Journal of Visualized Experiments

Design and development of a model to study the effect of supplemental oxygen on the cystic fibrosis airway microbiome

Asynch.CoverPage.ManuscriptDraft

Common.Labels.ArticleType	Invited Methods Collection - JoVE Produced Video
Common.Text.ManuscriptNumber:	JoVE62888R1
Common.SubmissionDetails.FullTitle:	Design and development of a model to study the effect of supplemental oxygen on the cystic fibrosis airway microbiome
Common.SubmissionDetails.CorrespondingAuthor:	Peggy Lai, M.D., M.P.H. Massachusetts General Hospital Boston, MA UNITED STATES
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Common.EditSubmissionSteps.AdditionalInformation:	
Common.Text.Question	**Common.Text.Response**
Please specify the section of the submitted manuscript.	Biology
Please indicate whether this article will be Standard Access or Open Access.	Standard Access ($1400)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Charlestown, MA, USA
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TITLE:

Design and Development of a Model to Study the Effect of Supplemental Oxygen on the Cystic Fibrosis Airway Microbiome

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KEYWORDS:

artificial sputum medium, culture, people with cystic fibrosis (pwCF), hyperoxia, metagenomics sequencing, airway microbiome

SUMMARY:

The goal of this protocol is to develop a model system for the effect of hyperoxia on cystic fibrosis airway microbial communities. Artificial sputum medium emulates the composition of sputum, and hyperoxic culture conditions model the effects of supplemental oxygen on lung microbial communities.

ABSTRACT:

Airway microbial communities are thought to play an important role in the progression of cystic fibrosis (CF) and other chronic pulmonary diseases. Microbes have traditionally been classified based on their ability to use or tolerate oxygen. Supplemental oxygen is a common medical therapy administered to people with cystic fibrosis (pwCF); however, existing studies on oxygen

and the airway microbiome have focused on how hypoxia (low oxygen) rather than hyperoxia (high oxygen) affects the predominantly aerobic and facultative anaerobic lung microbial communities. To address this critical knowledge gap, this protocol was developed using an artificial sputum medium that mimics the composition of sputum from pwCF. The use of filter sterilization, which yields a transparent medium, allows optical methods to follow the growth of single-celled microbes in suspension cultures. To create hyperoxic conditions, this model system takes advantage of established anaerobic culturing techniques to study hyperoxic conditions; instead of removing oxygen, oxygen is added to cultures by daily sparging of serum bottles with a mixture of compressed oxygen and air. Sputum from 50 pwCF underwent daily sparging for a 72-h period to verify the ability of this model to maintain differential oxygen conditions. Shotgun metagenomic sequencing was performed on cultured and uncultured sputum samples from 11 pwCF to verify the ability of this medium to support the growth of commensal and pathogenic microbes commonly found in cystic fibrosis sputum. Growth curves were obtained from 112 isolates obtained from pwCF to verify the ability of this artificial sputum medium to support the growth of common cystic fibrosis pathogens. We find that this model can culture a wide variety of pathogens and commensals in CF sputum, recovers a community highly similar to uncultured sputum under normoxic conditions, and creates different culture phenotypes under varying oxygen conditions.

INTRODUCTION:

Cystic fibrosis (CF) is a genetic disease characterized by an inability to clear thick mucus from the lungs, leading to repeated infections and progressive lung function decline that often results in the need for lung transplantation or death. The airway microbiome of people with cystic fibrosis (pwCF) appears to track disease activity[1], with the reduction in microbial diversity associated with adverse long-term outcomes[2,3]. In clinical studies of pwCF, supplemental oxygen therapy has been associated with more advanced disease[4,5], though traditionally, the use of oxygen therapy has been viewed as simply a marker for disease severity[6]. Recent studies from a clinical trial of patients with respiratory failure have shown that higher patient oxygen levels are paradoxically associated with an increase in serious bacterial infections and higher mortality[7], suggesting that supplemental oxygen may contribute to disease pathogenesis. The effect of supplemental oxygen on the cystic fibrosis lung microbiome and associated lung and airway microbial communities has not been well studied.

Mechanistic studies often cannot be performed directly on human subjects due to logistical difficulties and potential ethical issues associated with interventions of unknown medical benefit or harm. Translational approaches that integrate human biospecimens into model systems can offer important biological insights in these cases. While the ability to use or tolerate oxygen has traditionally been an important component of microbial classification, little is known about how the therapeutic introduction of supplemental oxygen to the environment might perturb airway microbial communities. To shed light on the unknown effects of supplemental oxygen on the lung microbiomes of patients with CF, we needed to address two major challenges; first, the creation of a culture medium that physiologically approximates the composition of CF sputum; second, the creation of a model system that allows the maintenance of elevated oxygen concentrations in culture over extended periods of time.

Artificial sputum media (ASM) are widely used to emulate lung sputum *ex vivo*[8–10], but there is no clear consensus on a specific recipe. This protocol describes an artificial sputum medium recipe and preparation strategy carefully designed to physiologically approximate sputum from pwCF. **Table 1** outlines the chosen recipe values based on published Lature. Basic chemical components and pH were matched to values identified by studies of human CF sputum[11–13]. Low concentration physiological nutrients were added using egg yolk, which was included as 0.25% of the final volume[10], as well as vitamin and trace metal mixes[14,15]. Mucin, the key protein in sputum[16], was included at 1% w/v[14]. Although more labor-intensive, filter sterilization was chosen over the more conventional practice of heat sterilization to reduce potential problems from heat-induced denaturation of essential media components[10]. An additional benefit of filter sterilization is that it generates media that are transparent (heat-sterilization creates turbid media due to precipitation and coagulation of salts and proteins), allowing this artificial sputum media to be used to follow microbial growth based on increases in turbidity.

This model system for the hyperoxic culture is based on anaerobic culturing techniques where oxygen is added rather than removed, creating a model for the effect of supplemental oxygen use for pwCF. **Figure 1** and the associated oxygen sparging protocol outlines the components of an oxygen sparging system, which can be obtained at low cost from general laboratory and hospital suppliers. This system enables the mixing of compressed oxygen and air to fixed concentrations ranging from 21%–100% oxygen. The integration of an oxygen sensor allows for the verification of the concentration of the output gas mixture, as well as checking the outflow gas composition of previously sparged serum bottles to verify that the oxygen conditions have been maintained within the desired range.

This protocol outlines procedures to create an artificial sputum medium, the construction and use of an oxygen sparging system, and the application of both to culture CF sputum under differential oxygen conditions.

PROTOCOL:

This study received approval from the Partners Institutional Review Board (Protocol # 2018P002934). Inclusion criterion included adult patients with cystic fibrosis who provided written informed consent for the study. There was no exclusion criterion. According to protocol guidelines, all sputum samples were collected from patients with cystic fibrosis during a scheduled outpatient visit with their clinical provider.

1. Artificial sputum medium preparation

NOTE: Quantities listed here are for the production of 1 L of final artificial sputum medium, and assume the specific reagents listed in the **Materials Table.** Numbers must be adjusted for other volumes or for the use of different reagents to ensure the same final product. See **Table 1** for target concentrations.

1.1. Artificial sputum chemical mix (ASCM)

NOTE: ASCM makes up 25% of the final medium volume. It is shelf-stable and can be prepared in bulk or in advance. If being prepared for later use, autoclave the chemical mix and safely store it at room temperature.

1.1.1. Mix the constituent chemical stock solutions.

1.1.1.1. Prepare 1 M NaCl stock: Add 58.44 g of NaCl per liter of sterile water.

1.1.1.2. Prepare 1 M KCl stock: Add 74.55 g of KCl per liter of sterile water.

1.1.1.3. Prepare 1 M $MgSO_4$ stock: Add 246.47 g of $MgSO_4 \cdot 7H_2O$ per liter of sterile water, or 120.37 g of anhydrous MgSO4 per liter of sterile water.

1.1.1.4. Prepare 1 M glucose stock: Add 180.16 g of glucose per liter of sterile water.

1.1.2. Autoclave sterilize the chemical stock solutions, as well as an empty 250 mL bottle. Perform the autoclaving steps to at least standard values of 121 °C and 15 PSI for 30 min.

1.1.3. Add 80.59 mL of sterile water to the empty 250 mL bottle.

1.1.4. Add 152.30 mL of 1 M NaCl stock to the mix.

1.1.5. Add 15.8 mL of 1 M KCl stock to the mix.

1.1.6. Add 610 µL of 1 M $MgSO_4$ stock to the mix.

1.1.7. Add 700 µL of 1 M glucose stock to the mix.

1.2. Artificial sputum mucin mix (ASMM)

NOTE: ASMM makes up 50% of the final medium volume. Ensure that it is prepared on the same day as the final medium batch.

1.2.1. Add 450 mL of sterile water to an empty 1 L bottle.

1.2.2. Add 50 mL of 10x Phosphate-Buffered Saline (PBS) to the bottle.

1.2.3. Add a disposable magnetic stir bar to the bottle.

1.2.4. Autoclave the bottle containing PBS and the stir bar.

1.2.5. Measure out 10 g of mucin powder and add it to the PBS.

1.2.6. Shake the bottle vigorously for preliminary mixing.

1.2.7. Place the bottle onto a hot plate with a magnetic stirrer. Set heat to medium-high targeting 50 °C and stirring speed to 1100 rpm. Ramp up the speed gradually so that the bar does not fly off the magnet.

1.2.7.1. Allow to heat and stir for 15 min.

1.2.7.2. Pick up the bottle with heat-resistant gloves. Observe to check if mucin powder settles out of the solution.

1.2.7.3. If mucin powder is not fully dissolved, return the bottle to heat/stirrer for 5-min intervals until it is completely dissolved.

1.2.8. Allow the mucin mix to cool to room temperature.

1.3. Artificial sputum biological mix (ASBM)

NOTE: ASBM is 25% of the final medium volume. Prepare it on the same day as the final medium batch, and unlike the other mixes, do not expose its components to any heat.

1.3.1. Thaw the 100x vitamin stock in a 4 °C fridge or on ice.

NOTE: Pre-portion the vitamin stock into 10 mL aliquots to minimize the number of freeze/thaw cycles.

1.3.2. Add 124.24 mL of sterile water to the empty autoclaved 250 mL bottle.

1.3.3. Add 25.76 mL of 50x essential amino acid stock to the mix.

1.3.4. Add 80.14 mL of 100x non-essential amino acid stock to the mix.

1.3.5. Add 10 mL of (thawed) 100x vitamin stock to the mix.

1.3.6. Add 1 mL of 1000x trace metals stock to the mix.

1.3.7. Add 8.33 mL of 30% egg yolk emulsion to the mix.

1.3.8. Add 400 µL of 10 g/L ferritin stock to the mix.

1.3.9. Mix the solution well via manual shaking.

1.4. Artificial sputum medium (ASM)

1.4.1. Add 250 mL of ASCM to the 1 L bottle containing ASMM.

1.4.2. Add 250 mL of ASBM to the medium bottle.

1.4.3. Titrate the medium with basic MOPS buffer (1 M) to reach a pH of 6.3 on a narrow range pH paper. Prior to titration, the medium mix will be too acidic.

1.4.4. Refrigerate the resulting artificial sputum medium at 4 °C until it is ready for filtration.

1.4.5. To start the filtration process, transfer 200 mL of unfiltered artificial sputum medium to a vacuum filtration system with a 0.22 µm pore size filter.

1.4.6. Connect the filtration system to the vacuum pump, turn on the vacuum pump, set it to 70 mbar, and then place the chamber on an orbital shaker shaking at 90 rpm in a cold room at 4 °C.

1.4.6.1. Top off with an additional 150 mL of the medium as an appreciable amount is filtered. It takes 1–2 days to filter 1 L of medium completely.

1.4.6.2. Repeat with additional chambers until all the media is filtered.

NOTE: Try not to filter more than 350 mL of the medium through the same 0.22 µm filter since mucin will plug the filter over time.

1.4.7. Refrigerate filtered artificial sputum medium at 4 °C until ready for use. Use ASM within one month of preparation for best results.

2. Oxygen sparging

2.1. Sparging station setup

NOTE: This protocol should only need to be done in full once, after which point the setup can be maintained through simple maintenance as necessary. See **Figure 1** for a visual schematic of the oxygen sparging system.

2.1.1. Obtain and properly secure the compressed air and oxygen tanks.

CAUTION: High pressure makes the tanks extremely dangerous when mishandled. Ensure that the tanks are completely sealed and secured, there are no leaks when the tank is closed, and that all handling personnel is fully trained in their use.

2.1.2. Attach an air regulator to the compressed air tank with a wrench. For optimal flow reading on the regulator, attach the regulator as close as possible to an upright position.

2.1.3. Attach an oxygen regulator to the compressed oxygen tank, attaching as close as possible to an upright position. Depending on the oxygen tank, one may need to invert the direction to tighten.

2.1.4. Connect the tubing from the regulators to a Y-connector to combine the gas flow from the two tanks.

2.1.5. Connect the output of the Y-connector to the central T-junction valve.

2.1.6. Connect one side of the central T-junction valve to a gas pressure gauge.

2.1.7. Connect the other side of the gas pressure gauge to a 25 mm diameter sterile syringe filter with a 0.22 µm pore size.

2.1.8. Attach a second 25 mm diameter syringe filter to a syringe without a plunger to be used as a gas release during sparging.

2.1.9. Connect the final side of the central T-junction valve to a second T-junction valve for the oxygen monitor.

2.1.10. Connect a 25 mm diameter syringe filter to one side of this second T-junction valve, along with tubing to attach 18 G needles.

2.1.11. Connect the final side of the second T-junction valve to the oxygen monitoring apparatus.

2.1.12. Connect a cut-off tube to the other side of the oxygen monitoring apparatus to be used as a gas release during monitoring.

CAUTION: When testing/using the oxygen sparging system, take careful note of the position of the T-junctions and ensure it matches the intended path through the system. Failure to do so will result in pressure buildup inside the system and cause components to fail and come apart**.**

2.1.13. For the maintenance of the system and to keep it working at optimal performance, the following practices are beneficial.

2.1.13.1. Reinforce the connections with liberal amounts of Teflon tape to greatly improve their seal and reduce the chance of components coming apart from the internal pressure.

2.1.13.2. Keep combined flow rate under 10 L/min to mitigate maximum pressure and prevent failures.

2.1.13.3. If a leak is suspected, use a detergent solution such as commercially available liquid leak detectors to identify its location easily, as it will bubble above any gas leaks. Patch

leaks using a Polytetrafluoroethylene tape (e.g., Teflon).

2.1.13.4. Replace the 25 mm diameter syringe filters in the oxygen sparging system bi-weekly, but this varies with use frequency. Over time, particles caught in the filter reduce the gas flow rate and cause pressure buildups.

2.1.13.5. Calibrate the oxygen monitor to 21% oxygen compressed air prior to carrying out measurements.

2.1.13.6. Upon completion of the system use, turn off the tanks and bleed the excess gas from the regulators until the flow completely stops.

2.2. Serum bottle culture sparging

2.2.1. Label 500 mL autoclaved serum bottles with sample identifiers, date/time of inoculation, and target oxygen percentage.

2.2.2. In a biological hood, add 24 mL of the artificial sputum medium to each serum bottle being set up.

2.2.3. Add 1 mL of the patient sputum homogenized with an 18-G needle (diluted with sterile saline if necessary to obtain sufficient volume of sample for each culture condition) to each serum bottle.

2.2.4. Using sterile tweezers, place the autoclaved rubber stoppers onto the top of each serum bottle.

2.2.5. Press down the rubber stoppers, take care not to touch the underside of the stopper with hands.

2.2.6. Remove the bottles from the hood, apply and crimp the aluminum seals. Remove the center piece from the seals.

2.2.7. Wipe down the top of bottles with an alcohol wipe and pass them through a Bunsen burner flame.

2.2.8. Affix a sterile 18-G needle to a plunger-less syringe with a filter. Insert this gas release into the bottle first.

2.2.9. Affix a sterile 18-G needle to the gas output from the system and insert the gas output needle into the bottle as well.

2.2.10. Route the T-junctions from the tanks through the oxygen monitor. Verify that the target oxygen concentration is flowing through the system. Target approximately 5 L/min of gas flow.

2.2.11. Reroute the T-junctions from the tanks through to the gas output. The gas starts to flow through the serum bottle.

CAUTION: Pay close attention to the pressure gauge during oxygen sparging. If pressure increases unexpectedly, shut the system off immediately.

2.2.12. Run oxygen sparge through the serum bottle for 1 min. At 5 L/min, this allows for 10 air exchanges and ensures the internal atmosphere reaches the desired concentration.

2.2.13. Remove the gas release 18-G needle.

2.2.14. Allow the pressure in the serum bottle to build to +1 atmosphere (2 atmospheres at sea level) and then immediately remove the gas output needle.

NOTE: Maintaining pressure aids retention of hyperoxic conditions over time.

2.2.15. Place the serum bottle into a 37 °C incubator shaker at 150 RPM. Incubate the samples for three 24-h intervals. At each 24-h interval, take an aliquot for downstream analysis, re-sparge the samples and return them to incubation for a total incubation time of 72 h.

2.3. Outflow oxygen measurement

2.3.1. Calibrate the oxygen meter to 21% compressed air, and then turn off the tank.

2.3.2. Route the serum bottle intake through the oxygen meter and affix a sterile needle to the end.

2.3.3. Insert the needle into the serum bottle.

2.3.4. Wait for the outflow reading to stabilize. A low flow rate out of the serum bottles means this may take up to 2 min. Report the peak difference from room air (number furthest from 21%).

2.3.5. If performing multiple readings, flush the system with compressed air between readings.

REPRESENTATIVE RESULTS:

These protocols were applied to 50 expectorated sputum samples from pwCF presenting for routine care to an outpatient cystic fibrosis clinic at Massachusetts General Hospital in Boston, Massachusetts. Each patient's sputum was cultured under 21%, 50%, and 100% oxygen conditions using the artificial sputum medium, with 0.5 mL aliquots taken from each culture at 24 h, 48 h, and 72 h of culture time for testing. Cultures were photographed when extractions were made to track visual changes. In addition, a 0.5 mL aliquot of each primary sputum sample was taken prior to culturing. This resulted in 10 discrete samples per patient and a final N of 500 samples. Of these, sputum from 11 patients (11 uncultured sputa, 11 cultured sputa from 21%

oxygen at 48 h of incubation) underwent nucleic acid extraction[17], sequencing libraries were generated using a commercial DNA library preparation kit, and metagenomic sequencing was performed on a whole genome sequencing platform targeting ~ 5 Gb of sequence per sample with 150 base pair, paired-end reads. Raw reads were processed using the bioBakery suite of tools[18], which includes quality control and removal of human "contaminant" sequences and taxonomic profiling with the MetaPhlAn3 profiler[19]. At the time of nucleic acid extraction, 10 million cells of *Imtechella halotolerans*, a halotolerant species normally found in estuary ecosystems and not in human microbial communities, were spiked into each sample, allowing the quantification of absolute microbial load for each sample[20].

Figure 2 shows individual and average outflow oxygen measurements and pH levels over the course of the culture process for 50 sputum samples cultured under each oxygen condition and an example of a visual differential culture phenotype. Cultures were maintained at 37 °C except during brief periods when sparging and removal of sample aliquots was performed. With both sparging intervals of 12 h and 24 h, elevated oxygen concentrations were maintained, although a drop over time was observed for all three oxygen conditions, with 100% oxygen falling to approximately 85%, 50% oxygen falling to 40%, and 21% oxygen falling to 18%. Oxygen conditions remained distinct, and importantly, elevated oxygen concentrations were maintained throughout the process for hyperoxic samples. pH measurements showed a greater degree of variability but stayed well within a physiologically normal range with no statistically significant changes over time. These measurements indicate that these methods maintain discrete differential oxygen conditions throughout the culture process. Lastly, an example of one of many visual culture phenotypes that differentiated across oxygen concentration is shown. This sample had marked turbidity differences after 72 h of culture, with higher oxygen associated with lower visual turbidity. Differential culture phenotypes support the presence of hyperoxia-induced effects on culture communities.

Figure 3 compares microbial load, microbial diversity, and microbial community composition between uncultured sputum and cultured sputum (21% oxygen condition for a period of 48 h). Measurements reveal the only major difference introduced by the act of culturing to be an approximately 20-fold increase in microbial load as compared to uncultured sputum. The immune system and the typical mechanical sputum clearance mechanisms such as coughing normally serve as a regulatory process limiting the microbial load in the lung, even in cases of dysfunction and infection like those seen in pwCF. *Ex vivo* culture has no such regulatory mechanisms, and microbial communities are instead free to proceed toward cellular saturation. Alpha and beta diversity metrics indicate that despite this difference in microbial load, the underlying community composition remains well-preserved, with minimal global differences introduced by the culture process.

Figure 4 expands on the comparison between uncultured and cultured sputum samples, looking at the binary presence/absence of the 120 microbial species conclusively identified by shotgun metagenomics sequencing from cultured and uncultured sputum obtained from 11 patients. Microbes are clustered based on phylogenetic similarities. 46 (38.3%) of these species were identified in both uncultured and cultured samples (cyan color), while 35 (29.2%) were exclusively

identified in uncultured samples (yellow) and 39 (32.5%) were exclusively identified in cultured samples (blue). It is likely that there is greater parity than what we identified using sequencing in terms of what is present and what is absent, but some taxa fall below the sequencing detection threshold in some cases. The differences do indicate that the culture process introduces some bias in cultured compared to uncultured sputum. Most notably, culturing increases the presence of fungi such as *Candida* and *Aspergillus*, as well as *Enterobacterales* members including *Escherichia, Serratia*, and *Streptococcus* members. Conversely, *Bacteroidetes* members such as *Prevotella* and *Clostridiales,* which are anaerobes, were present in cultured samples but not present in cultured samples. This may be attributed to the lack of an anaerobic condition in our experimental model.

Figure 5 shows absorbance-based growth curves of common CF lung pathogens isolated from sputum obtained from 50 different pwCF. These isolates represent phenotypically different clinical isolates obtained using enrichment culture procedures from the Massachusetts General Hospital Clinical Microbiology Laboratory, and include *Pseudomonas aeruginosa* (N = 53), *Staphylococcus aureus* (N = 37), *Stenotrophomonas maltophilia* (N = 12), *Klebsiella pneumoniae* (N = 3), and *Achromobacter sp* (N = 7). Growth curves were obtained by culturing each isolate in artificial sputum media at 37 °C in the dark, with ASM sans bacterial inoculation serving as a negative control. The transparent quality of ASM (which is due to filter rather than heat sterilization) allows for conducting optical measures to estimate growth curves. Absorbance readings of 600 nm light were taken every 10 min, and the first 24 h of each curve are shown. The absence of changes in absorbance in the ASM-only negative control indicates cultures free of contamination. The demonstrative curves shown here follow typical growth curve patterns indicating the viability of this ASM recipe as a medium for the absorbance-based generation of growth curves.

FIGURE AND TABLE LEGENDS:

Table 1: Artificial sputum medium recipe derived from review of media and physiology Lature. (**Column 1**) Reagents and key values in the formulation of artificial sputum medium. (**Columns 2–7**) Comparison of recipes from extant Lature[8–10,12,14,15]. (**Columns 8–9**) Artificial sputum medium recipe detailed in this protocol and the corresponding sources that informed each selected value[10–15].

Table 2: Amino acid concentrations previously described in cystic fibrosis sputum and in artificial sputum medium recipe detailed in this protocol. (**Column 1**) Key amino acids. (**Column 2**) Amino acid concentrations of sputum from people with cystic fibrosis[12]. (**Column 3**) Amino acid concentrations in artificial sputum medium detailed in this protocol

Figure 1: Connection schematic of oxygen sparging system components. Flow diagram of the connections between components of the system used to sparge serum bottles to desired oxygen concentrations between 21% and 100%. The system has 3 modes of use determined by the position of the two T-junction valves. The system can route gas from the tanks through the gas output or through the oxygen percentage monitor, as well as route outflow gas from previously sparged serum bottles through the monitor to check concentration after time has elapsed.

Figure 2: Target oxygen concentrations are approximately maintained with both 12 h and 24 h sparging intervals, and pH remains in the physiological range during culture. (**A**) Outflow oxygen readings from 12 h and 24 h oxygen sparging intervals over a 72-h period. (**B**) pH readings for samples measured every 24 h. (**C**) An example image of cultured sample CFB010 after 72 h, displaying differential turbidity across oxygen concentrations. The color indicates target oxygen percentage; error bars denote 95% confidence intervals. Critical thresholds are emphasized with dashed lines.

Figure 3: Culturing increases microbial load, but underlying community composition is preserved. Uncultured sputum (yellow) and cultured sputum (blue) using artificial sputum medium at 21% oxygen for 48 h. Aliquots underwent nucleic acid extraction and shotgun metagenomics sequencing to detect possible bias introduced from culture conditions. (**A**) Absolute microbial load (determined by spike-in controls) and alpha diversity metrics. Using linear mixed effects models, uncultured vs. cultured sputum predicted microbial load but not alpha diversity. (**B**) Ordination of first two components of beta diversity metrics, controlling for difference in microbial load. No significant difference in either metric after PERMANOVA.

Figure 4: The majority of identified taxa are present in both source sputum and culture, while others only appear in source sputum or culture. Shotgun metagenomics sequencing used to compare differences in microbial community composition between uncultured and cultured sputum samples. Phylogenetic tree of all identified microbial species in sequenced samples (N = 120). Species marked with yellow (N = 35, 29.2%) were only seen in uncultured sputum samples. Species marked with blue (N = 39, 32.5%) were only seen in artificial sputum medium culture samples. Species marked with cyan (N = 46, 38.3%) were seen in both uncultured and cultured samples.

Figure 5: Artificial sputum medium is sufficiently transparent to be used as a growth curve medium for culturing clinical isolates. Optimal density readings at 600 nm were taken every 10 min, and the first 24 h of each curve are shown. Gray lines represent individual readings, and orange lines represent the mean absorbance for each taxon. Artificial sputum medium blank included as a control.

DISCUSSION:

In this study, an *in vitro* model was developed to study the effect of hyperoxia on lung microbial communities. This model, based on artificial sputum medium and daily sparging of serum bottles, maintains elevated oxygen concentrations and supports the growth of microbes identified in sputum from pwCF.

There are several critical steps of this approach. First is the choice to use filter-sterilization rather than heat-sterilization of the artificial sputum medium prevents denaturing of mucin and other heat-sensitive components of the medium and yields a clear medium that can be used for optical measures of microbial growth. While filter-sterilization has been proposed in other protocols[10], we have found that the addition of orbital shaking during the filtration process was essential to

prevent clogging of the filter that otherwise occurred when a minimum amount of the prepared medium had been filtered. While the filtered artificial sputum medium may have a lower-than-intended final mucin concentration due to mucin impaction in the filter, sputum from pwCF has been shown to have lower mucin concentrations than sputum from people without cystic fibrosis[21]. The starting mucin concentration of 1% used in this protocol is higher than other approaches that have used filter-sterilization, with one group using a starting mucin concentration of 0.5%[10], while typical recipes use mucin concentrations ranging from 0.5%–2% (**Table 1**). Thus, even with a loss of mucin in the filter-sterilization process, the final medium prepared using this protocol is likely to have mucin concentrations within the physiological range[22].

The second is the composition of artificial sputum medium. The recipe for artificial sputum medium was chosen based on existing physiologic studies of sputum from pwCF (**Table 1**). Using shotgun metagenomics sequencing, we were able to verify that sputum cultured with this artificial sputum medium broadly recapitulates the microbial community composition of uncultured sputum (**Figure 3**). At normoxic conditions, this medium also supported the growth of 112 different clinical isolates representing common pathogens isolated from the sputum of cystic fibrosis patients. Thus, these data demonstrate that this formulation of artificial sputum medium supports the growth of airway microbiota from pwCF. Salmon sperm DNA, a common addition to existing recipes (**Table 1**), was omitted. One intended application of this model is metagenomics sequencing, and thus salmon sperm DNA was not included in order to reduce the addition of non-microbial nucleic acids as these reads would be filtered out after sequencing, thus decreasing our effective sequencing depth. While sputum from pwCF has high concentrations of extracellular DNA[23], a significant proportion of it is microbial in origin[24], and it is unclear if the addition of salmon sperm DNA to the artificial sputum medium makes it more physiologic or whether the culture approach described in this protocol leads to high levels of microbially-derived extracellular DNA; we did not distinguish between extra- and intra-cellular DNA concentrations in our studies. Future studies may wish to verify the concentration of extracellular DNA generated by this culturing method.

Third, to our knowledge, no published studies on cystic fibrosis lung microbial communities have addressed hyperoxic conditions. This model uses inexpensive and commonly available equipment from general laboratory or hospital suppliers to build an oxygen sparging system. Important considerations for the maintenance of hyperoxic conditions include the volume of the culture medium relative to the available headspace in the serum bottles. In initial attempts during protocol development, 125 mL serum bottles were used. However, the use of 500 mL serum bottles (allowing for a 475 mL headspace to 25 mL culture ratio) allowed the maintenance of the desired concentration for up to 24 h, thus reducing the frequency of oxygen sparging. This approach generates discrete oxygen conditions, thus allowing the simultaneous culture of different patient samples across multiple oxygen conditions. Other tools from anaerobic culture can be leveraged for hyperoxic culture, including the use of anaerobic jars or Balch-type tubes sparged with oxygen. Analyses of lung microbial communities using metagenomics sequencing indicate that overall alpha and beta diversity are comparable between cultured and uncultured sputum. When evaluating the differential abundance at the species level, culturing at 21%

oxygen, enriched for the growth of aerobes and facultative anaerobes, including *Enterobacterale, Streptococcus,* and fungi. This is likely due to the exclusion of an anaerobic condition which has been observed in the airways of pwCF[25,26]. Future studies could consider the inclusion of compressed nitrogen into this model as an anaerobic condition to study of a range of anoxic and oxic conditions and corresponding aerobic and anaerobic microbiota found in airway microbial communities.

The key principles outlined in these protocols may be instructive for the conduct of similar studies related to the influence of oxygen on complex microbial communities or common lung pathogens. Oxygen is the most common therapy used in treating all advanced lung diseases, and a better understanding of how it might lead to collateral unanticipated effects on airway microbial communities and common respiratory pathogens will be important for the care of pwCF and other chronic lung diseases.

ACKNOWLEDGMENTS:

Part of this work was performed at Marine Biological Lab with support from Marine Biological Lab, DOE (DE-SC0016127), NSF (MCB1822263), HHMI (grant number 5600373), and a gift from the Simons Foundation.

DISCLOSURES:

The authors have no conflicts of interest to disclose.

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Figure 1

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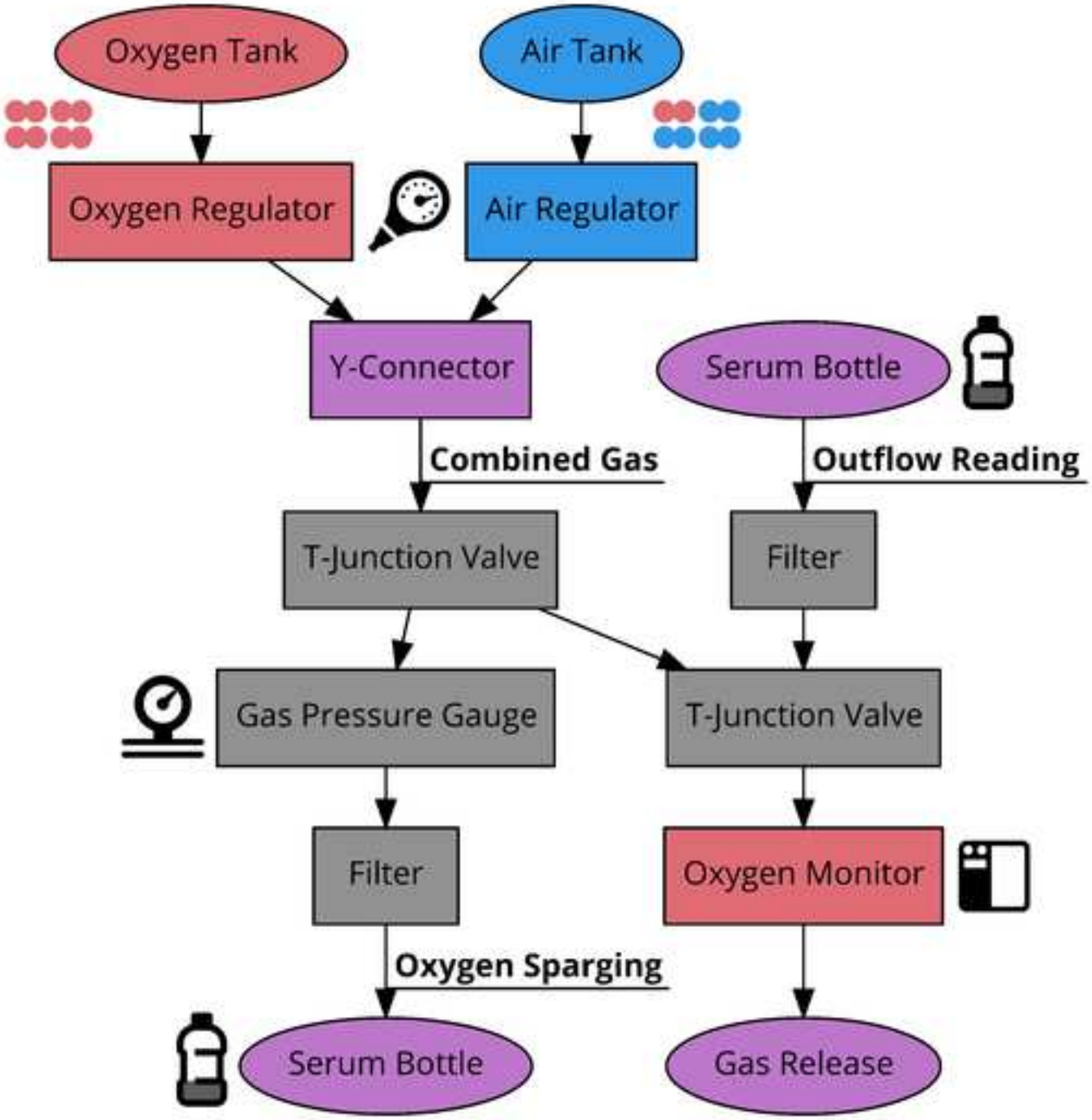

Figure 2

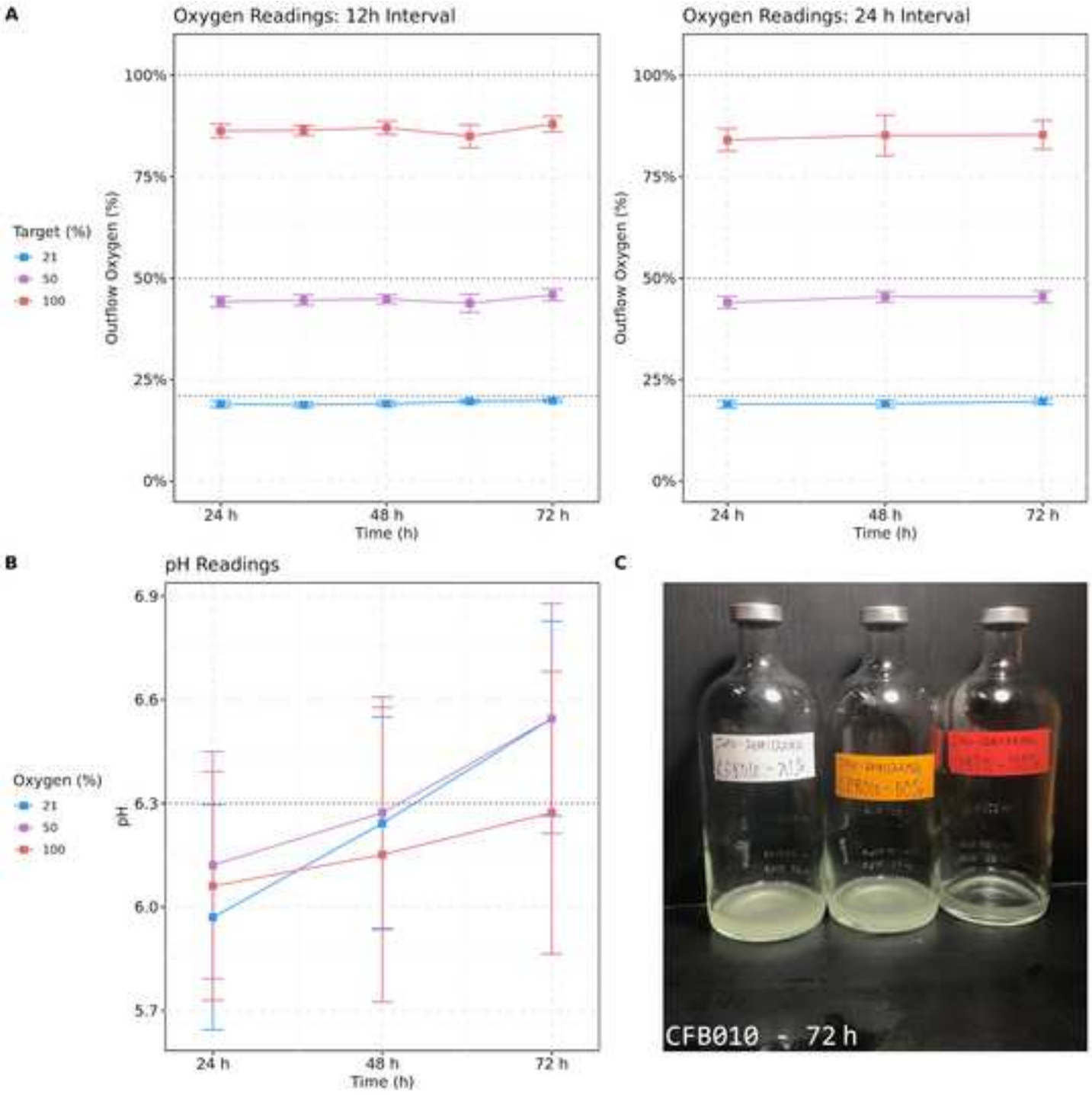

Figure 3

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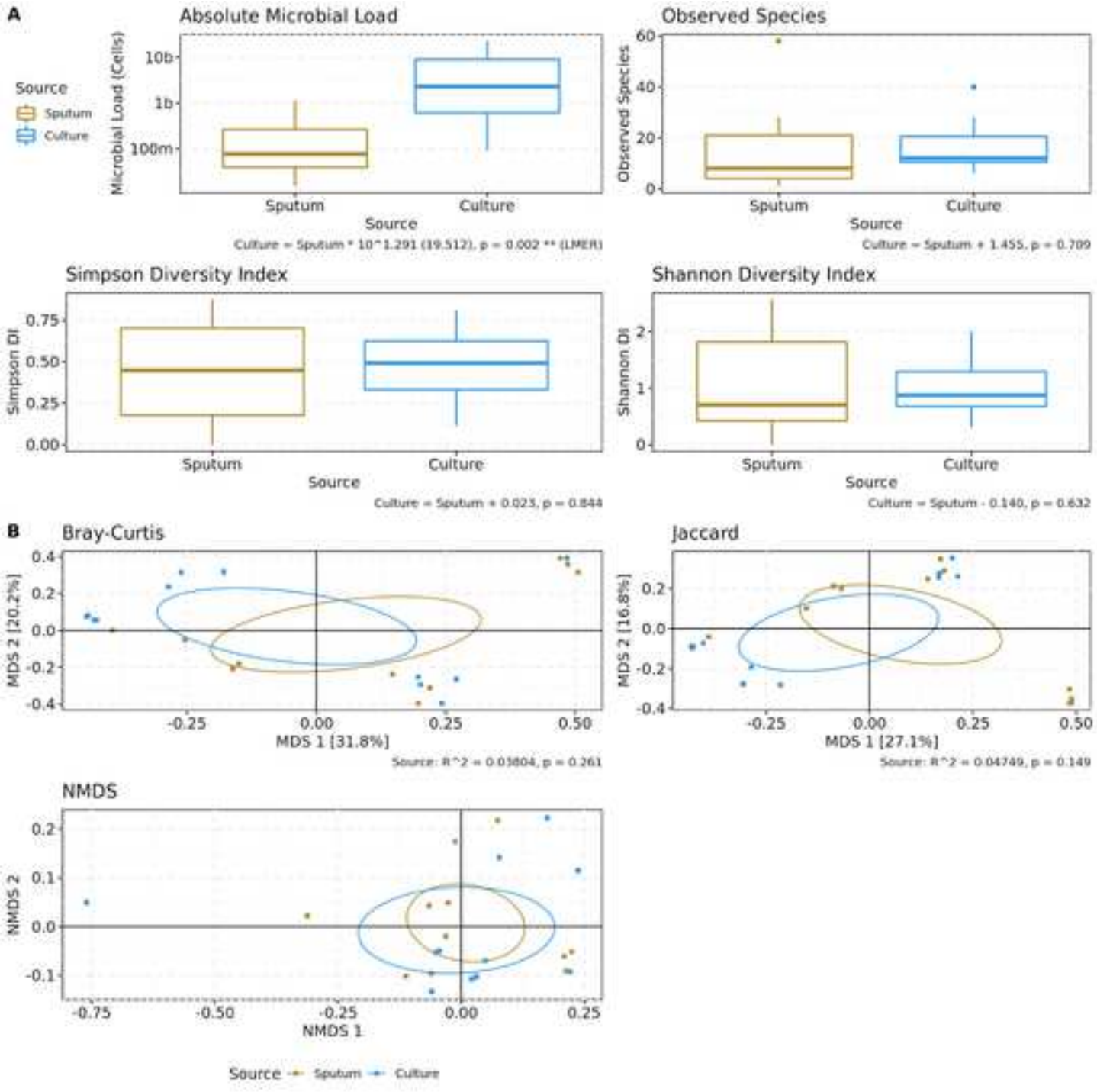

Figure 4

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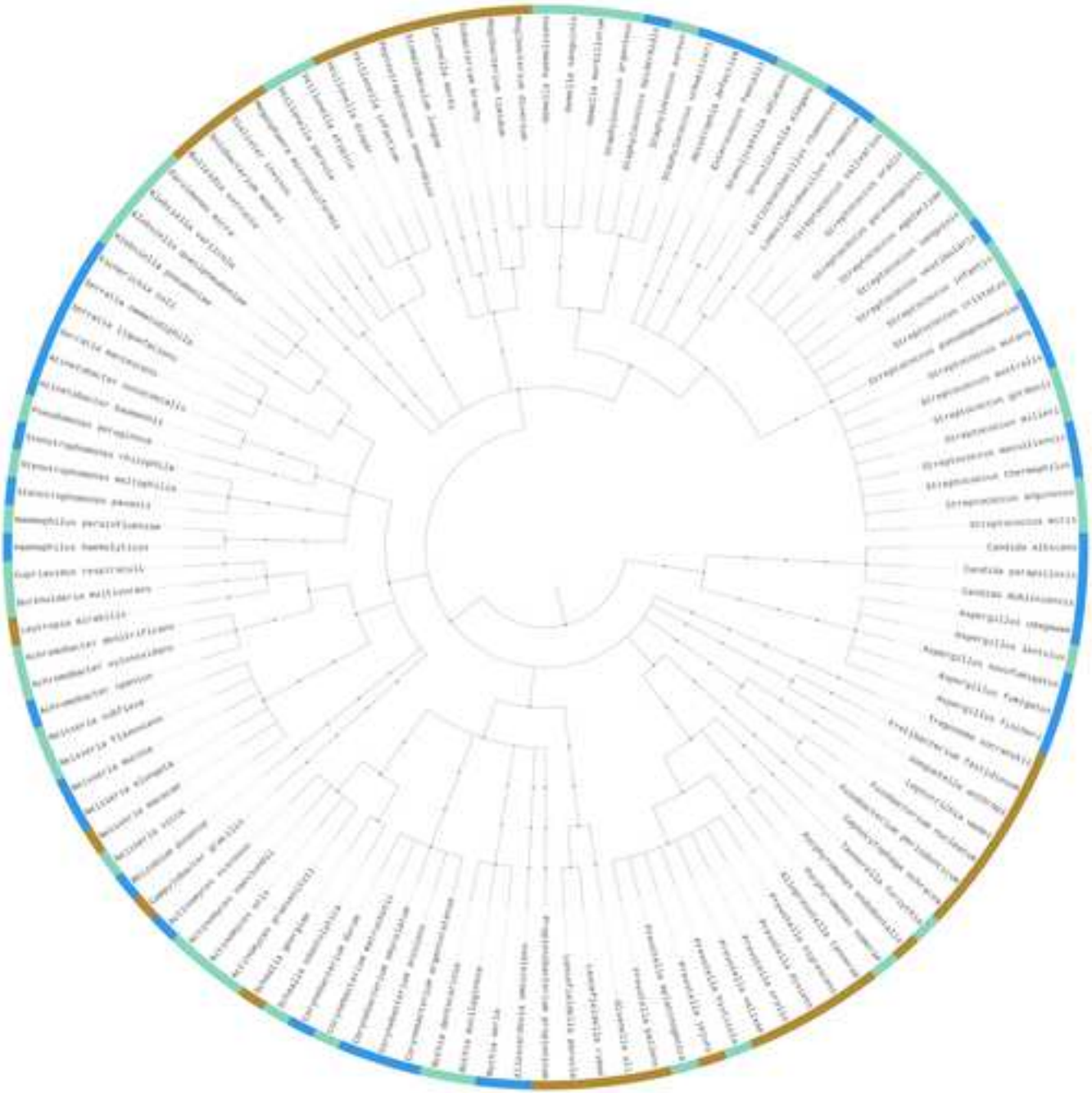

Figure 5

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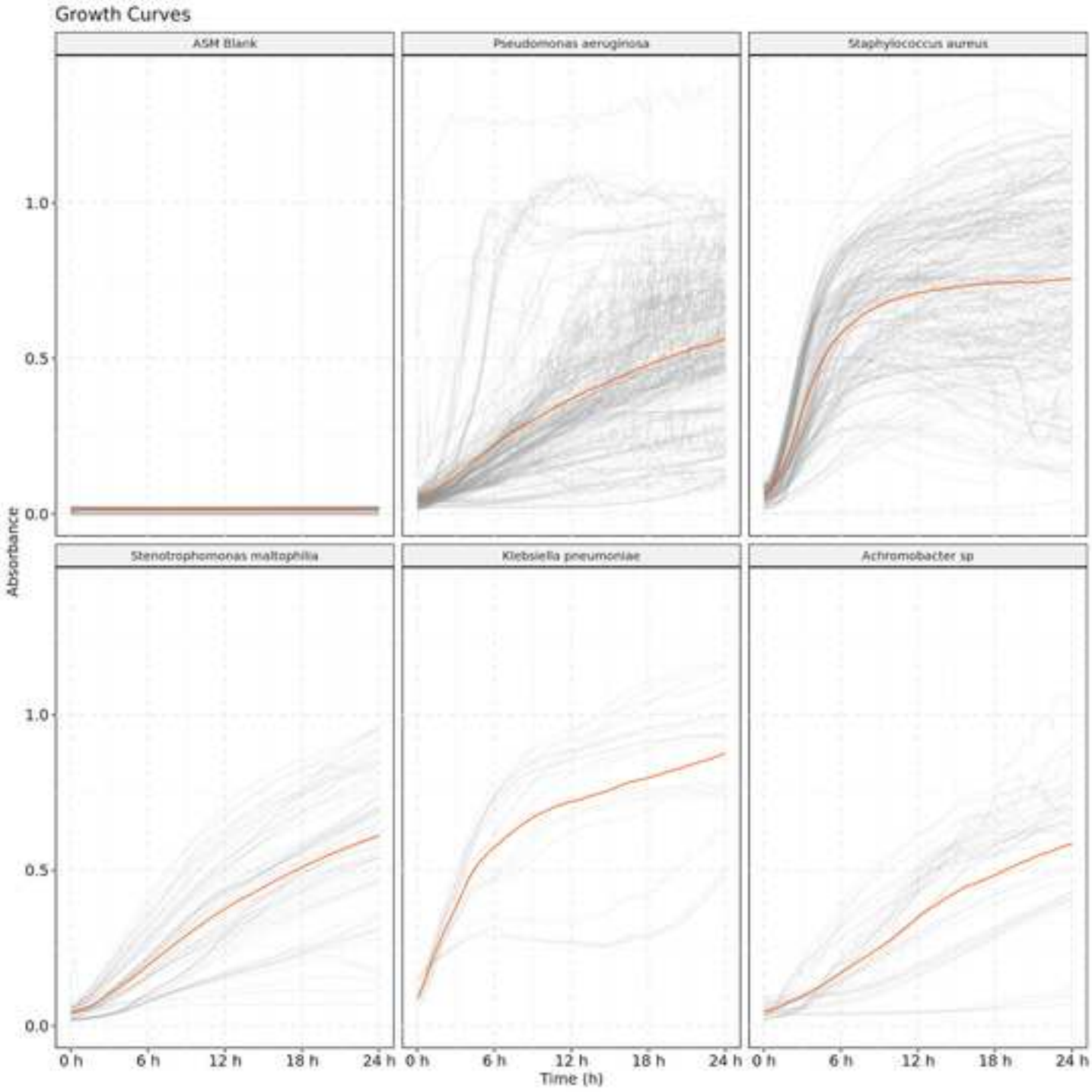

Table 1

Column 1	Column 2	Column 3	Column 4	Column 5	Column 6
Value	**Comstock**	**Kirchner**	**Sriramulu**	**Palmer**	**Flynn**
Mucin	2% w/v	0.5% w/v	0.5% w/v	-	1% w/v
Sodium Chloride	85.5 mM	85.5 mM	85.5 mM	66.6 mM	89.8 mM
Potassium Chloride	29.5 mM	29.5 mM	29.5 mM	15.8 mM	-
Magnesium Sulfate	-	-	-	0.6 mM	1 mM
Iron Sulfate	-	-	-	0.0036 mM	-
Ammonium Chloride	-	-	-	2.3 mM	60 mM
Monopotassium Phosphate	-	-	-	2.5 mM	60 mM
Glucose	-	-	-	3.2 mM	13 mM
Lactate	-	-	-	9 mM	-
Essential Amino Acids	14.45x	0.25 g/L	0.25 g/L	Per Acid	0.5x
Non-Essential Amino Acids	28.9x	0.25 g/L	0.25 g/L	Per Acid	0.25x
Vitamins	-	-	-	-	-
Trace Metals	-	-	-	-	1x
Egg Yolk	0.25%	0.25%	0.25%	-	-
Ferritin	0.0003 g/L	-	-	-	-
Salmon Sperm DNA	1.4 g/L	4 g/L	4 g/L	-	-
DPTA	-	-	0.0059 g/L	-	-
pH	-	6.9	-	6.8	-
Storage	0	4°	-	-	4°
Sterilization	Autoclave	Filter	Autoclave	-	Autoclave

Column 7	Column 8	Column 9
Gallagher	**Lai**	**Source**
2% w/v	1% w/v	Flynn
85.5 mM	152.3 mM	Lapierre
2.95 mM	15.8 mM	Palmer
1 mM	0.61 mM	Palmer
-	-	-
-	-	-
-	-	-
40 mM	0.7 mM	Sambeek
-	-	-
0.375x	1.29x	Palmer
0.5x	8.01x	Palmer
1x	1x	Gallagher
1x	1x	Flynn
0.25%	0.25%	Kirchner
0.0004 g/L	0.0004 g/L	Gallagher
1.4 g/L	-	-
-	-	-
-	6.3	Lapierre
4°	4°	Kirchner
Autoclave	Filter	Kirchner

Table 2

Column 1	Column 2	Column 3
	CF Sputum	ASM
Total Amino Acids	10.25 mM	10.76 mM
Alanine	0.96 mM	0.80 mM
Arginine	0.17 mM	0.94 mM
Asparagine		0.91 mM
Aspartic Acid	0.45 mM	0.80 mM
Cysteine	0.09 mM	0.33 mM
Glutamic Acid	0.84 mM	0.80 mM
Glycine	0.65 mM	0.80 mM
Histidine	0.28 mM	0.35 mM
Isoleucine	0.60 mM	0.52 mM
Leucine	0.87 mM	0.52 mM
Lysine	1.15 mM	0.64 mM
Methionine	0.34 mM	0.13 mM
Ornithine	0.36 mM	
Phenylalanine	0.29 mM	0.26 mM
Proline	0.90 mM	0.80 mM
Serine	0.78 mM	0.80 mM
Threonine	0.58 mM	0.52 mM
Tryptophan	0.07 mM	0.06 mM
Tyrosine	0.43 mM	0.26 mM
Valine	0.60 mM	0.52 mM

Dear Editor,

We appreciate the thoughtful comments. Below we address them in a point-by-point format.

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Response: This was done as suggested.

2. Please remove the citations from the Abstract and include them in the Introduction section.

Response: This was done as suggested.

3. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Response: This was done as suggested.

4. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets or dashes.

Response: Substeps have adhered to the number formatting that was suggested.

5. Please include a one-line space between each protocol step and then highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol and should be written in imperative tense.

Response: This was done as suggested.

6. Please include an ethics statement before the numbered protocol steps, indicating that the protocol follows the guidelines of your institution's human research ethics committee.

Response: This was done as suggested.

7. Please add more details to your protocol steps. In addition, please ensure you answer the "how" question, i.e., how is the step performed?

Line 157: Please specify the heating temperature and the stirring speed

Response: 50°C at 1100 rpm.

Line 187: How long can the solution be refrigerated?

Response: Up to one month. This was described in protocol but further emphasized.

Line 188/219/261: Please specify the filtration membrane.

Response: 0.22 μm filtration membrane pore size was previously detailed in the protocol but further emphasized.

Line 190: How much vacuum pressure to be applied?

Response: Vacuum pump reduces vacuum chamber pressure to 70 mbar (0.069 atm). This was added to the manuscript.

Line 120: This filter is of the same type as before? Please specify.

Response: The filter used for making ASM is a Stericup Quick Release-GP Sterile Vacuum Filtration System with a 0.22 µm pore size. The filter used as part of the oxygen sparging system is a to Millex 25 mm diameter sterile syringe filter with a 0.22 µm pore size. This has been emphasized in the protocol.

Line 254: Please include any patient inclusion/exclusion criteria.

Response: This was done as suggested.

Line 261/280/281: Is the needle diameter critical here? If so, please specify.

Response: Needles should be at least 18 gauge in size to facilitate sparging. This has been specified in the protocol.

Line 276: Please mention the incubation time.

Response: Samples are incubated for three 24-hour intervals. After each aliquot set is taken, samples are re-sparged and returned to incubation, for a total incubation time of 72 hours.

8. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials. Please sort the Materials Table alphabetically by the name of the material.

Response: This was one as suggested.

9. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

Response: The discussion was completely revised as suggested.

10. Please spell out the journal titles in the References.
Response: This was done as suggested.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
The aim of this paper is to propose an adaptation of the artificial sputum media to study the effect of oxygen concentration on cystic fibrosis airway microbiomes. While such technical paper is important for the community, I am confused by the way this paper is written. The abstract sounds promising but not really representative of the paper itself.

The paper is organized in 3 parts. The first one is about the elaboration of the culture medium and the oxygen sparking. It is well described, however unfortunately some information is lacking to reproduce the experiments.
The second part is/should be the results of hyperoxic conditions on sputum microbiome. This part sounds promising, but I found it hard to read and unclear. My main question being what experiments had been carried out and on how much patient sputum

The final part is a discussion which is mostly centered on the preparation of the medium. There is no real discussion on the effect of oxygen concentration on the sputum microbiote.

Response: We apologize for the lack of clarity in the manuscript. JoVE is a methods journal and this manuscript was meant to focus on the approach to studying the effect of supplemental oxygen on the lung microbiome rather than on the biology of such effects. We have revised both the abstract and the discussion to better reflect this.

Major Concerns:
Part 1 :
ASSM preparation : "set heat to medium-high". "Medium-high" do not mean something. What is the real temperature used? Do you verify the effect of the temperature on the stability of mucin ? Since you heat ASSM, could it be autoclaved for storage?
Response: The Artificial Sputum Mucin Mix (ASMM) is heated to 50°C to facilitate dissolving of the mucin; while proteins may be subject to denaturation at temperatures above 40°C, studies suggest that this is not the case with mucin[1]. The temperatures we use for heating the ASMM is far below the standard autoclaving temperatures are 120°C thus we would recommend preparation of the ASMM at the time of preparing artificial sputum medium.

ASBM preparation : According to Palmer, the amino-acid composition of artificial sputum media is important. However, you do not detail the composition of your essential amino acid and non-essential amino acid stock solutions. This information should be well detailed.
Response: The used essential and non-essential amino acid stocks have amino acid ratios designed for the creation of minimum essential media, and the overall concentration of essential and non-amino acids are tailored as a group to the overall identified concentration in CF sputum. We now include **Table 2** where the final per-amino-acid concentrations are listed for the ASM detailed in this protocol, as well as the concentrations previously described in CF sputum by Palmer *et al*[2] for comparison.

	CF Sputum	**ASM**
Total Amino Acids	10.25 mM	10.76 mM
Alanine	0.96 mM	0.80 mM
Arginine	0.17 mM	0.94 mM
Asparagine		0.91 mM
Aspartic Acid	0.45 mM	0.80 mM
Cysteine	0.09 mM	0.33 mM
Glutamic Acid	0.84 mM	0.80 mM
Glycine	0.65 mM	0.80 mM
Histidine	0.28 mM	0.35 mM
Isoleucine	0.60 mM	0.52 mM
Leucine	0.87 mM	0.52 mM
Lysine	1.15 mM	0.64 mM
Methionine	0.34 mM	0.13 mM
Ornithine	0.36 mM	

Phenylalanine	0.29 mM	0.26 mM
Proline	0.90 mM	0.80 mM
Serine	0.78 mM	0.80 mM
Threonine	0.58 mM	0.52 mM
Tryptophan	0.07 mM	0.06 mM
Tyrosine	0.43 mM	0.26 mM
Valine	0.60 mM	0.52 mM

ASM : The duration of the filtration runs over 1 to 2 days. Is the filtration done at room temperature in a cold room or do you use a refrigerated orbital shaker?
Response: Filtration is done in a cold room at 4°C. This detail has been added to the protocol.

Serum bottle culture sparging : In which case, is it necessary to dilute the patient sample? If a dilution do you use 1mL of patient sputum or 1mL of the diluted sputum?
Response: Dilution of patient sputum was necessary in cases where the volume of sputum provided was insufficient to create all of the necessary aliquots for each culture condition. The same volume of sputum (diluted or undiluted) is used regardless of whether dilution was necessary, and we control for dilution factor in calculation of absolute abundance.

Part 2 :
You used 50 sputum from patients. Could you please give some precision, like the age of the patients, how long have patients been followed for cystic fibrosis ?, are sputum from 50 patients or not?…? The microbiote of the sputum depends on the age of the decease and should be different for younger than older patients.
Response: This is a methods paper and so we did not include the patient characteristics of the 50 patients from whom sputum was obtained. This information will be presented in a separate paper focused on the biological effect of supplemental oxygen on the cystic fibrosis lung microbiome.

If I understand well, you used "only" 11 sputum patients for nucleic acid extraction. How were chosen these 11 sputum? Is this restriction only done for these extractions?
Response: Each sputum generated 10 potential samples for sequencing; 1 uncultured sputum sample, 9 cultured sputum samples corresponding to each oxygen (21%, 50%, 100%) and time (24 hour, 48 hour, 72 hour) condition, thus a total of 110 samples underwent metagenomics sequencing. Cultured and uncultured samples from the first 11 patients enrolled in our study underwent nucleic acid extraction and sequencing. We were limited by the available budget for this study as metagenomics sequencing is significantly more costly than amplicon sequencing. The limited number of sequenced samples will limit the power for differential abundance testing, but not for omnibus measures of the microbiome such as measures of alpha and beta diversity.

Figure 2 : Do the data correspond to the mean measurements for the 50 sputum?
Response: Yes, the data correspond to mean measurements for the 50 sputum samples. This has been clarified in the figure legend.
Figure 3 : Same remark as figure 2. Why do you show results for only one condition? Did you do the measurements for the 8 other conditions?

Response: The purpose of this manuscript is to validate or methodological approach, thus we compare uncultured sputum with sputum cultured under the 21% oxygen condition for 48 hours.

Figure 4 : Were the 120 identified microbial species, isolated from 11 or 50 sputum? This is unclear.
Response: Aliquots of cultured and uncultured sputum were obtained from 11 patients and sequenced. This has been clarified in the figure legends.

Figure 5 : Same remarks as in figure 4, do you get these isolates from the 11 or the 50 sputum?

Response: Clinical isolates were obtained from 50 different patients.

Does the 53 isolates for Pseudomonas aeruginosa correspond to 53 different strains or to 53 phenotypically different clinical isolates (same remarks for the other bacteria isolates)?
For Pseudomonas aeruginosa, measuring optical density at 600nm may be tricky because of the produced pigments which will have an impact on the measurements. This is important since different isolates may produced different amount of pigments with time leading to misinterpretation of the culture growth curves. To avoid this, it is better to culture Pseudomonas aeruginosa in the dark which usually limits the pigment production. Did you make the bacteria cultures in the dark or not?
Response: We thank the reviewer for this comment. Each isolate was a phenotypically different clinical isolate. All growth curves were obtained by culturing each isolate in the dark. This detail has been added to the Figure 5 legend as detailed below.

"**Figure 5** shows absorbance-based growth curves of common CF lung pathogens isolated from sputum obtained from 50 different pwCF. These isolates represent phenotypically different clinical isolates obtained using procedures from the Massachusetts General Hospital Clnical Microbiology Laboratory, and include *Pseudomonas aeruginosa* (N = 53), *Staphylococcus aureus* (N = 37), *Stenotrophomonas maltophilia* (N = 12), *Klebsiella pneumoniae* (N = 3), and *Achromobacter sp* (N = 7)."

Part 3 :
While the discussion on the creation of your own artificial sputum medium is well documented, the discussion on the effect of oxygen on the cultured sputum is in my opinion lacking. Please develop your experimental results in the discussion… Or change the title and the abstract of your paper focusing on the creation of this artificial medium for use in studies of the oxygen effects on cultured bacteria.
Response: We have changed the title of this methods paper to "Design and development of a model to study the effect of supplemental oxygen on the cystic fibrosis airway microbiome" and modified the abstract as suggested.

Minor Concerns:
line 294 : One should read "In addition, 0.5mL aliquot..."

Response: Thank you for this comment, the typo has been corrected as suggested.

Reviewer #2:
Manuscript Summary:
The authors have developed a model system which allows to examine the effects of supplemental oxygen on cystic fibrosis lung microbial communities. They have shown the associated oxygen sparging protocol. This system enables the mixing of compressed oxygen and air to fixed

concentrations ranging from 21 to 100 percent oxygen. They outlined the procedures to create artificial sputum medium (ASM) and the construction and use of an oxygen sparging system, and the application of both to culture CF sputum under differential oxygen conditions.

Major Concerns:

1) In the artificial sputum medium preparation methods (lines 188 - 196), the authors explained how they sterilized the medium by using a filtration technique, in which a 0.22 µm filter was used, resulting in a relatively transparent medium afterward. The authors stressed that one filter was supposed to be used for approximately 350 mL. Otherwise, mucin plug would occur. This is the primary concern. The medium's transparency is clear evidence indicating that some components (e.g., mucin) have been clogged on the filter, which raises an uncertainty of the concentration of the medium's components. For example, 1% mucin was assigned in the first place, but after filtration, the mucin concentration may be lower, which might have impact on bacterial behavior (see the paper by Sriramulu et al., J Med Microbiol 2005). Authors should discuss this point.

Response: We thank the reviewer for this comment. Based on the editor and this reviewer's comment, we have completely revised the discussion including addressing the possibility that the filtered artificial sputum medium has mucin concentrations lower than the unfiltered 1% as detailed below:

"While filter-sterilization has been proposed in other protocols[3], we have found that the addition of orbital shaking during the filtration process was essential to prevent clogging of the filter that otherwise occurred when a minimum amount of the prepared medium had been filtered. While the filtered artificial sputum medium may have a lower-than-intended final mucin concentration due to mucin impaction in the filter, sputum from pwCF has been shown to have lower mucin concentrations than sputum from people without cystic fibrosis[4]. Our starting mucin concentration of 1% is higher than other approaches that have used filter-sterilization, with one group using a starting mucin concentration of 0.5%[3], while typical recipes use mucin concentrations ranging from 0.5% to 2% (**Table 1**). Thus even with loss of mucin in the filter-sterilization process, our final medium is likely to have mucin concentrations within the physiological range[5]"

2) Usually ASM contains high amount of salmon-sperm DNA (1.4 - 4 g/L). Why did the authors omit this component?

Response: We have revised the discussion to address the rationale for omitting salmon-sperm DNA from our recipe.

"Salmon sperm DNA, a common addition to existing recipes (**Table 1**), was omitted. One intended application of this model is metagenomics sequencing, and thus salmon sperm DNA was not included in order to reduce the addition of non-microbial nucleic acids as these reads would be filtered out after sequencing thus decreasing our effective sequencing depth. While sputum from cystic fibrosis patients has high concentrations of extracellular DNA[6], a significant proportion of it is microbial in origin[7], and it is unclear if the addition of salmon sperm DNA to the artificial sputum medium makes it more physiologic, or whether the culture approach described in this protocol leads to high levels of microbially-derived extracellular DNA; we did not distinguish between extra- and intra-cellular DNA concentrations in our studies. Future studies may wish to verify the concentration of extra-cellular DNA generated by this culturing method."

3) Why did the authors use 21%, 50%, and 100% O2 concentrations? How do these concentrations correlate with those used in the clinical practice?

Response: We wanted to use a range of oxygen concentration seen in clinical settings. 21% oxygen corresponds to no supplemental oxygen administration (i.e. room air), while 100% oxygen is the

maximum amount of oxygen administered which is commonly given using a high flow oxygen setup or by mechanical ventilation. 50% was chosen as an intermediate value commonly seen in clinical settings.

4) Why does the pH increase with time at all O2 concentrations (Fig. 2)?

Response: Using mixed effects models, the pH increase over time was not found to be statistically significant, with an estimated increase of 2.820E-3 per hour and a p-value of 0.292.

5) Is any correlation between pH changes and microbial load and/or diversity?
Response: There is no correlation between either pH changes and microbial load (Estimate = 4.779E-5, p = 0.543), pH changes and alpha diversity (Shannon Alpha Diversity: Estimate = 2.441E-5, p = 0.858), nor pH changes and beta diversity (Bray-Curtis Beta Diversity: R^2 = 0.00098, p = 0.462
Jaccard Beta Diversity: R^2 = 0.00066, p = 0.614).

Minor Concerns:
1) Jaikumpun et al. (Int. J. Mol. Sci. 2020) have recently used ASM to investigate the growth of P. aeruginosa and S. aureus. They used different methods to study bacterial growth (CFU, flow citometry) because of the turbidity of the medium.
The authors might consider to compare the current data with these previous findings.

Response: We thank the reviewer for this helpful suggestion. We use filter rather than heat-sterilization and thus the resulting artificial sputum medium is not turbid and is therefore amenable to optical-based measures of growth. In this manuscript the growth curves were used to demonstrate that our artificial sputum medium recipe can support the growth of a large number of clinical isolates from CF sputum; in future studies focused on microbial growth, CFU or flow-cytometry-based measures of microbial growth would indeed be important to verify the optical measures used in this protocol.

2) The abstract is too long especially its introductory part. Furthermore, it is also too general and does not contain specific data.

Response: We have shortened the abstract and added additional experimental detail as suggested.

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