplicon sequencing, which indicated that specific bacteria might mediate the development of NSCLC by inducing chronic inflammation.

When categorizing based on the lung cancer subtypes, *Bacillus* and *Castellaniella* were significantly enriched in the BALF samples of patients with ADC, whereas *Brucella* was enriched in the SCC group. Another study reported the enrichment of *Acinetobacter* in BALF samples of patients with ADC, whereas *Bacillus* and *Brucella* showed no significant differences between the ADC and SCC groups [10]. These conflicting associations of microbiomes with different pathological subtypes could be attributed to the significant variations across individuals and sampling methods.

The lung microbiome is similar to other microbiomes from other parts of the

human body, and there are significant individual differences. Although we screened some differential microorganisms via culture or sequencing, the sample size was small, and more clinical samples are needed for verification. The resolution of 16S rDNA sequencing is limited since only bacterial identification at genus level was relatively accurate, causing our comparison between the two methods with only genus information, giving up the species information from culturomics method. Metagennomic sequencing combined with culturomics should be preferable for the parallel comparison at the species level. Moreover, the causal relationship between the screened microorganisms and the occurrence and development of lung cancer remains uncertain, and follow-up studies are also required.

In conclusion, we examined the pneumonic and oral microbiota of lung cancer patients using culturomics and 16S rDNA sequencing. We found alterations in the microbial community of patients with lung cancer, whose diversity might be site and pathology dependent. Using culturomics, we found that *Streptococcus* and *Veillonella* were highly dominant in both pneumonic and oral samples of patients with lung cancer, which suggests the possible deleterious effects of airway microbial dysbiosis originating from the oral cavity. We showed that *Prevotella oralis* was isolated only from the H group and that *Gemella sanguinis* was isolated only from the NSCLC group, which was consistent with the findings of 16S rDNA sequencing. This study provides basic data on the microbiota diversity in pneumonic and oral samples from patients with lung cancer. These might serve as potential bacterial biomarkers and new targets for lung cancer diagnosis and treatment, and the causative relationships can be explored using these isolated strains.

AUTHOR CONTRIBUTIONS

Ethics approval and consent to participate

All procedures performed in studies involving human participants or animals were approved by the Institutional Review Board of the Peking University School of Oncology (No. 2018KT89). All patients gave their written consent before inclusion in the study.

Consent for publication

Not applicable.

Data availability

All the data generated or analyzed in this study are included in this published article (or its Supplementary Information files). The read sequences obtained from Illumina NovaSeq were submitted to the NCBI Sequence Read Archive (SRA) under accession number PRJNA904049 (BioProject ID) (http://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA904049).

Competing Interest

The authors declare no competing interests.

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Authors' contributions

Y.J.B. designed research and project outline. R.F.Y and Z.P.W. directed the research. Y.F.S, J.J.L., Y.J.L., A.T.T, Z.M.L, M.M.L, J.B, and Z.H.W. performed isolation, deposition and identification. Y.F.T. and Z.Y.P. performed genome analysis. Y.F.S. and Y.J.B. drafted the manuscript. All authors read and approved the final manuscript.

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Fig 1. Summary of culturomics methods and workflow.

Fig 2. Bacteria identified from the BALF samples. **A)** Phylogenetic tree and **B)** proportion of 198 bacterial species isolated from the BALF samples listed according to their phylum. **C)** UpSet plot showing the shared cultured species among human oral/respiratory, gut, urine, and vagina.

Fig 3. Variations in microbial composition in different anatomical sites. **A)** Taxonomic composition at the phylum level in BALF and oral samples based on 16S rDNA amplicon sequencing (Top 30). **B)** The Shannon diversity index of BALF and oral samples. *p* values were calculated using the Wilcoxon test. *$p < 0.05$. **C)** PCoA analysis of samples from three different anatomical site in patients with lung cancer. ANOSIM was performed to statistically evaluate significant difference. **D)** Notched box plots illustrating the differences of the significantly 2 genus relative abundances in three different anatomy sites. * $p < 0.05$. **E)** Proportions of bacterial species isolated from C, H, and O samples listed according to their phylum. **F)** Venn diagram of the culturable bacterial species from the three different anatomical sites. Abbreviations: C, H, O: samples from the cancerous site and contralateral healthy controls from lungs and the oral site, respectively, of patients with lung cancer.

Fig 4. Differentially abundant taxonomy between cancer and paired healthy lung. **A)** LEfSe was used to identify the bacterial microbiota that significantly differed between the cancerous and healthy lung. Only taxa meeting a significant LDA threshold value of >3.5 and $p < 0.05$ are shown. **B)** Heatmap analysis of lung microbiota at C and H sites of the patients with lung cancer based on culturomics (n = 15). Each row represents an individual species with the higher isolated rate in the two groups. Top 25 bacterial species were recovered from either group. Transition from blue to red represents the frequency of culturomics recovery from 0 to 1. Statistical comparisons were made using the Fischer's exact test with FDR correction. * $p < 0.05$

Fig 5. Characterization of lung microbiota in different lung cancer subtypes. **A)** Taxonomic composition at the genera level in NSCLC and SCLC lung samples. **B)** Venn diagram of the culturable bacterial species isolated only from NSCLC and only from SCLC lung samples. **C)** Differences in the relative abundance of the genera corresponding to the species isolated from NSCLC and SCLC samples. Differential microbial taxa were identified using paired t-test, and the *p*-values were adjusted for multiple comparison using the FDR. **D)** Taxonomic composition at genera level in ADC and SCC lung samples. **E** The LEfSe was used to identify the bacterial microbiota that significantly differed between NSCLC and SCLC. Only taxa meeting a significant LDA threshold value of >4 and $p < 0.05$ are shown.

Table 1. Clinical characteristics of patients.

Variable	NSCLC	SCLC	Non
N	16	7	2
Age-mean (SD)	65 (7.0)	67 (12.8)	69 (8.2)
Gender			
Male, n (%)	9 (56%)	5 (72%)	2
Female, n (%)	7 (44%)	2 (14%)	0
Smoking			
Current or former Smoker, n (%)	12(75%)	6(86%)	1
Never smoker, n (%)	6(25%)	1(14%)	1
Pathological diagnosis			
Adenocarcinoma, n (%)	8(50%)	——	——
Squamous cell carcinoma, n (%)	7(44%)	——	——
Unidentified	1(6%)	——	——
Distant			
MO	4(25%)	2(29%)	——
M1	12(75%)	5(71%)	——

Table 2. Summary of the proportion of the TOP20 bacterium in BALF samples and the related proportion in oral cavity.

Genera	Species	C	H	O
Streptococcus	***Streptococcus salivarius***	60.00%	60.00%	73.33%
	Streptococcus pseudopneumoniae	53.33%	40.00%	33.33%
	Streptococcus parasanguinis	93.33%	86.67%	100.00%
	Streptococcus oralis	93.33%	93.33%	100.00%
	Streptococcus mitis	93.33%	86.67%	100.00%
	Streptococcus gordonii	53.33%	53.33%	80.00%
	Streptococcus constellatus	66.67%	73.33%	93.33%
	Streptococcus anginosus	66.67%	66.67%	100.00%
Veillonella	***Veillonella parvula***	60.00%	60.00%	73.33%
	Veillonella atypica	66.67%	80.00%	66.67%
Solobacterium	***Solobacterium moorei***	66.67%	60.00%	73.33%
Slackia	***Slackia exigua***	60.00%	73.33%	60.00%
Parvimonas	***Parvimonas micra***	80.00%	80.00%	93.33%
Mogibacterium	***Mogibacterium diversum***	53.33%	46.67%	53.33%
Granulicatella	***Granulicatella adiacens***	66.67%	53.33%	46.67%
Gemella	***Gemella morbillorum***	66.67%	53.33%	66.67%
Fusobacterium	***Fusobacterium nucleatum***	40.00%	66.67%	80.00%
Dialister	***Dialister invisus***	73.33%	53.33%	73.33%
Anaeroglobus	***Anaeroglobus geminatus***	53.33%	60.00%	73.33%
Actinomyces	***Actinomyces odontolyticus***	80.00%	80.00%	66.67%

Abbreviations: C, H, O: samples from the cancerous site and contralateral healthy controls from lungs and the oral site, respectively, of patients with lung cancer.