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Optimization of lung tissue pre-treatment by bead homogenization for subsequent culturomics

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The discovery that the lung harbors a diverse microbiome, as revealed by next-generation sequencing, has significantly altered our understanding of respiratory health and disease. Despite the association between the lung microbiota and disease, the nature of their relationship remains poorly understood, and culture isolation of these microorganisms could help to determine their role in lung physiology. Current procedures for processing samples from the lower respiratory tract have been shown to affect the viability of microorganisms, so it is crucial to develop new methods to improve their survival. This study aimed to improve the isolation and characterization of lung microorganisms using a bead-beating homogenization method in a mouse model. Microsphere diameter and bead-beating time affected the survival of the microorganisms (*E. coli*, *S. aureus* and *C. albicans*). Using 2.3 mm diameter microspheres for 60 s of bead-beating promoted the survival of both bacteria and yeast strains. After intratracheal instillation of these microorganisms in mice, approximately 70% of the cells were recovered after the tissue homogenization. To assess the efficiency of the proposed method, the diversity of bacteria was compared between the homogenate and lung tissue samples. Ninety-one genera were detected in the lung tissue, and 63 in the homogenate. Bacterial genera detected in the homogenate represented 84% of the total abundance of the microbiota identified in the lung tissue. Taken together, these results demonstrate that the tissue homogenization process developed in this study recovered the majority of the microorganisms present in the lung. This study presents a bead-beating homogenization method for effective cultivation of lung tissue microorganisms, which may help to improve the understanding of host–microbe interactions in the lung.

Keywords Microbiota, Next-generation sequencing, Microspheres, 16S rRNA gene, Lung

Abbreviations

NGS	Next-generation sequencing
COPD	Chronic obstructive pulmonary disease
BHI	Brain heart infusion broth
ASVs	Amplicon sequence variants

Since the lung is no longer considered sterile, there has been great research interest in studying its microbiome. Next-generation sequencing (NGS) has revealed that the microbial community inhabiting the lung is more complex than previously thought, consisting of different phyla, such as Firmicutes, Bacteroidetes, Proteobacteria, Fusobacteria and Actinobacteria[1–5]. While these studies confirmed the presence of facultative anaerobic microorganisms such as *Pseudomonas* sp., *Haemophilus* sp., and *Streptococcus* sp., they also revealed the presence of strict anaerobic bacteria, including *Veillonella* sp., *Prevotella* sp., and *Porphyromonas* sp[1–5]. Changes in the composition of the lower respiratory tract have been observed in several respiratory diseases, including chronic

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obstructive pulmonary disease (COPD), asthma, and lung cancer, suggesting a possible role of these microbes in their development and/or severity[1–5]. However, little is known about the relationship between the lung microbial community and disease. Studies have suggested that lung-dwelling microbes can influence the host immune system and subsequently shape local inflammatory responses[6].

However, investigating the lung microbiome often requires invasive studies that can raise ethical concerns in human research. Animal models provide an opportunity to conduct mechanistic studies that are challenging to perform in humans[7]. Additionally, the airway microbiome is undoubtedly influenced by the environment[8], which is easier to control in animal experiments than in human studies. Overall, murine models of the lung microbiome have great potential to elucidate the causal relationship between the microbiome and disease[7]. Understanding these interactions is crucial for advancing our knowledge of respiratory disease pathophysiology and discovering new therapeutic targets. Culture isolation of these microorganisms could help to determine their role in lung physiology.

Lung tissue samples are among the most valuable samples for characterizing the lower respiratory tract microbiome. However, recovery of microorganisms from tissues is challenging because the methods commonly used to process low airway samples can affect microbial survival. In fact, all tissue homogenization methods based on sonication, pressure cycling technology, liquid nitrogen treatment, osmotic shock, detergents or enzymes affect the viability of microorganisms present in the tissue[9,10]. Finally, mechanical tissue lysis remains the best alternative for maintaining microbial viability[11].

Bead-beating homogenization is one of the most useful method for tissue disruption methods[9]. It consists of shaking the tube containing the tissue at high speed in presence of zirconium microbeads, which causes its mechanical disruption and subsequent release of tissue-attached microorganisms[12]. However, the viability of microorganisms has been shown to depend on the diameter of the microspheres and the bead-beating time[11,13]. In this study, we propose to develop a tissue homogenization process based on the bead-beating method to characterize and isolate the microbial community inhabiting the lung using mice as a model.

Methods

Microbial strains and culture media

Planktonic cultures of microorganisms were subjected to bead beating homogenization, and the effect of this process on microbial viability was evaluated. Suspensions of *Staphylococcus aureus* ATCC25923, *Escherichia coli* (previously isolated in the laboratory) and *Candida albicans* ATCC29 strains were prepared in brain heart infusion broth (BHI, MilliporeSigma®, Germany) or Spider medium (for bacteria and yeast, respectively). Prior to each experiment, concentrations were checked by light absorbance and plating of serial dilutions.

The homogenates obtained from mouse tissues were cultivated in BHI broth supplemented with or without mucin under anaerobic and aerobic conditions. The growing microorganisms were subsequently identified by sequencing their entire 16S rRNA gene.

Bead-beating homogenization protocol

Planktonic cultures collected during the exponential growth phase (10^5 CFU in 1 mL) were homogenized using either 0.1 or 2.3 mm beads (zirconium/silica, BioSpec®, USA) for 30, 60 or 180 s. The beads were sterilized by autoclaving and UV exposure prior to each experiment, and the bead-beating homogenization process was performed in a Mini-Beadbeater 16 homogenizer (BioSpec®, USA). One hundred microliters of each of the processed and unprocessed (control) suspensions of *E. coli*, *S. aureus* and *C. albicans* were plated in triplicate on MacConkey, mannitol salt, and Spider agar respectively, since these media are considered optimal for cultivating these microorganisms. Subsequently colonies were enumerated after overnight incubation. Three replicates of the experiment were performed for each culture. To evaluate the effect of the bead-beating homogenization process on the survival of microorganisms, the CFU concentrations of processed and control microbial suspensions (determined after overnight incubation) were compared[14].

Intra-tracheal instillation of microbes in mice.

All animal experiments were approved by the Ethics Committee of the Faculty of Medicine of the University of Chile (No. 1067) and all methods were carried out in accordance with relevant guidelines and regulations. The study report follows recommendations in the ARRIVE guidelines.

To determine the influence of lung tissue on bacterial viability during processing, BALB/c mice (n = 3) were intratracheally instilled with a "pool" of microorganisms (*E. coli*, *S. aureus* and *C. albicans*) in BHI broth. As a control, BALB/c mice (n = 3) were administered BHI broth only. An amount of 10^5 CFU was instilled into mice, because the concentration of bacterial communities inhabiting the lung is close to 10^3–10^5 bacteria per gram of lung[15,16].

Previous studies using intra-tracheal instillation have shown that this procedure results in a homogeneous distribution of the administered solution in the lungs, with less variability between treated animals[17]. Animals were anesthetized with 2–3% isoflurane/oxygen mixture and a cannula was subsequently inserted into the trachea through the mouth using an operating otoscope (Welch Allyn Li-Ion Handle, Hallowell®, USA). A 30-µL volume of the microbial "pool" or BHI vehicle (control) was administered through the cannula. Mice were euthanized by cervical dislocation in deep anesthesia (Ketamin 150 mg/kg and Xylazin 15 mg/kg) and the lungs removed by surgery. After washing in PBS (pH 7.5), the lungs were cut into small pieces under sterile conditions and stored in a cryotube on ice until bead-beating. Lung tissue was homogenized under the conditions determined in this study using 2.3 mm diameter beads and a homogenization time of 60 s. As previously described, microbial stability was determined by plating 100 µL of the suspensions on MacConkey, mannitol salt and Spider agar in triplicate for the detection of *E. coli*, *S. aureus* and *C. albicans* respectively.

Comparison of the bacterial diversity in the homogenate and the tissue of mouse lung by 16S ribosomal RNA gene sequencing

The lungs of BALB/c mice (n = 4) were harvested and cut into small pieces. All procedures were performed under a laminar flow hood using sterile equipment to limit airway contamination. Half of the lungs were subjected to bead-beating homogenization according to the protocol described above. Identification of microorganisms was directly performed in the lung tissue from the other part of the lung section using a previously validated procedure that aims at enriching microorganisms[18]. Briefly, small lung tissue pieces in sterile PBS were subjected to magnetic stirring to separate microorganisms from tissue. The material was then filtered to reduce host tissue[18].

DNA was isolated from lung tissues filtrates and homogenates using previously validated procedures[18], which involved the QIAamp DNA Mini Kit protocol (Qiagen®, Germany) supplemented with bead-beating steps using 0.1 mm zirconia beads.

To analyze the bacterial population, 16S rRNA gene sequencing was performed using PacBio sequencing technology (Fasteris®, Switzerland). The full-length 16S rRNA gene was amplified using degenerate versions of the universal bacterial 16S rRNA gene primers 27 F (5′-AGRGTTYGATYMTGGCTCAG) and 1492 R (5′-RGYTAC CTTGTTACGACTT). We employed the QIIME 2 version 2022.8 for bioinformatic analysis[19]. Initially, FASTQ files were imported using the 'qiime tools import' command, followed by quality summarization of demultiplexed sequences via 'qiime demux summarize'. Subsequently, we used DADA2 for denoised with specified parameter. Taxonomic assignment of denoised sequences was conducted using the sklearn classifier with the Silva 138 database. Finally, amplicon sequence variant (ASV) counts were obtained and collapsed to different taxonomic levels.

Statistical analysis

Statistical analysis was performed using GraphPad 8 software. The Shapiro–Wilk test was used to determine whether the variables followed a normal distribution. The results were expressed as mean ± SD and analyzed by two-way ANOVA test. A p value of < 0.05 was considered significant. Data analysis and visualization corresponding to the microbiota analysis were performed in the R statistical computing environment (version 4.1.0). The R package Phyloseq (version 1.38.0) was used for microbiome analysis, and the data were visualized using ggplot2 (version 3.4.4) and the Venn package (version 1.7.3).

Ethics approval

All animal experiments were approved by the Ethics Committee of the Faculty of Medicine of the University of Chile (No. 1067) and all methods were carried out in accordance with relevant guidelines and regulations.

Results

Validation of the lung tissue homogenization process

The homogenization protocol based on the bead-beating method was set-up using microbial cultures for evaluating its effect on microbial mortality. For this, the effect of microsphere diameter (0.1 and 2.3 mm) and bead-beating time (30, 60 and 180 s) on microbial survival was evaluated. The impact of these parameters was tested on three microbial strains cultured in exponential phase (approximately 10^8 CFU/mL), which are easily cultivated and commonly observed in dysbiotic events associated with lung disorders[20–22], (i.e., *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans* as Gram-negative and Gram-positive bacteria, and yeast respectively). Figure 1 shows the percentage of surviving microbial strains (compared to the initial concentration) after the application of the different homogenization methods. Higher mortality of *E. coli* (Fig. 1A) and *S. aureus* (Fig. 1B) was observed when smaller microspheres (0.1 mm) were used for a longer bead beating time of 180 s ($p = 0.026$ and $p = 0.004$, respectively), compared to 30 s and 60 s ($p = 0.014$ and $p = 0.016$, respectively). In contrast, the larger microspheres (2.3 mm) did not affect the survival of the bacteria. As shown in Fig. 1C, the survival of *C. albicans* does not appear to be affected by either microsphere diameter or bead beating time. Together, these results indicate that a bead-beating time of 60 s using 2.3 mm diameter microspheres maintain the survival of both bacteria and yeast strains.

In a second step, the tissue homogenization process was evaluated in mouse lung tissue using the previously established parameters (2.3 mm diameter microspheres and bead-beating time of 60 s). We evaluated the recovery rate of mixed cultures (*E. coli*, *S. aureus* and *C. albicans*) administered intratracheally into the lungs of the animals (n = 3). An equivalent dose of 10^5 CFU was administered, which is similar to the concentration of the microbiota recovered in the lungs. The recovery of the inoculated microorganisms was estimated by comparing the number of microorganisms determined after lung tissue homogenization with that present in the inoculated dose (Fig. 2). To ensure that the resident microbiota did not impact our results, we previously determined the concentrations of commensal *E. coli*, *S. aureus* and *C. albicans* inhabiting the lungs in control mice (n = 3). *S. aureus* and *C. albicans* were recovered (mean ± SD) at 221 ± 314 CFU/mg and 98 ± 170 CFU/mg of lung, respectively, while *E. coli* was not detected. Although the lung bacterial community can vary significantly among mice based on environmental factors (strongly clustering by cage)[15], these results suggest that resident microorganisms were present at lower concentrations than those inoculated, which should not affect our results. The administered dose of microorganisms (10^5 CFU/mouse or 500.000 CFU/mg of lung) was statistically higher than the residents (Wilcoxon test, $p = 0.05$ for *S. aureus*; $p = 0.04$ for *E. coli*; $p = 0.05$ for *C. albicans*). On average, 70% of the intratracheally-administered microorganisms were recovered, ranging from 60 to 75%. These data show that our tissue homogenization process recovered the majority of microorganisms from the lung tissue for subsequent cultivation.

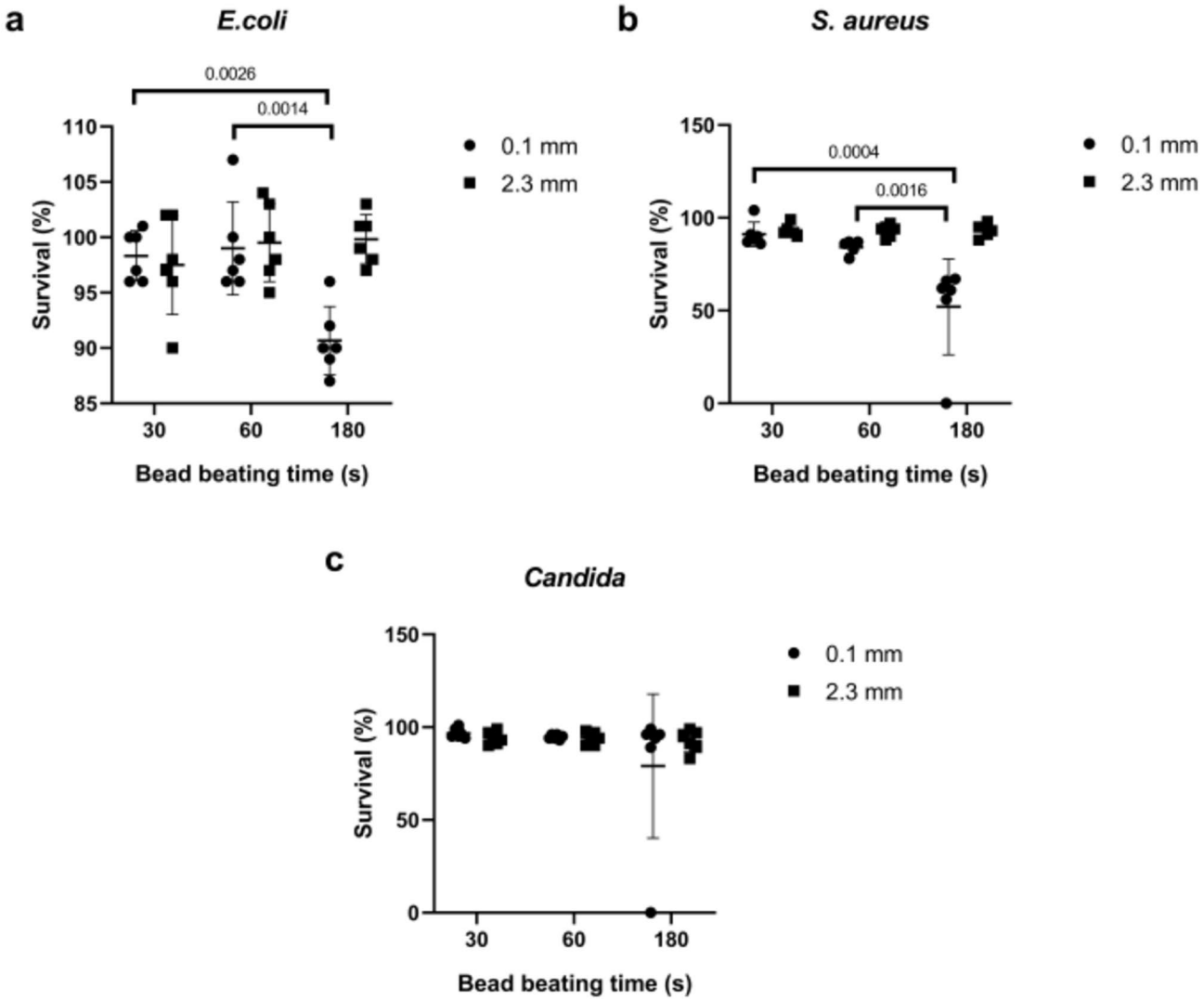

Figure 1. Effect of bead beating time and microspheres diameter on the viability of microorganisms. The effect of bead beating time (30, 60 and 180 s) and the diameter of the microspheres (0.1 and 2.3 mm) on the viability of *E. coli* (**a**), *S. aureus* (**b**) and *C. albicans* (**c**) cultures was assessed. The viability was estimated by comparing the concentration of microorganisms after the homogenization protocol with the initial concentration of culture ($\approx 10^8$ CFU/mL). Each bar represents the mean of three experiments ± SD. Statistical differences were tested using two-way ANOVA ($p < 0.05$).

Performance of the tissue homogenization process on mouse lungs

To evaluate the loss of microorganisms caused by the previously developed tissue homogenization method, we compared the bacterial diversity of the mouse lung with and without bead-beating tissue homogenization by sequencing the entire 16S rRNA gene. For each mouse, part of the sectioned lung was used for the direct characterization of the tissue-associated bacteria by magnetic stirring, and the other part was used for tissue homogenization.

The phylum/genus taxonomic profiles of the lung microbiome were determined by sequencing (n = 4) (Fig. 3A,B). This analysis did not include the microorganisms identified in the homogenate. At the phylum level, *Firmicutes* (53.7 ± 5.9%), *Proteobacteria* (27.7 ± 20.1%), *Actinobacteriota* (8.3 ± 7.6%), *Bacteroidota* (7.7 ± 6.1%) and *Myxococcota* (1.8 ± 3.6%) were the most abundant, while *Verrucomicrobiota, Patescibacteria, Bdellovibrionota* and *Planctomycetota* abundances were less than 1% (Fig. 3A). At the genus level, *Lactobacillus* and *Curvibacter* were the dominant taxa in the mouse lung microbiome, accounting for 33.6 ± 25.2% and 18.2 ± 27.3% of the total bacteria, respectively. In addition, other genera such as *Escherichia*, *Cutibacterium*, *Muribaculaceae*, *Massilia*, *bacteriap25*, *Faecalibacterium*, *Sphingomonas*, *Rothia* and *Ruminococcus*, were detected at abundances between 1 and 10% (Fig. 3B). Interestingly, some *Lactobacillus* species, *Muribaculaceae*, *Cutibacterium*, *Faecalibacterium* and *Ruminococcus* are obligate anaerobes. Other strict anaerobic bacteria were also identified in lesser abundance (Table 1). Together the strict anaerobic microorganisms represent 52.8 ± 36.9% of the total abundance.

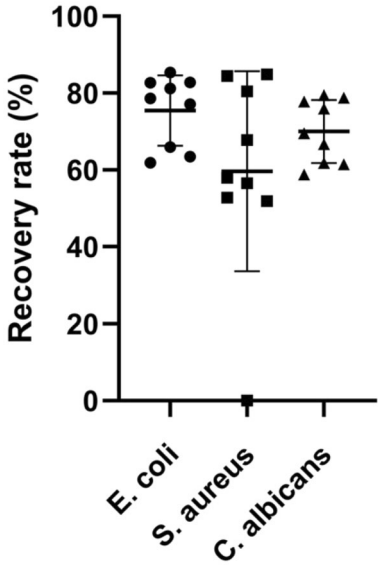

Figure 2. Recovery (%) of the inoculation of mixed culture in the lung tissue of mice. Mixed cultures (*E. coli*, *S. aureus* and *C. albicans*) were intratracheally instilled in the lungs of the mice. The counts of the different microbial strains were determined by culture after bead-beating homogenization of the lung tissue (2.3 mm diameter microspheres and bead-beating time of 60 s). The recovery (%) was estimated by comparing the dose of microorganisms determined following the homogenization of lung tissue with the concentration of inoculated mixed culture (≈ 10^5 CFU). Each bar represents the mean of four experiments ± SD.

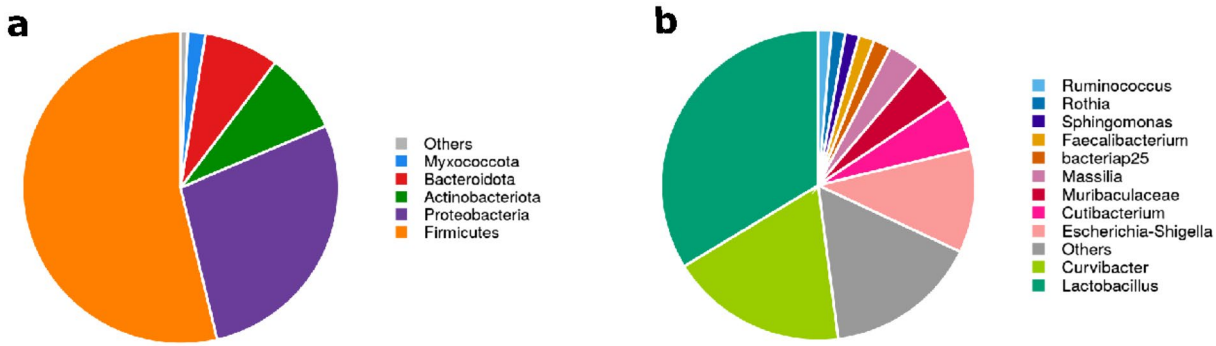

Figure 3. Bacterial composition in the lungs of mice at the phylum and genus levels. Pie charts show the cumulative relative abundance of phyla (**a**) and the 11 most abundant genera (**b**) retrieved exclusively in the lung tissue of mice (n = 4).

Interestingly, 13 genera identified in this study (listed in the Table 2) have the capacity to degrade mucin and/or harbor glycan-degrading enzymes (glycosyl hydrolases)[23,24], representing 37.5 ± 25.8% of the total abundance.

The sequencing reads from this study were then compared to public GenBank sequences to determine if the identified taxa were lung specific. Indeed, these indigenous microorganisms could lead to greater co-evolution with the host, thereby potentially exerting a greater influence on host physiology and disease. Isolating and investigating these microorganisms could be of great interest in the future to understand host-microorganism interactions in the pathophysiology of respiratory diseases. Our reads shared more than 98% identity with sequences from the GenBank database (from uncultivated or cultivated species). The matching sequences were associated with sequences identified in the gut (i.e., *Lactobacillus* sp., Faecalibacteriaceae, *Ruminococcus* sp., Muribaculaceae, *Odoribacter splanchnicus*, and *Enterobacter* sp.), mouth (*Rothia dendoriosa* and *Streptococcus* sp.), nasal cavity (uncultured bacteria p25 and *Staphylococcus xylosus*), indoor air (*Rhodopseudomonas*) and environment (*Pseudomonas* sp. and *Curvibacter* sp.). None of the reads identified in this study showed similarity to sequences previously detected in the lung. However, studies of the murine lung microiome remain scarce.

Sequencing analysis revealed 91 genera in the lung tissue and 63 genera in the homogenate. Approximately 38%[35] of the 91 genera detected in the lung tissue were also recovered in the homogenate (Fig. 4). However, these bacterial genera present in the homogenate represented 84% of the total abundance of the microbiota identified in the lung tissue. The Bead-beating homogenization process rescued 67% (24/36) of obligate anaerobes and 85% of mucin-degrading bacteria (11/13). We noted that the 56 bacterial species identified in the lung tissue and not retrieved in the homogenate, ranging in abundance from 0.01 to 3.5% (representing 16% of the total).

Genera	Relative Abundance (%, mean ± standard deviation)
*Lactobacillus**	33.56% (± 25.18)
*Cutibacterium**	5.59% (± 4.92)
*Muribaculaceae**	4.43% (± 6.92)
*Faecalibacterium**	1.57% (± 2.82)
*Ruminococcus**	1.35% (± 2.54)
*Faecalibaculum**	0.69% (± 1.39)
*Roseburia**	0.49% (± 0.99)
*Odoribacter**	0.49% (± 0.99)
*Subdoligranulum**	0.47% (± 0.93)
*Dialister**	0.45% (± 0.90)
*Prevotella**	0.43% (± 0.54)
vadinBE97	0.26% (± 0.53)
*UCG-005**	0.27% (± 0.53)
Flavobacterium	0.25% (± 0.51)
Monoglobus	0.25% (± 0.50)
Paraprevotella	0.23% (± 0.46)
*Dubosiella**	0.23% (± 0.46)
*Christensenellaceae**	0.22% (± 0.44)
*Lachnoclostridium**	0.18% (± 0.37)
*Collinsella**	0.19% (± 0.37)
*CAG-56**	0.18% (± 0.21)
UBA1819	0.17% (± 0.34)
*Blautia**	0.17% (± 0.34)
*Fenollaria**	0.14% (± 0.28)
Coprococcus	0.12% (± 0.25)
*Finegoldia**	0.11% (± 0.22)
Saccharimonadaceae	0.11% (± 0.21)
*Agathobacter**	0.08% (± 0.16)
Peptoniphilus	0.06% (± 0.12)
Bacteriovorax	0.06% (± 0.11)
*Dorea**	0.05% (± 0.11)
Terrimonas	0.05% (± 0.09)
*Lawsonella**	0.03% (± 0.06)
Actinomyces	0.03% (± 0.06)
Bdellovibrio	0.02% (± 0.04)
*Bacteroides**	0.02% (± 0.04)

Table 1. Strict anaerobe genera identified in the lung of mice. *Genera retrieved in the homogenate.

Genera	Relative Abundance (%, mean ± standard deviation)
*Lactobacillus**	33.56% (± 25.18)
*Escherichia**	10.78% (± 20.40)
*Ruminococcus**	1.35% (± 2.54)
*Staphylococcus**	0.72% (± 0.90)
*Pseudomonas**	0.65% (± 1.22)
*Prevotella**	0.43% (± 0.54)
*Collinsella**	0.19% (± 0.37)
*Lachnoclostridium**	0.18% (± 0.37)
*Blautia**	0.17% (± 0.34)
Coprococcus	0.12% (± 0.25)
*Acinetobacter**	0.07% (± 0.14)
Actinomyces	0.03% (± 0.06)
*Bacteroides**	0.02% (± 0.04)

Table 2. Bacterial genera with mucin-degrading capacity identified in the lung of mouse. *Genera retrieved in the homogenate.

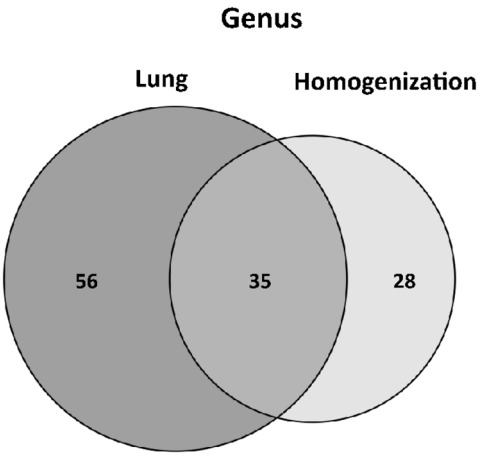

Figure 4. Venn diagram of the common bacteria identified in lung tissue and homogenates. Common bacteria between lung tissues and homogenates were identified by sequencing the full-length 16S rRNA gene using PacBio sequencing technology.

Taken together, these results indicate that the tissue homogenization process developed in this study recovered the majority of the microorganisms present in the lung and particularly the obligate anaerobes.

Culture identification of the bacterial communities inhabiting the lung

To ensure the feasibility of culturing microorganism from the tissue homogenization process, the homogenate was cultivated in BHI broth supplemented with or without mucin under anaerobic and aerobic conditions. As we previously identified potential mucin-degrading bacteria in the lung of mice, we hypothesized that the microorganisms colonizing or persisting in the lung should use airway mucus as a nutrient source. The growing microorganisms were subsequently identified by sequencing their entire 16S rRNA gene. We isolated 6 bacterial strains identified as *Lactobacillus reuteri*, *Lactobacillus johnsonii*, *Bifidobacterium pseudolongum*, *Staphylococcus lentus,* and *Parasuterella* sp. According to the relative abundance of microorganisms identified in the lung tissues by 16S rRNA sequencing, these 6 cultured bacterial species represented 34% of the total bacterial composition identified in the lung tissues of the mice. Interestingly, *Lactobacillus reuteri*, *Lactobacillus johnsonii* and *Bifidobacterium pseudolongum* were shown to grow in mucin-enriched environments.

Discussion

The exploration of the lower respiratory tract microbiome has become increasingly crucial. Numerous studies have utilized culture-independent metagenomic DNA sequence analysis to reveal significant alterations in the microbiome associated with various respiratory diseases such as chronic obstructive pulmonary disease, asthma, idiopathic pulmonary fibrosis, lung cancer, and acute respiratory distress syndrome[20,25]. These alterations could therefore play a crucial role in influencing the onset and advancement of respiratory diseases, underscoring the critical importance of comprehending the biology of the lung microbiome in clinical practice. The isolation and cultivation of these microbes remain crucial for their direct study and confirmation of their metabolic and physiological functions in the lung. Simple direct streaking of tissue onto agar plates is commonly used, but this method may have limited sensitivity and is likely to isolate mainly surface microbes. However, the majority of microorganisms are not expected to be on the outer surfaces of the collected tissue samples[26]. Therefore, streaking tissues on agar plates or immersing them in culture broth it is not sufficient, and there is a clear need to improve tissue processing to increase the sensitivity of lung tissue culture. Accordingly, the aim of this study was to evaluate a tissue homogenization method based on a bead-beating procedure for the isolation and culture of microbes inhabiting the lung.

The first steps of tissue sample processing have a major impact on the subsequent steps in the microbiology workflow. Bead-beating homogenization is an interesting method for releasing microorganisms attached to lung tissue. An additional advantage is the standardized way of processing tissue samples[27].

In all the experiments of this study, lung tissue was maintained at 4 °C from homogenization to plating, as the kinetic energy generated by the homogenizer has been reported to promote microbial growth[28]. This step must have the ability to release microorganisms from the tissue while maintaining microbial survival. Microsphere diameter and homogenization time are important factors in tissue homogenization and microbial survival[11,13]. Examples of gram-positive and gram-negative bacteria (*S. aureus* and *E. coli,* respectively) and yeast (*C. albicans*) commonly obtained from the lung were tested. The effect of processing can vary among different bacterial species and strains, probably due to their cell envelope structure and hence resistance to mechanical stress[26]. In the present study, a smaller diameter (0.1 mm) of microspheres was associated with greater mortality for *E. coli* and *S. aureus*, while a larger diameter (2.3 mm) was associated with greater bacterial survival. Homogenization time increases this phenomenon. In fact, tissue processing should always be kept to the minimum necessary, as the longer the processing, the lower the viability of the released bacteria[26]. Microspheres with a diameter of

0.1–0.5 mm disrupt the bacterial cell wall and affect the viability of the microbes[11,13]. On the other hand, *C. albicans* was not affected by microsphere diameter. Compared to bacteria, yeast cell walls need microspheres with diameters between 0.5 and 1.25 mm to be disrupted[13]. Our study showed that bead-beating homogenization with 2.3 mm diameter microspheres and a homogenization time of one minute maintained the survival of the bacteria and yeast.

Because the toughness of lung tissue can affect microbial recovery[26], we evaluated the efficiency of our bead-beating homogenization process. Lung tissue from mice previously intratracheally inoculated with microorganisms (*E. coli*, *S. aureus* and *C. albicans*) was processed prior to culture. To ensure that the resident microbiota did not confound our results, we previously confirmed that the inoculated microorganisms were absent or present at low concentrations in the resident microbiota of the mouse lung. Our bead-beating homogenization protocol shows good efficacy in recovering the inoculated microorganisms, with approximately 70% of the microorganisms recovered. Taken together, these results demonstrate that our bead-beating homogenization protocol can recover living microbes that inhabit the lung.

To assess whether our protocol results in loss of microorganisms, we determined the bacterial community by NGS sequencing in lung tissue subjected to magnetic stirring and in the homogenate obtained by bead-beating. Approximately 38% of the bacterial species identified in the lung tissue were recovered from the homogenized tissue, representing 84% of the abundance of the recovered lung microbiota. The microorganisms not rescued by bead beating homogenization were low in abundance in the lung microbiome (between 0.01 and 3.5% in abundance). It cannot be excluded that some of these microorganisms are contaminants. The degree of sample complexity, such as high amounts of host DNA, could also affect the taxonomic identification in the microbiome analysis[29].

Interestingly, our study revealed that a significant part of the microorganisms identified in the lung of mice corresponded to obligate anaerobes (52.8 ± 36.9% of the total abundance). Our homogenization process recued 67% of them. The culture of the homogenized tissue allowed the identification of 5 bacterial species (i.e., *L. reuteri*, *L. johnsonii*, *B. pseudolongum*, *S. lentus* and *Parasuterella* sp.) that are among the most abundant microbes in the lung microbiome of mice. The genus *Lactobacillus* was previously reported to be the most abundant bacterial genus in the mouse lung[30,31]. Intriguingly, *L. reuteri*, *L. johnsonii* and *B. pseudolongum* were cultivated in the presence of mucin, which is the major nutrient source for microorganisms in the lung[32,33]. In vitro studies showed that *L. reuteri*, *L. johnsonii* and *B pseudolongun* showed cell adhesion capacities to mucus, allowing them to outcompete[34,35]. Moreover in silico studies revealed that *L. reuteri*, *L. johnsonii* and *B. pseudolongun* strains harbor glycosyl hydrolases (GHs), suggesting that these species have the capacity to enzymatically degrade mucin glycans[35,36]. However this capacity could be strain-dependent since media additionned with mucus did not enhance their growth[35,36].

Although all the bacteria identified in this study were from the upper respiratory tract, some of them could colonize and/or persist such as mucin-degrading bacteria. In our study, we detected 13 genera that could have mucin-degrading capacity, representing 37.5 ± 25.8% of the total abundance. Since nutrients for bacteria and yeast are rather scarce in the lung, this ability may provide an interesting competitive advantage for these bacteria over those that cannot use these substrates. Some microorganisms could not just transit in the lung and particularly those capable of adapting to the lung environment. Nevertheless, our study failed to grow microorganisms present in low abundance in the lungs of mice, which could be due to the lack of optimized culture media and/or growth inhibition by the dominant bacteria. Selective culture media may favor the selective growth of low-abundance microorganisms (Supplememtary information 1).

Conclusion

In conclusion, the bead beating homogenization process developed in this study is effective to facilitate subsequent culture of lung microorganisms. In addition, tissue homogenization may be beneficial for the processing of lung tissue samples. The isolation of microorganisms inhabiting the lung and particularly the obligate anaerobes could help to improve knowledge of the ecological relationship between the host and the microbial communities present in the lung.

Data availability

Raw data and metadata are available on the open data repository Zenodo (DOI:https://doi.org/10.5281/zenodo.10908536).

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Author contributions

F.M., M.G., C.T., P.P. and C.P. conceived and designed the experiments; L.A., A.Z., and H.M. performed the experiments; F.M. and L.A. analyzed the data; O.C., S.C., P.P. and F.M. performed bioinformatic analysis; F.M. prepared the figures; J.C. formatted tables, figures, the manuscript and applied corrections as advised by other authors; F.M. and L.A. wrote the manuscript. All authors read and approved the final manuscript.

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Competing interests

The authors declare no competing interests.

Additional information

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