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Inhaled Microbiomics: Targeted Delivery of Prebiotics and Antibiotics in Patients with Cystic Fibrosis

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BY

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THESIS

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DEDICATION

I dedicate this work to my grandparents, Jack and Carol McChesney, and Charles and Margaret Gaddy.

Before the age of significant consciousness, so 4 or 5, Margaret (Granny) found me strategically dissecting my brand-new toy. Granny was fast to let me know that I was misbehaving with my new item. However, I too was fast to let her know "hang on, I'm having an experience." My vocabulary may not have been equipped to describe the 'experiment' I was executing, nonetheless, Charles (Granddad) came to my rescue as a handy lab aid in my research and to defend my endeavors. From that day on, my whole family has offered consistent and wholehearted support in my pathway into research. While I sometimes remain unsure if I am having an 'experiment' or an 'experience,' I know for certain that I make them proud and am honored to do so.

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ABSTRACT

Adults with Cystic Fibrosis (CF) are a unique and expanding patient population in the context of massive progress in disease screening and treatment. However, exacerbations remain a significant complication of the disease associated with its decline. CF pathophysiology promotes chronic respiratory bacterial infections, which are implicated in exacerbations. An important finding in research surrounding the respiratory microbiome has correlated the loss of diversity being with a worse prognosis in chronic lung diseases. We developed an aerosol formulation towards supporting the existing, diverse respiratory microbiome. Our formulation of prebiotics with and without antibiotics was designed to fit within the complex, multistep, inhaled therapy used by CF patients. The excipients used were safe and cost-effective, offering an optimal SD process. The formulation was seen to be of the appropriate size characteristics for pulmonary delivery. Further, in-vitro data highlights preclinical safety and efficacy in bacterial and human cell models.

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CHAPTER 1: INTRODUCTION

CF and Public Health Considerations

Cystic Fibrosis (CF) is an incredibly unique disease state within the current healthcare climate. This population has shown tremendous expansion over recent decades, as reported by the Cystic Fibrosis Foundation (CFF) 2021 Patient Registry (1). Growth in CF patient population can be attributed to the downstream effects of substantial progress in CF screening, diagnostics, and treatments. These advancements in practice are reflected in a decrease in all-cause mortality in CF from 1.53% to 0.7%, increased median age of death from 25.6 years to 33.9, and an increase in five-year survival from 36.3% to 53.1% (1). Over the last 30 years, the number of individuals with CF that make it to adulthood has doubled, and it is expected to continue rising.

These groundbreaking advancements in practice have created an uncommon phenomenon in the current healthcare realm: an unprecedented and expanding CF patient population. To briefly explain, CF has been a chronic disease with detrimental outcomes for decades. When first described in 1938, the disease was found postmortem in infants who had died at average ages of less than 18 months (2). As recent as 1978, the life expectancy had only just begun to exceed 11 years old (2). In the early 2000s, life expectancy had climbed massively to just under 30 years old (2), and the current estimates speculate that patients live beyond 50 years (1). The massive improvements in patient healthcare and downstream CF patient outcomes have allowed these patients to live fuller and healthier lives than ever in the history of this disease. However, this has created a patient population that was fundamentally non-existent just a few decades earlier: an aging adult population with CF.

While patient outcomes have seen impressive progress, there still is a significant complication of CF that is associated with morbidity and progression of disease: exacerbations (1, 3, 4). Exacerbations can be briefly defined as a sudden increase in respiratory symptoms with a marked decrease in lung function (often permanent). Exacerbations are frequently associated with bacterial and viral infections (5), and CF patients are often chronically inhabited by pathogenic organisms (6). Per the CFF report, in 2021, an estimated 20,000 pulmonary exacerbations occurred (1), and these episodes have previously been significantly correlated with CF disease progression and severity (4).

Thus, we theorize that reducing the incidence and severity of these exacerbations could be a mainstay in retarding the disease and promoting the overall well-being of this expanding patient population.

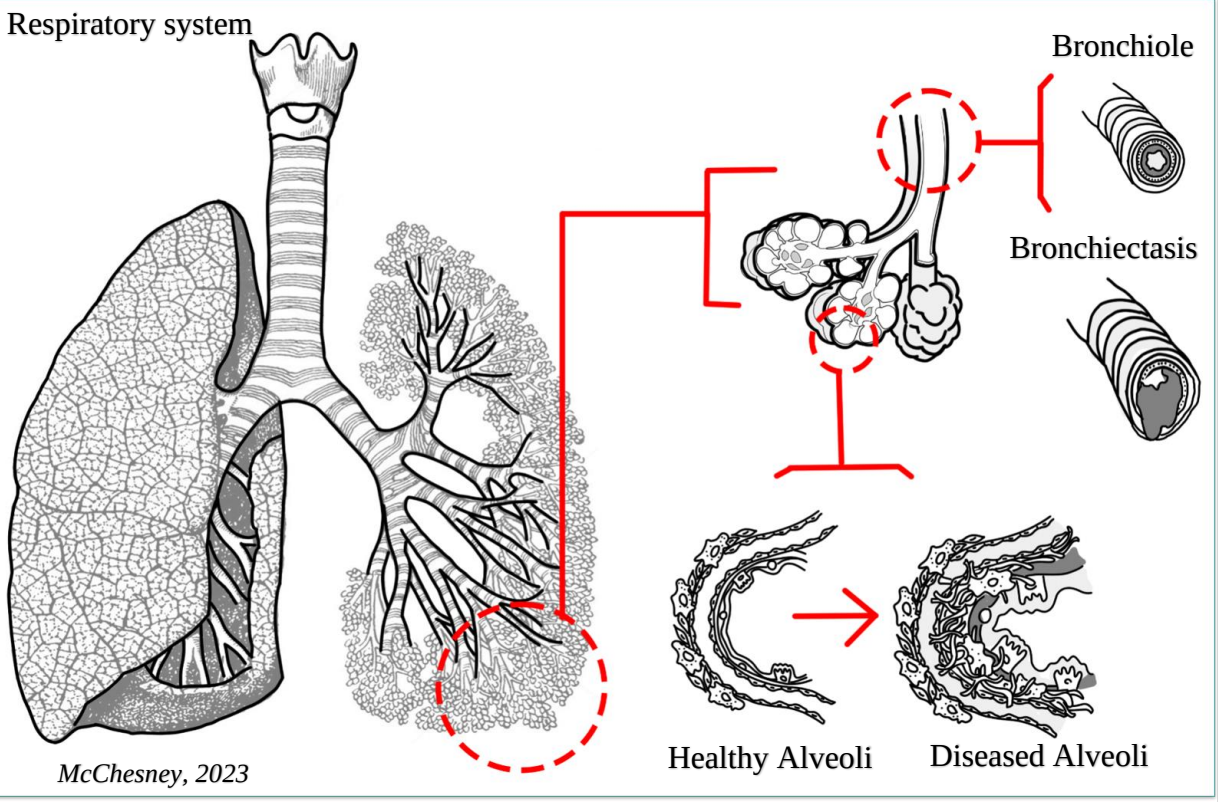


Fig 1: Artwork highlighting the pathological changes in the respiratory system of a CF lung, with focus on bronchiole and alveolar changes.

CF Pathophysiology and the Respiratory Microbiome

CF is a chronic, progressive, autosomal recessive genetic disease caused by the abnormal functioning of the cystic fibrosis transmembrane conductance regulator protein (CFTR) that affects all the body's organ systems (7). The lung is most often implicated in morbidity and mortality (3, 8), as respiratory/cardiorespiratory pathologies account for more than 44.4% of all-cause mortality (1). **Fig 1** visualizes pertinent physiological factors in CF that play a role in respiratory microbiome concepts. The respiratory microbiome, as the name implies, is the ecosystem of bacteria living within the respiratory tract (9). CF patient lung

factors create microenvironments, which are key to the incredibly complex relationship between the disease state and the existing bacterial inhabitants. Briefly, some of these factors include the formation of a biofilm, increased viscosity of mucus, widening and thickening of the airways (bronchiectasis), and altered lung pH (11).

On the biochemical scale, this pathophysiology of the CF lung provides a landscape for chronic bacterial infections as the tissues are more easily exposed to, inhabited by, and less able to clear pathogenic bacteria (6). This promotes chronic inhabitation of bacterial species not common to a healthy respiratory system. Bacterial colonization has been seen in 63.8% of sputum cultures of CF patients as of 2021, with the most common inhabitants being *P. aeruginosa* and *S. aureus* (1, 4, 12). CF exacerbations caused by these pathogenic bacteria are associated with an increased risk of pro-inflammatory cascades, expedited decline in lung function, and a worse prognosis (4). The negative health outcomes with these pathogenic bacterial inhabitants are further shown by the induction of epithelial cell death (13), suppression of the host immune system (14), and antagonistic effects against antibiotic treatments (15).

Thus, the role of the respiratory microbiome in CF has hit the forefront of clinical research. The moderation of these negative effects of these pathogenic bacterial species would theoretically offer critical improvement to exacerbation management, improving morbidity and mortality downstream. Before discussing the antibiotic treatments for CF exacerbations and their clinical outcomes, it is important to understand the other inhabitants of the respiratory microbiome and their potential commensal effects. Current literature has focused on these potentially beneficial species given the recurrent, and significant findings about the state of the disease and its relationship with the respiratory microbiome. To summarize these findings in one sentence: a loss in microbiome diversity with increased bacterial abundance has been associated with a worse prognosis in chronic lung diseases such as CF (16, 17, 18). The negative findings of these trials equate to a respiratory microbiome, which is often overrun with pathogenic bacterial colonization and lacking bacterial diversity. Further, investigations have shown that dysbiosis (imbalance or disruption) of the respiratory microbiota ecosystem is associated with accelerated loss in lung function, increased inflammation, and expedited progression of the disease state (9). Expanding further on the commensal effects of certain respiratory bacteria, includes the modulation of immune responses, changes in gene expression

and biotransformation of endogenous and exogenous compounds (including macromolecules, viruses, and bacteria) (17, 19).

Anaerobic bacterial species are often the focus of current studies of the commensal microbiome in CF. Below are brief summaries of the beneficial as well as the potentially negative effects of these bacteria within CF patients. *Prevotella* has been a prominent organism described in the literature. Some of the contributions of *Prevotella sp.* include decreased loss in FEV_1 and inflammation during exacerbations (20), prevention of *P. aeruginosa* from dominating the endogenous microbiota (20), and the secretion of small chain fatty acids (SCFAs). SCFAs are key macromolecules to healthy respiratory function and only synthesized through bacterial metabolism (21). Other bacterial species often seen in a similar light to *Prevotella*, are *Veillonella* and *Porphyromonas* (22). Like Prevotella, these bacteria play a key role in respiratory health (23). However, all aforementioned commensal bacteria also show the potential to induce harm to the respiratory physiology. Some of the established risks include opportunistic infections (24), secretion of beta-lactamases (25), and a pro-inflammatory response if the secretion of SCFAs is excessive (22).

For this reason, our dry powder formulation will aim to support the existing, diverse respiratory microbiome – and not to add or remove any species.

The Respiratory Microbiome Treatment Conundrum

Many of the recent improvements in CF patient morbidity, mortality, and exacerbation frequency can be attributed the use of Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) modulators (1). However, bacterial considerations remain critical in the management of CF based on the etiology discussed above.

Within CF management, antibiotics are used as both maintenance medications as well as for the acute treatments in exacerbations. Tobramycin as a maintenance therapy is used in nearly 60% of patients, as it is recommended for patients with evidence of *P. aeruginosa* colonization (1). Tobramycin, for this indication, is used in 28-day cycles towards preventing the pathogenic expansion of P. aeruginosa. For the treatment of acute exacerbations, sputum cultures are used towards guiding antibiotic therapy for the specific pathogen (4). However, data of antibiotic treatments highlights a limitation in the ability to decrease the microbial

density within the respiratory tract (26) while inducing a significant loss in microbial diversity (27). The failure to decrease the quantity of bacterial load while inducing dysbiosis of the existing microbiome is a key consideration in the limitations of antibiotic treatments for CF exacerbation. This bleaching of the microbiome (depletion of the bacterial diversity and composition) while retaining a copious total bacteria burden are factors associated with worse prognoses in lung diseases (17, 18).

The notion that current antibiotic treatments may deepen the individual's susceptibility to future infections and potentiate the progression of the disease creates a conundrum in CF management. Above, we discussed the pathogenic bacteria's role in CF progression, alongside increasing evidence of the beneficial role of a diverse lung microbiome. These data highlight the complexity of CF treatment. On the one hand, there is increasing knowledge on the importance of a diverse microbiome and its critical function on a pathophysiological scale. On the other hand, bacterial inhabitants are known to worsen infections, which could be opportunistic and may induce various complications.

Thus, we hypothesize that preventing a loss in the diverse microbiome may offer a mechanism to slow CF disease progression. Due to the conflicting microbiome data, our formulation will aim to support a diverse respiratory microbiome rather than introducing new bacterial species into the lungs. Our investigational formulation will serve as a prebiotic rather than a probiotic. Prebiotics are the macromolecules critical for bacterial survival, while probiotics are the living bacterial species themselves. As there is uncertainty over the pathogenic versus mutualistic roles of the microbiome, providing specific nutrients to feed the existing diverse bacterial inhabitants in the lung is a lesser risk than a targeted treatment of **potentially** beneficial bacteria.

Therefore, we hypothesize that targeted inhaled delivery of prebiotic powders into the deep lung (alveolar deposition) will promote and support the bacterial diversity within the existing respiratory microbiome, thereby decreasing morbidity and disease progression in CF patients.

CF Standards of Care

The pharmaceutical design investigated within this research will fit into the already existing, complex, multistep therapy process used by CF patients many times per day (1, 12, 28). Existing treatment consists of pretreatment with a bronchodilator, followed by an osmotic agent, a mucus lysing agent, and finally, inhaled antibiotics or other maintenance therapies. Many patients use Airway Clearance Techniques immediately after the use of the mucus lysing agent. The most common clearance technique use percussion vests and/or exercise (1). Following the clearance of mucus, the patient takes the final treatment consisting of inhaled antibiotics – which is the point of care our treatment will focus on.

The current standards of CF inhaled treatments are summarized in greater detail in **Table 1**. These summaries include treatment classifications, general formulation considerations, dosing, cost, and frequency of use in CF patients by percentage. This current standard of care highlights the burden of effort and treatment cost to CF patients. Based on the per-dosing cost estimates (without insurance), treatments using the most common medications would result in a daily cost of $610.41 to $900.38, not including the $2,000-$15,000 percussion vest often used. Thus, any new pharmaceutical developments for CF must consider the existing complex treatment care plan so as not to further complicate an excessively complex regimen.

For this reason, our formulation is designed around macromolecules that are cost-effective, have proven human safety data within the respiratory tract, and fit directly into the current CF treatment regimens.

Table 1: Common treatments utilized in the management of CF. Formulation, dosing, and administration were retrieved from IBM Micromedex®. These pharmaceutical considerations were verified using Lexicomp®, which wa able to provide additional information in the form of cost. Frequency of utilization is based on the CF Foundation Annual patient reporting, as of 2021 (5).

Drug class	Example	Formulation / Administration	Dosing	Cost / dose	Frequency of use
Bronchodilator	Albuterol	Aerosol Solution, metered dose inhaler	PRN, and 2 doses prior to ACT	$0.37	93%
Osmotic Agent	Hypertonic Saline HyperSal®	Nebulized solution	4 mL twice daily	$0.88	70%
	Mannitol Bronchitol®	Dry powder inhaler	400 mg via 10 caps twice daily	$78.3	No data
Mucolytic Agent	Dornase Alfa Pulmozyme ®	Jet nebulizer ™ system	2.5 mL once daily	$60.53	88.4%
Airway Clearance	Percussion Vest	Nonpharmacological	Penultimate step in airway clearance	$2k - $15k	76.3%
Inhaled Antibiotics	Nebulized Tobramycin	Nebulized solution	300 mg / 4mL twice daily	$141.2	59.6% (no data per dosage form)
	TOBI®	Dry powder inhaler, Podhaler ™	4 caps (28mg each) twice daily	$243.8	
Modulators*	Ivacaftor	Oral treatments, taken daily to target CFTR mutation	Combination products based on patient genomics	$250 - $530	85.1%
	Lumacaftor				
	Tezacaftor				
Anti-Inflammatory	Azithromycin	Oral tablet	250 mg daily, or 250 - 500 mg three times weekly	$7.77 - $15.54	56.4%
	Ibuprofen	Oral capsule	25 – 30 mg / kg per day	~$0.01 per kg	No data
Pancreatic Replacement Therapy*	Lipase, Protease, Amylase	Oral capsule	25,000 – 50,000 units / meal	$7.21 - $9.41	> 80%

Hypothesis and Formulation Design

Based on the discussion above, promoting diversity in the existing respiratory microbiota of CF patients is hypothesized to be of substantial clinical benefit. We will directly deliver our nutrient-rich cargo to the existing microbiota of the respiratory tract using targeted delivery of spray-dried (SD) inhalable powders. Other investigations have relied upon the gut-lung axis to affect the respiratory microbiome (29). However, enteral treatments have shown minimal impact on restoring the lung microbiome (30). For this reason, targeted inhaled delivery directly into the deep lungs using SD powders may offer a novel mechanism to affect the respiratory microbiome (positively) in CF patients. Towards this goal, our study has the following three aims:

- Formulate an inhalable prebiotic powder with the antibiotic tobramycin and without, as outlined (**Table 2**).
- Characterize the dry powder formulation for particle size, composition, and stability.
- Evaluate cytotoxicity and immunogenicity of the powder formulation using *in-vitro* bacterial and human co-culture cells.

Table 2: Formulation design for dosing of each experimental group. For SD all formulations were solvated in aqueous media at 1% w/v. Components include tobramycin, small chain fatty acids (SCFA), Arginine (Arg), and Leucine (Leu)

ID	**Tobramycin**	**SCFA**	**Arg**	**Leu**
Tobramycin	112 mg			
T(+)	112 mg	10 mg	64 mg	64 mg
T(-)		10 mg	64 mg	64 mg

Our proposed formulation will fit in at the step of the “inhaled antibiotics” treatment regimen shown in **Table 1**. Formulation components are summarized in **Table 2**. The dose of

tobramycin within our formulation is based on that used in the FDA-approved treatment (112 mg per dose; TOBI PODHALER® from Novartis) (31). In current practice, patients who use inhaled tobramycin do so on 28-day cycles. During the 28 days that they are not taking the inhaled antibiotic, there is no treatment they use in its place (i.e., they take nothing). In our proposed treatment regimen, T(+) would replace the 28-day cycle with tobramycin, and T(-) would replace the 28 days without treatment.

To summarize the formulation groups (**Table 2**), tobramycin refers to the antibiotic without prebiotics, a positive or negative control for our studies. Within the prebiotic-containing samples, T(+) contains tobramycin and T(-) without tobramycin. It is critical to note that throughout our studies, tobramycin may be referenced as a component of T(+) or as a sole antibiotic (when used as an experimental control).

To further explain the investigational formulations, T(+) and T(-) contain the "prebiotic blend" that is referenced throughout this research and is the novelty of the investigational formulation. This prebiotic blend is composed of SCFAs (small chain fatty acids), L-Arginine (Arg), and L-Leucine (Leu). These macromolecules are selected for multiple reasons beyond just the function as a prebiotic to supply nutrients to the endogenous lung flora. SCFAs are key macromolecules to respiratory function and health, and they are exogenous compounds only made through our natural flora (21). The SCFAs utilized in our formulation include calcium acetate, calcium propionate, and sodium butyrate at a 3:1:1 ratio, respectively, reflecting their endogenous concentrations (32). In addition to being a key amino acid in many metabolic processes, Arg also has mucolytic properties (33). Recent clinical trials have tested inhaled dry powder Arg in CF patients with noted improvement of pulmonary function (34). Leu is used in many bacterial metabolic processes and is a common excipient used in SD for pulmonary delivery (35).

The novelty of our investigational formulation includes providing concurrent prebiotics while administering the respiratory antibiotic tobramycin and then interrupting the antibiotic treatment with prebiotics alone to promote a diverse and healthy lung microbiome.

CHAPTER 2: MATERIALS AND METHODS

Materials

Calcium acetate (C1000), sodium butyrate (303410), calcium propionate (21230), L-arginine (A8094), L-leucine (61819), and tobramycin (PHR1079) were obtained from Sigma-Aldrich. Ultrapure water was obtained in-house via ThermoScientific Barnstead E-PURE, 4-Module System.

Formulation Development

Dry powders were formulated using the Buchi B-290 Mini spray dryer (Buchi Corporation, Flawil, Switzerland) equipped with a two-fluid nozzle, with fluid transport via the inbuilt peristaltic pump and a 6mm silicone tube. SD solutions were prepared within VWR Ultra-High Performance Centrifuge Tubes, combining the various powders by weight (2% w/v at the ratios outlined in **Table 2**) and dissolved using water; with only gentle vortex-mixing needed to dissolve all solutes. The solutions were SD using a continuous flow rate of 4 mL/min, an outlet temperature between 50-55 °C, an atomizing gas pressure of 742 L/h, and an aspirator setting of 100%. These SD parameters were kept constant for all the spray drying experiments. The SD results will be expressed as the manufacturing process yield and any important process parameters related to the specific SD operation. For powder yields after SD, a yield greater than 50% is workable for lab scale assessments and yields greater than 70% reflect an "excellent" manufacturing process (36).

Formulation Assessment

Thermal gravimetric analysis for residual moisture content was performed on SD powders following manufacture. Using the VWR 1370 FM Forced Air Oven and VWR-220TC high-precision balance, loss in powder weight was made following incubation at 110°C for 48 hours at roughly 200 mg per test. Changes in total powder mass were measured before and after the incubation step; loss in mass was attributed to the evaporation of residual moisture from the SD powders. This type of assessment is commonly performed within pharmaceutical

spray drying research (10). The percentage of residual moisture is a key factor in dry powder formulations due to their impact on formulation stability and sterility (37). When using gravimetric analyses, a common target for residual moisture content is less than 5% weight by volume (38).

Volumetric particle size [Dx(50)] and particle size distribution (PSD) were determined using laser diffraction via the Malvern Mastersizer 3000, equipped with the Aero S dry dispersion apparatus. For PSD analyses, 5-10 mg of powder was used in duplicate (N=2) for all samples manufactured. Particle sizing was performed at a pressure of 4 bar and 100% feed rate. Particle sizes are reported as Dx(50), which equates to the median diameter of the particle size distribution; Dx(10) represents the size below which 10% of the total volume of particles is present; Dx(90) correlates to the particle size below which 90% of the total volume of particles are found; and span reflects the variability or width in the PSD and reflects the homogeneity of these SD samples (39). In addition to the numeric distributions of sizes, graphical representations are included for all studies. PSD measurements are employed to highlight considerations of the samples size, homogeneity, and clumping characteristics. These are essential factors for formulations for pulmonary delivery, as it has been established that sizes less than 5 microns improve drug delivery into the deep lung compared to larger and/or clumpier particles (39). Assessments of PSD were repeated following storage at 40 °C and 4 °C for 6 months to detect any preliminary stability concerns as it relates to storage conditions and sizing characteristics thereafter.

A Next-Generation impactor (NGI) was used to determine the aerodynamic particle size distribution (APSD) of SD samples. APSD is reported as the mass median aerodynamic diameter (MMAD) and the fine particle fraction of less than 4.6 microns ($FPF_{4.6}$) at a flow rate of 60 L/min. A flow rate of 60 L/min was used for all NGI experiments as this flow rate collects more particles within the size range reflective of deep lung deposition (39, 40). In addition to the numerical summaries of data, graphical depictions of the APSD are included. MMAD and $FPF_{4.6}$ are vital markers in respiratory drug delivery, as they offer further insight to the particle characteristics that may affect their movement through the aerodynamically complex respiratory tract. The aerosolized movement of particles through bronchioles of decreasing diameter will show any impact particle density could have on their deposition into the deep

lung (41). For each assessment, 10 capsules (Size 3; Capsugel; Hypromellose Capsules Vcaps Plus) of roughly 200 mg of SD samples were introduced stepwise to the NGI using an Aerolizer® inhaler. During each NGI run, the flow rate was checked using a digital flowmeter every 5 capsules to ensure it remained consistent at 60 L/min. Following administration of all 10 capsules into the NGI, the powder deposition at the various stages was measured gravimetrically using the VWR-220TC high-precision balance.

Pharmaceutical Assessment

Using the SpectraMax ID5 spectrophotometer, the SD formulation was assessed for tobramycin loading. The T(+) samples were solvated in ultrapure water and analyzed at the 280 nm wavelength based on the findings of preliminary spectra (**Supplemental Figure 1**). Absorbance data of SD T(+) samples was compared to standards of known quantities of pure tobramycin, and calculations against these standards allowed for quantification of tobramycin within the T(+) sample. Drug loading, as the name implies, is the percent of the active pharmaceutical ingredient per unit of total mass of powder. Within SD, certain API (active pharmaceutical ingredients) may be preferentially lost within manufacturing processes. Thus, verification of API loading per powder mass is a critical assessment. Without this information, the amount of API in the SD powder would be unknown – and patient dosing would be unreliable.

MIC Assessment of SD powders

The minimum inhibitory concentration (MIC) of the T(+) sample was evaluated in gram-positive (G+) and gram-negative (G-) bacterial species, against a positive control of pure tobramycin. For the determination of MIC towards a G+ species, *S. aureus* (MRSA) (USA300 methicillin-resistant *Staphylococcus aureus* AH1263) was used based on literature, which has shown its applicability to pathogenic MRSA within the healthcare setting (42). *E. coli* (*Escherichia coli* BL21DE3, New England Biolabs, Ipswich, MA, USA) was studied to determine the MIC towards a G- species. This strain of *E. coli* has been extensively studied in investigations of bacterial genomics and transcription (43). However, the use of *E. coli* within our investigation was to test for MIC against a well-known G+ bacterial strain.

Briefly, MIC assessments of our SD powders were made per CLSI M07-A9 protocols (44) with slight modifications as follows. Bacterial colonies were prepared overnight in Trypticase Soy Broth (TSB; BD and Co., Franklin Lakes, NJ, USA) and placed on a shaking incubator at 37 °C for 12-18 hours. After incubation, to prepare the inoculum, bacteria from the overnight stock were diluted in TSB until the optical density range was between 0.1 and 0.3 when read at 600 nm on a SpectraMax ID3 spectrophotometer (Molecular Devices, San Jose, CA, USA); this dilution equates to a range of 9×10^3 to 2.7×10^4 CFU/mL. Using these diluted bacterial samples, round bottom 96-well plates were prepared using TSB and exposed to serial dilutions of the tobramycin-containing SD samples. These plates were placed within an incubator (set at 37 °C) for 16-20 hours to allow bacterial growth. In addition to the MIC plates, bacterial colonies were also plated on blood agar media (BD and Co.) to determine colony forming units (CFU).

For MIC determination, both visual metric and spectrophotometry were used. Visually, following the CSLI guidelines, the MIC was detected as the last column within the plate that lacked visible growth (>2 mm button or definite turbidity). For spectrophotometric analysis, the plate was read at 600 nm, and growth was defined as samples with greater than 3 times the absorbance readings of the negative control. MIC determination was only made if there were successful positive and negative controls; otherwise, the experiment was determined to be a failure due to contamination. CFU counts provided secondary validation to ensure that plates included sufficient bacteria for MIC assessment.

Cell Studies

Human cell lines were studied using a co-culture of A549 and THP-1 cells for assessments of cytotoxicity and immunogenicity. The decision to assess cytotoxic and immunogenic effects on human cells was due to the potential for toxicities as seen in literature for the various compounds contained within our formulation (20, 21, 45, 46). A549 cells were selected to model cells within the respiratory microbiome as they are derived from human lung epithelial adenocarcinoma cells (47). THP-1 cells are dendritic cells derived from leukemia monocytes, and they are used to model inflammatory and infectious effects as demonstrated in cell research (48). This co-culture, incorporating A549 and THP-1 cells, offers a novel

mechanism for assessing the cytotoxic and immunogenic effects for human cells of the respiratory tract. The co-culture was seeded at a 5:1 ratio of A549 cells to THP-1 within a 96 well plate (Corning™, NY) for all assays, equating to 15,000 and 3,000 cells respectively, and confirmed via hemocytometer (Fisher Scientific, PA).

Sample exposure to the co-culture occurred over a 48-hour window for varied concentrations of T(+), 100ug/mL of T(-), and tobramycin (50ug/mL) as a positive control and the diluent media as a negative control. For these studies, the diluent media used was RPMI1640 and F-12K media (10% fetal bovine serum, 1% pen/strep, 0.5mM sodium pyruvate, 0.025mM 2-mercaptoethanol). The concentration of the T(+) sample used in these experiments ranged from 1 to 100 ug/mL. The preliminary tests were focused on T(+) as it held all components of the other samples (i.e., having tobramycin in addition to the prebiotic blend).

For assessments of cytotoxicity, using the MTT [3-(5,4-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] and LDH (lactate dehydrogenase) assays were performed per product protocols following exposure to samples. The MTT (Promega; CellTiter 96 Non-Radioactive Cell Proliferation Assay, MTT) was used to assess markers of cytotoxicity based on metabolic activity of mitochondria and/or interruptions to cell proliferation (49). The LDH (Promega; LDH-Glo Cytoxicity Assay) assay was used as a secondary marker for cytotoxicity, as it is an enzyme released from cells upon the rupture of plasma membrane correlating to apoptosis, cellular damage, or other etiologies of cellular death (50). The LDH and MTT assays were analyzed via SpectraMax iD5 (Molecular Devices) and SpectraMax 340PC (Molecular Devices), respectively.

Towards assessing the potential for immunogenicity following exposure to our samples, the supernatant of the co-culture was collected for ELISA (ThermoFisher; Luminex xMAP MAGPIX) for detection of the target antigens IL-6 and IL-8 (Bio Techne; Human XL Cytokine Luminex® Performance Panel). The presence of these specific antigens was picked given their known role as broad spectrum cytokines with implications throughout the inflammatory cascade (51). For both the cytotoxic and immunogenetic analyses, all assessments incorporated a one-way ANOVA, with Bonferroni's test for correction of multiple comparisons.

CHAPTER 3: RESULTS, DISCUSSION, AND FUTURE DIRECTIONS

Spray Drying Manufacturing

In total, 11 SD runs were performed using the process parameters mentioned in the **Methods** section. Of these 11 runs, 7 were for the T(-) samples, and 4 were for the T(+). The average product yield and PSD data for all the SD runs are summarized in **Table 3**. The average yield for the T(+) and the T(-) sample was 76.6% ± 3.7 and 78.1 ± 4.9, respectively. Thermal gravimetric analysis determined the water content for SD samples to be 5.6% ± 0.6% (N=3) with no significant difference between the residual moisture content between the T(+) and the T(-) formulations. Gravimetric analysis was not performed on every SD batch since this is a destructive analytical method which required a large powder volume per assessment. In addition to the yield and residual moisture finding of impartiality between samples, no complications related to the SD of these products were found during manufacture. This ease and consistency of manufacture for these samples reflects the potential for practical scalability from the benchtop scale.

Table 3: Summary of SD manufacturing and characterization data for the T(-) (N=7) and T(+) (N=4) samples and their standard deviations.

ID	Yield	Dx(10)	Dx(50)	Dx(90)	Span
T(-)	78.1% ± 4.9	0.27 ± 0.02	1.61 ± 0.25	3.78 ± 0.50	2.18 ± 0.11
T(+)	76.6% ± 3.7	0.24 ±0.01	1.33 ± 0.30	3.22 ± 0.44	2.28 ± 0.17

Formulation Characteristics

Particle size distribution (PSD) data were consistent across the different experimental groups, as shown in **Table 3**. PSD analyses were performed on all eleven SD samples. The

data show consistent size ranges across all batches, further supported in the graphical data shown in **Fig 2**. The PSD, with a Dx(50) ranging from 1.33 to 1.61 microns, reflects the appropriate particle size range for inhaled delivery. The PSD shown in **Fig 2** further supports the SD powders' free-flowing and non-clumping nature. The graphical distribution of PSD highlights a considerable component of the powder sample of size less than 1 micron in size, as seen in the left-sided deviation of the otherwise normal curve. Further, stability testing of PSD showed that time and temperature (4 °C versus 40 °C for 6 months) had no impact on the distribution of sizes (**Supplementary Figure 2**).

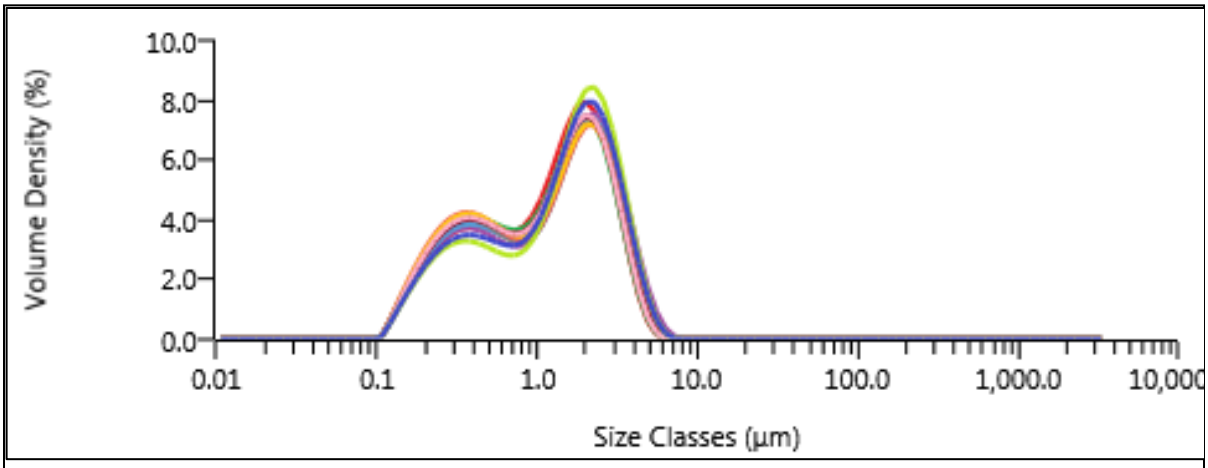


Fig 2: PSD graphs for all SD samples are depicted graphically. The x-axis depicts the size range of the particles, with an associated y-axis depicting the percent volume of particles of that size. A legend for each sample identifier is not included, given the similarity of all graphical data.

Aerodynamic sizing via the NGI highlighted the effect of particle composition, shape, and density on APSD. The presence of tobramycin in the SD powders increased the particle's density, thereby slightly altering their aerodynamic characteristics. This difference is clear in the larger MMAD and smaller $FPF_{4.6}$ for T(+) samples compared to T(-) samples. The APSD of T(+) and the T(-) powders can be visualized in the graphical representation and numerically in **Fig 3** and **Table 4**, respectively. However, the $FPF_{4.6}$ for samples only varied by an average of 2.2% between these samples; the impact on the particle penetration and deposition into the deep lung is unlikely to be affected significantly. We also saw slightly higher powder deposition in stage 3 (cut-off diameter 4.6µm) for T(+) samples compared to T(-) samples, though this did not adversely impact their MMAD. The percent deposition of the SD samples

within the NGI was approximately 32.0%, and no differences were seen in NGI deposition between T(+) and the T(-) powders. Although the total powder deposited in the NGI is low, the considerable loss of powder is associated with powders deposited in the NGI filters and is not due to the powder's properties (i.e., the powder being retained within the gelatin capsules or the mouth/throat/collecting tray of the NGI). Our results show that the MMAD is larger than the median geometric particle size (Dx50); which is often seen in aerosol particles based on the behavior of particles and the influence of their shape and density.

Table 4: Numerical summaries of APSD for the T(+) and T(-) samples, reported as MMAD and $FPF_{4.6}$ with associated standard deviations.

Sample ID	**T(-)**	**T(+)**
FPF(4.6)	79.1% +/- 0.735	81.3% +/- 10.12
MMAD	3.82 um +/- 0.06	2.72 um +/- 0.9

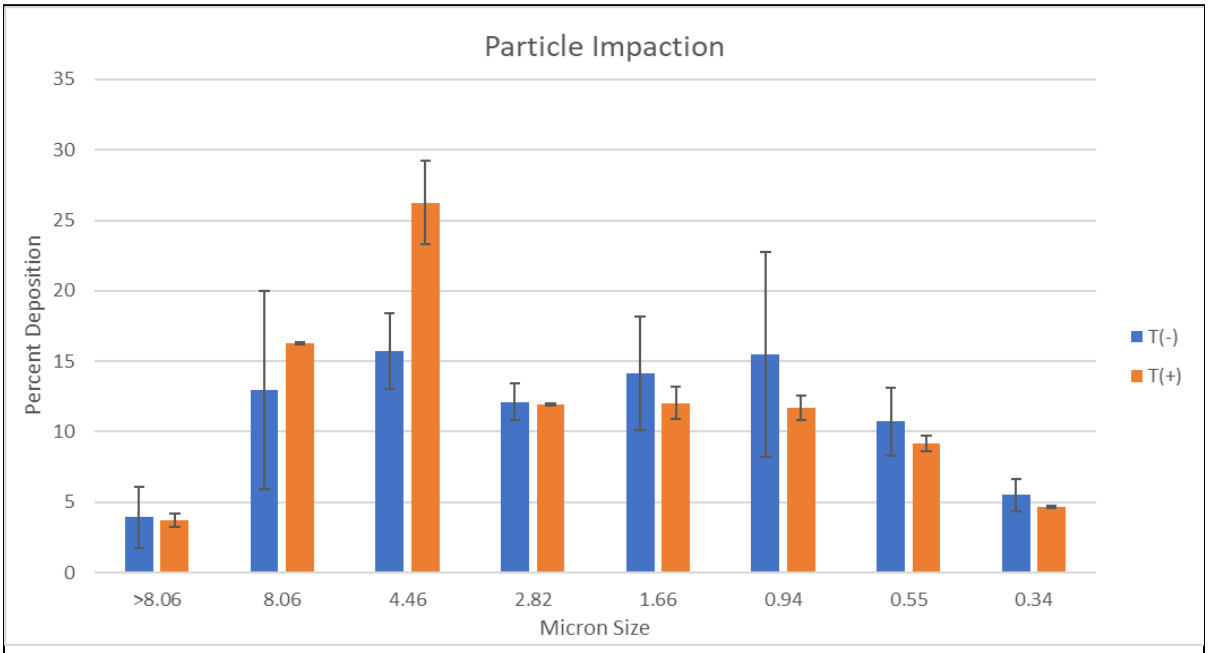


Fig 3: Depicts the graphical APSD for T(-) (blue), and T(+) (orange) samples. The x-axis represents the micron sized cut-off for the specific stage of the NGI. The y-axis is the percent of powder deposited in the particular tray, as a percentage of the total powder mass collected. Error bars are included for the standard deviation between experimental samples, T(-) N = 4 and T(+) N = 2.

Spectrophotometric Quantification of Tobramycin

Tobramycin loading efficiency was 99.4% for 3 different T(+) SD batches compared to the theoretical loading with a standard deviation of 18.9% (each sample ran in duplicate). This implies that tobramycin was successfully loaded into the particles after SD; however, the standard deviation implies the SD process may be sensitive to batch-to-batch variation. Thus, assessing the drug loading efficiency is a key parameter in characterizing the SD powders, especially during scale-up and repeated manufacturing studies.

Bacterial MIC After Exposure to SD Powders

MIC was determined for tobramycin and T(+) samples when exposed to MRSA and *E. coli*. For both iterations, the MIC was determined to be 2 ug/mL (**Supplemental Table 1**). Studies were performed in triplicate per assessment, and each experiment was performed twice. Our data show similar MIC values for the G+ and G- bacterial species when exposed to our SD samples, implying that prebiotics within the T(+) sample do not inhibit the antibacterial activity when compared to the tobramycin control.

Cytotoxicity and Immunogenicity Cell Studies

Tobramycin and T(+) samples were assessed for cytotoxicity and immunogenicity in a co-culture of THP-1 and A-549 cells. This co-culture of human cell line of monocytes and lung epithelial cells better mimics the complex cellular interactions within the lungs, thus providing a relevant *in-vitro* model for studying lung diseases, infection mechanisms, and drug efficacy and toxicity testing. MTT and LDH cytotoxic assays were conducted after exposing the cells to our samples at various concentrations (5, 10, 50, and 100 µg/ml of T(+), 100 ug/mL of T(-), and 50 µg/ml of tobramycin). These assays are commonly used in cell culture research to measure cell viability, cytotoxicity, and cell death. T(+) samples showed no significant difference when compared to tobramycin (positive control), 100 ug/mL of T(-), or the diluent (RPMI1640 and F-12K media) for the MTT and LDH assays (**Fig 4**).

Immunogenicity studies were also conducted using the co-culture of THP-1 and A-549 cells, towards representing the inflammatory responses seen in lung diseases. Specifically,

THP-1 cells differentiated into macrophage-like cells allow for studying immune cell activation and cytokine production in response to pathogens or inflammatory insults. We evaluated the cytokines IL-6 and IL-8 using ELISA after the co-culture cells were exposed to different concentrations of our samples, analogous to the cytotoxic studies mentioned above. The secretion of these cytokines by these cells could indicate cellular activation and inflammation, offering valuable insight into the interaction between immune cells (THP-1) and epithelial cells (A549) in the context of pulmonary diseases. The ELISA results showed no significant differences in IL-6 or IL-8 cytokine secretion from cells exposed to SD samples, including different T(+) concentrations, T(-), tobramycin, and the diluent alone (**Fig 5**). These data indicate that the investigational formulation causes no induction to cytotoxic or inflammatory effects on representative respiratory human cell lines.

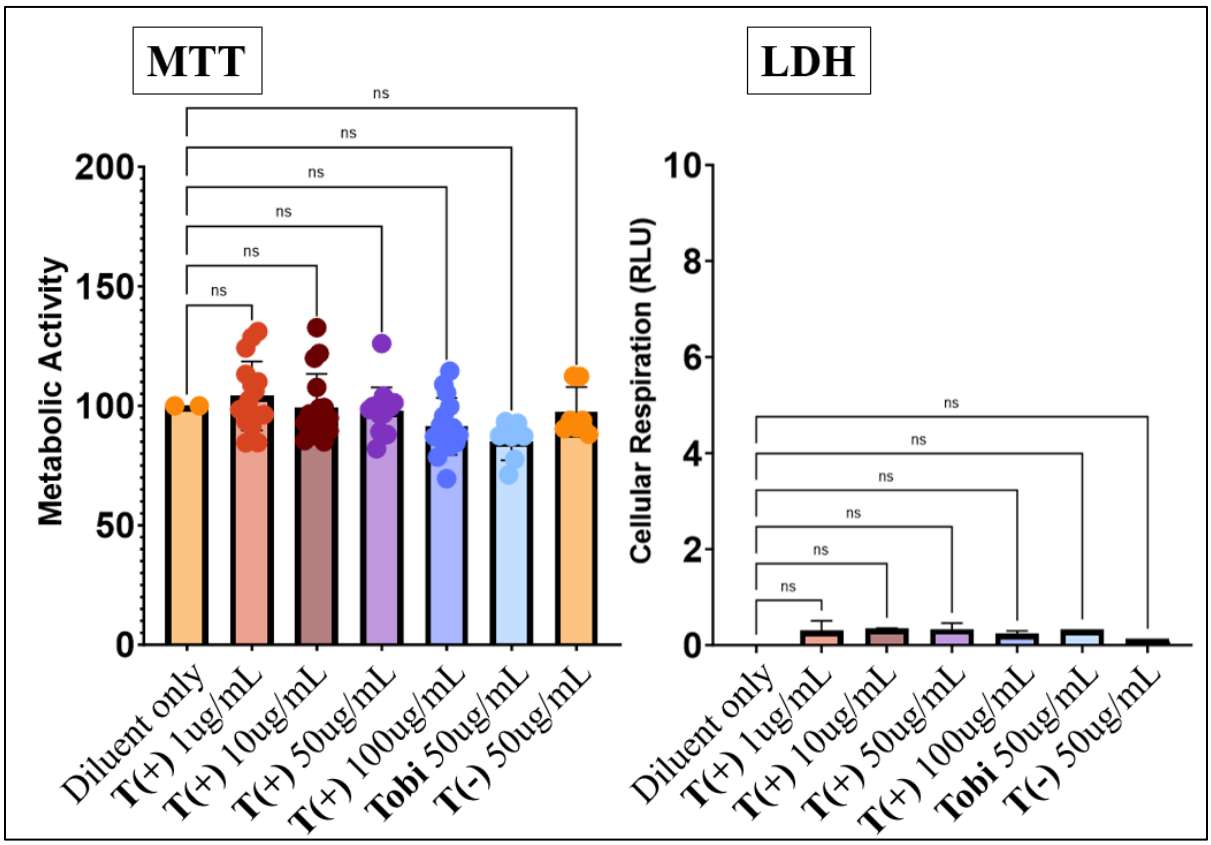


Fig 4: Graphical summaries for cytotoxic (MTT and LDH) assays. The y-axis summarizes the concentration of the various biomarkers, and the sample which the co-culture was

exposed to is listed across the x-axis. For media only, cells were exposed to the diluent used for all cell studies being the RPMI1640 and F-12K. All other samples include the experimental sample names and the concentration within this media. Error bars and tests for significance in between samples are included.

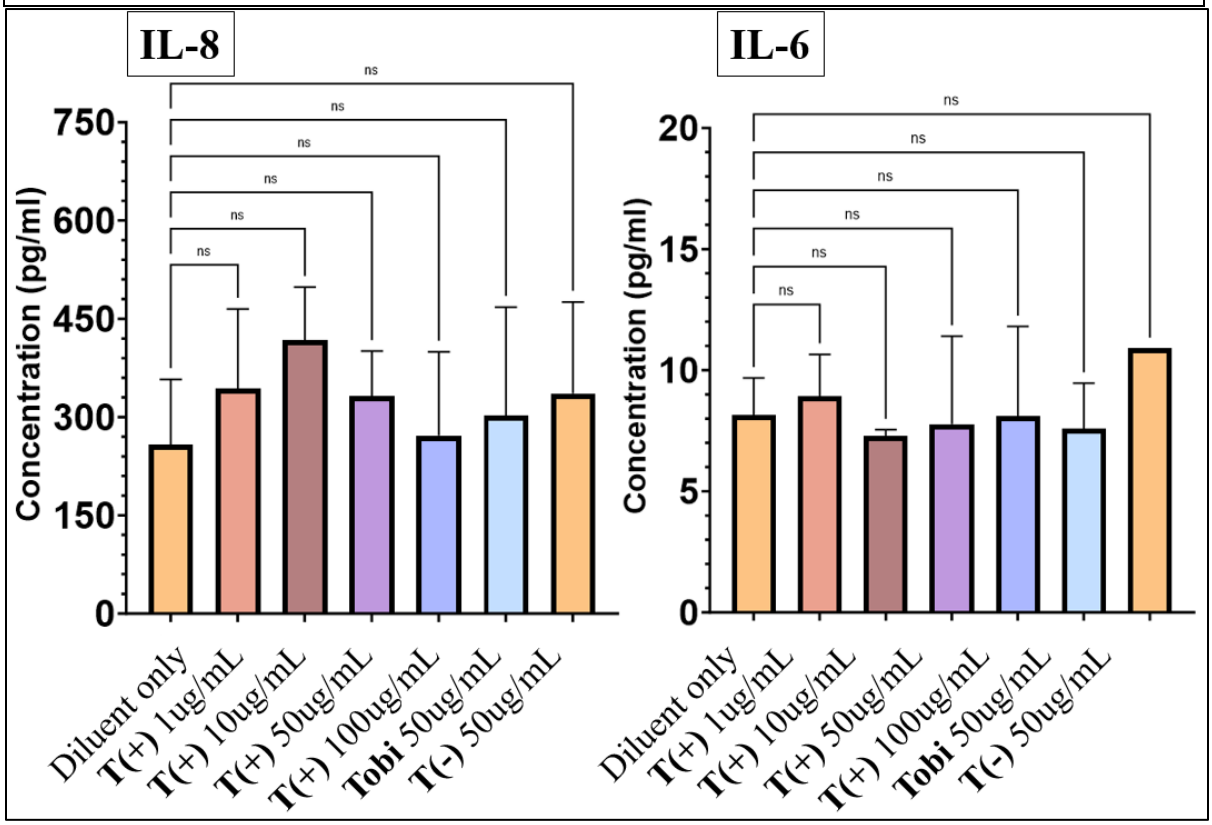


Fig 5: Immunogenic (IL-8 and IL-6) ELISAs which show no significant difference between experimental samples and controls. See **Fig 4** description for explanation of x and y-axis.

Discussion

Patients with CF will continue to improve in metrics of life quality and longevity, and treatments towards enhancing these outcomes are critical. In this research, we propose a mechanism to aid in the preservation of the complex, fragile respiratory microbiome that is prone to being disrupted in CF patients. At the current stage of care, this biota is constantly being bombarded with antibiotics, leading to patient lungs being progressively colonized with pathogenic bacteria. Towards abating these adverse effects, our novel inhaled formulation aims

to support bacterial diversity and health of the microbiome via provision of key macromolecules and nutrients.

Based on our literature reviews, this is the first instance in pharmaceutical formulation development wherein an investigational product is proposed to provide prebiotics directly to the deep lung. While the disease state of focus for the current project is CF, this mechanism of supporting the respiratory microbiota could prove beneficial across other lung pathologies. Worsened disease states with an increased abundance of specific respiratory bacteria while compromising on their diversity are seen across many lung diseases, including asthma and COPD (16, 17, 18).

In our formulation assessments, PSD and APSD are highly encouraging for inhaled delivery. Dry powders with a Dx(50) of 1.33-1.61, span 2.18-2.28, FPF4.6 79.1-81.3%, and MMAD of 2.72-3.82 microns are compatible with pulmonary delivery. An important consideration in PSD and APSD data, however, is the presence of particles less than 1 micron in size. This is seen in the left-sided deviation about the otherwise normal PSD curve (**Fig 2**), and a slight increase in APSD at the size cut off 0.94 microns (**Fig 3**). While graphically evident, the impact these nano-sized particles have on the overall PSD or APSD numerically was minimal (**Table 3, 4)**. These smaller ranges may be nano-sized particles generated during the SD process or the effects of particle shearing when propelled through the analytical sizing devices. As our formulation continues to be investigated, further testing of these smaller particles is necessary, as particles less than 0.5 microns may not be compatible with deep lung delivery. This is secondary to their small size limiting residence in the respiratory tract and increasing the chance that they are exhaled during expiration (39).

The focus of stability testing was to reassess the PSD of the dry powder after storage. Sizing is the first step in ensuring the product has remained stable, as it is the fundamental factor that may limit the particle's ability to penetrate the deep lung, i.e., if the powder has altered size characteristics after storage, it may no longer be delivered into the deep lung, and any further tests are futile as these particles will not reach the site of action. The sizing data (**Supplementary Figure 2**) showed that storage at extreme conditions caused no alteration in the size or distribution of particles within the SD sample. Based on these findings we can

conclude that these powders are highly stable from a sizing standpoint and should be investigated further to understand the temporal relationship to their safety and efficacy.

After PSD and APSD, drug loading evaluations of the T(+) samples were completed to estimate the quantity of tobramycin within the sample versus the theoretical loading. Data implied that tobramycin was loaded at a high proficiency with moderate variability, equating to an average loading of 99.4% +/- 18.9, N=3. This variability of loading (standard deviation 18.9%) may be due to batch-to-batch variability, or it could reflect limitations within the quantification protocol (as discussed further below in the limitations). If variability is proven to be inter-batch, and not simply analytical, measurements of drug loading are essential for all subsequent SD batches to control for variability in the API per manufactured mass. Drug loading data was not collected for the T(-) sample as it does not incorporate tobramycin. Future directions include the quantification of the various components of the prebiotic blend and secondary validation of the tobramycin loading using another analytical method.

Following pharmaceutical characterizations, assessments were made towards in-vitro efficacy of the T(+) sample on G+ and G- bacterial species. These MIC trials investigated the impact of the prebiotic blend on the antibacterial efficacy of tobramycin. Bacterial growth inhibitory testing was the focus of our trials, as the known risks of the pathogenic species outweigh the benefits surrounding the commensal species. Given this research's purpose of promoting respiratory microbiome’s diversity and health, it is essential to study the growth characteristics of commensal species in the future. There were two reasons inhibitory testing was the main investigation of this research. First, exposing the CF lung (inhabiting pathogenic bacteria) to prebiotics would be impractical without ensuring the antibiotic will still function appropriately. Second, the contentious data surrounding commensal bacteria makes studying the beneficial effects on these species somewhat futile. Nonetheless, bacterial MIC testing showed that the prebiotic blend had no impact on the efficacy of tobramycin towards MRSA or E. coli. Thus, we conclude that tobramycin maintained its efficacy on these bacterial species in the presence of our prebiotic blend.

In-vitro evaluations of our powder blend were done in A549 and THP-1 co-culture to model the human respiratory epithelia and monocytes. While the bacterial studies were focused on substantiating in-vitro efficacy, human cell line studies confirmed the preliminary safety of

our SD formulation. Analyses were focused on capturing any cytotoxicity or immunogenicity induced by SD samples, and thus broad markers of these cellular effects were selected. For cytotoxicity, MTT and LDH are well-established assays that provide markers of metabolic derangement, cellular damage, and death. Towards immunogenicity, the cytokines IL-8 and IL-6 were measured, given their implications throughout the inflammatory cascade. For both the cytotoxic and immunogenic studies, there was no significant difference between the various experimental groups tested. These studies were focused on varied concentrations of the T(+) sample, as it contains all components of the experimental groups (i.e., the prebiotic blend and tobramycin). As no significant difference was observed between samples and controls, the human cell studies offered preliminary insight into a lack of cytotoxicity or immunogenicity to human cells induced by the SD powders. Further, our data was conclusive that we felt further trials were not warranted to study varying concentrations of T(-) and tobramycin.

These proof-of-concept studies are promising for further pursuing inhaled prebiotic treatment to sustain a healthy, diverse respiratory microbiome in CF. However, our investigation has many limitations, which are summarized below. At the current scale, many limitations are due to funding and time constraints on the execution of further testing. It is also fair to say that these excuses (time and money) are obvious deflections when conducting graduate research. Therefore, for each limitation, I have provided a future direction on how to improve those limitations.

The first limitation is the quantification of tobramycin and the excipients within the SD formulation. Our protocol for quantifying tobramycin is not supported or established in the literature, and these assessments offered no insight into the specific composition of the prebiotic blend. The protocol for tobramycin quantification was based on preliminary trials, which showed spectral absorbance only at high concentrations (40 mg SD powder or 20 mg of tobramycin per mL water) at wavelengths less than 300 nm (**Supplemental Figure 1**). Analyses of the ‘prebiotic blend’ of the SCFA, Leu, and Arg did not show absorbance when analyzed at these conditions, supporting that the loading data reflected tobramycin contained within the sample. However, our preliminary testing must be supported by future high-performance liquid chromatography (HPLC) or gas chromatography-mass spectrometry (GCMS) protocols. These higher sensitivity analytical techniques will offer secondary

quantification of tobramycin and provide insight into the prebiotic blend composition. These loading trials are essential for future studies within this investigation. Without these more sensitive and specific tests, the current loading data for tobramycin within the formulation should be considered hypothetical.

Another relevant concern for the pharmaceutical powder characterizations of PSD (using laser diffraction) and APSD (using NGI), is their translation potential to actual CF patients. As the pathology of this disease affects the airway size, air pressure, surface characteristics, and overall volume of air moved per breath, the feasibility of the PSD data using these analytical devices for CF patients needs further investigation. However, the current industry standards for CF treatments using pulmonary delivery use similar analytical processes for PSD and APSD measurements. As analytical devices become more representative of the complex respiratory pathophysiology of CF, PSD and APSD characterization techniques should be reassessed.

Another limitation of this study is that we did not show that our dry powder formulation provided any beneficial effects on the bacterial colonizers of the respiratory microbiome. A barrier to investigating positive effects on the respiratory biome is the ambiguous data surrounding which bacteria are the beneficial species. Thus, to investigate if the formulation had a favorable effect on the beneficial bacteria – we would first have to determine what beneficial bacteria truly are. Assessments of bacterial commensalism in CF were not embedded in our study outline and thus outside the scope of this research. That said, it is highly likely that providing the prebiotics contained within our investigational formulation would support bacterial diversity within the lung microbiome.

The final limitation is using *E. coli* as a G- bacterial species for the MIC studies. This bacterial strain was selected as it did not require (temporally) extensive training and protocol approvals to work with the more representative lung pathogen *P. aeruginosa*. *P. aeruginosa* is the G- species most highly implicated in CF pathogenesis and should be the focus of future in-vitro testing. *E. coli* is not the appropriate bacterial species for respiratory pathogenesis and was used due to the experimental timeline constraints. Of note, *P. aeruginosa* has now been approved for future assessments of MIC that are more relevant to CF.

Our study represents a preliminary, proof-of-concept investigation that is both years and millions of dollars away from hitting the microbiota that needs it most. That said, the process toward pre-clinical animal toxicity studies and subsequent human trials would face fewer barriers when compared to other new investigational agents. Each component within our formulation has previously been tested for human safety after inhaled delivery (31, 33, 34, 35), except for SCFA. Therefore, establishing the safety of our combination product would potentially bypass many stages of approval within regulation and translational sciences.

In conclusion, through SD formulation and various pharmaceutical characterizations, we have successfully provided early proof of our hypothesis. Through this research, we have successfully formulated prebiotic powders with and without the antibiotic tobramycin. We characterized these powders for their PSD and APSD properties, which were ideal for pulmonary delivery. Bacterial MIC assessments showed that the prebiotic component of the powder did not inhibit the antibacterial effects of tobramycin against MRSA or *E. coli* in-vitro. Human cell lines using a novel co-culture of A549 and THP-1 cell lines showed no significant cytotoxicity or immunogenicity upon exposure to our investigational product. These data highlight the potential for continued pursuit of our novel investigational formulation at a clinical scale in CF.

Conclusion

We formulated our prebiotic powders with and without tobramycin using the SD process. We demonstrated that the powders were the proper size for deep lung deposition. *In-vitro* cell culture data supports this treatment toward pathogenic bacterial killing for G+ and G- species, without induction of toxicity or immunogenicity towards a representative respiratory co-culture of A549 and THP-1 cells. These preliminary findings support our hypothesis of pursuing respiratory prebiotic treatment as an adjunct to existing respiratory antibiotic therapy within CF patients.

APPENDICES

Supplemental Material

Supplementary Table 1 depicts the formulation data per SD sample for all manufacturing runs. All yields, PSD, and APSD are reported here.

Supplementary Table 1: Individual formulation data for all samples manufactured in SD, including yields, PSD, and APSD data. The different samples are identified based on the nomenclature used throughout the investigation, and the included superscript reflects the specific sample used in distinguishing between batches. Blank boxes indicate that the specific tests of APSD were not performed on those samples.

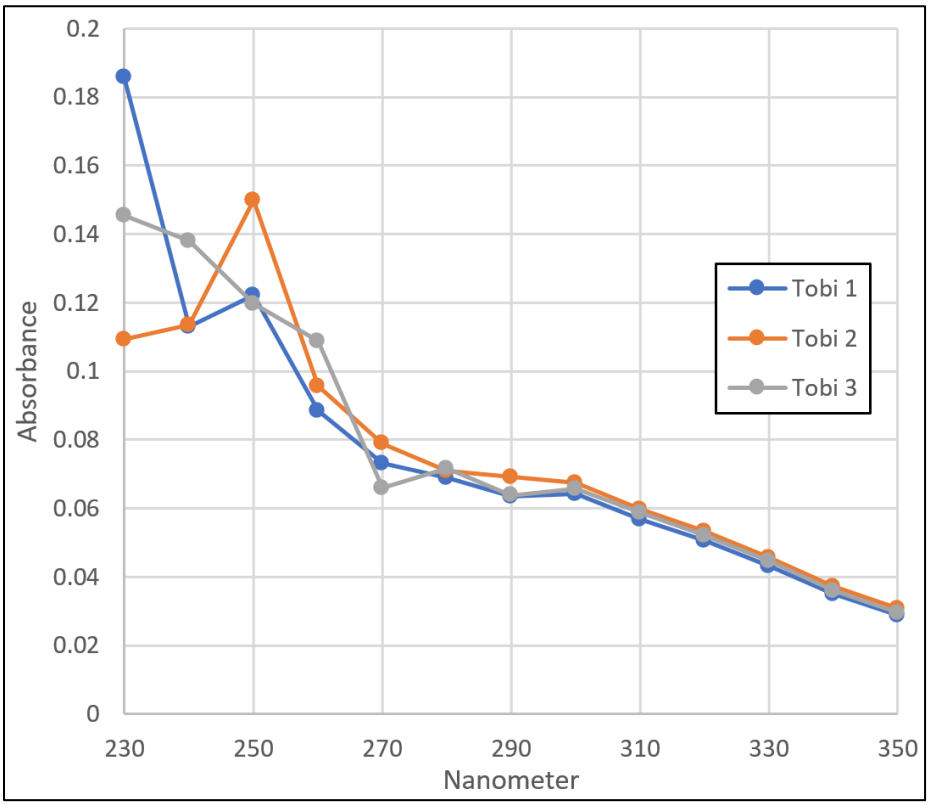
ID	Yield	Dx (10)	Dx (50)	Dx (90)	Span	FPF - 4.6	MMAD
T(-)1	76.4%	0.248	1.32	3.29	2.306	87.12	2.21
T(-)2	81.6%	0.245	1.27	3.23	2.357	87.17	2.33
T(-)3	68.2%	0.26	1.45	3.3	2.101	83.5	2.23
T(-)4	82.9%	0.272	1.73	3.95	2.214		
T(-)5	79.0%	0.271	1.77	3.97	2.094		
T(-)6	78.5%	0.298	1.83	4.41	2.08		
T(-)7	80.2%	0.274	1.87	4.28	2.134	69.62	4.1
T(+)1	78.9%	0.236	1.16	3.09	2.45	79.62	3.78
T(+)2	75.0%	0.247	1.19	2.91	2.319	78.58	3.86
T(+)3	72.3%	0.242	1.18	2.99	2.317		
T(+)4	80.3%	0.248	1.78	3.87	2.039		

Supplementary Table 2 includes the raw data of NGI as it relates to the T(+) and T(-) samples in regards to the deposition per tray. The first column, size, relates to which tray the powders deposited in. For example, size “>8.06 um” is the first tray of the NGI and the size is established based on the experimental flow rate.

Supplementary Table 2: Per stage deposition data for NGI analyses averaged for the experiments ran for the T(+) and T(-) samples. Included are the associated standard deviations.

Size (um)	**T(+)**	**Standard deviation**	**T(-)**	**Standard Deviation**
>8.06	3.71	0.48	3.66	1.85
8.06	16.26	0.10	12.55	5.79
4.46	26.25	2.96	14.66	3.04
2.82	11.94	0.10	11.89	1.18
1.66	12.01	1.15	13.60	3.45
0.94	11.67	0.86	15.64	5.94
0.55	9.18	0.57	10.40	2.06
0.34	4.66	0.10	7.04	3.19

(**Supplemental Fig 1**): absorbance values for tobramycin quantification via the SpectroMax ID5. Briefly, samples were solvated into ultrapure water targeting tobramycin concentrations of approximately 40mg/mL with serial dilutions down to 10mg/mL. Further serial dilutions were determined to be outside the reading range for absorbance analysis, being undetectable on the spectral readings.



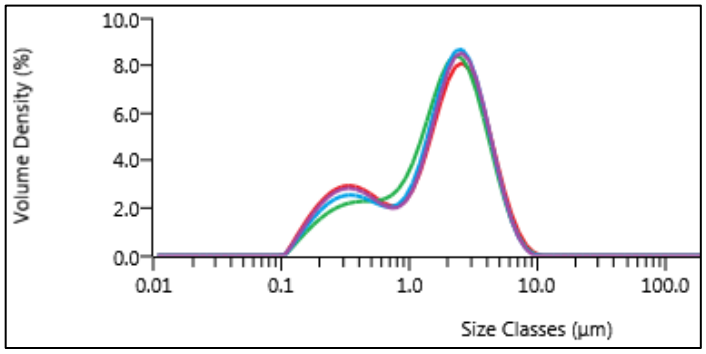
Supplemental Figure 1: Absorbance spectra for tobramycin 20 ug/mL, performed in triplicate and associated legend per experiment (identified as Tobi 1, 2, and 3).

Supplementary Table 3 provides the graphical summaries of MIC testing for the G+ and G- species when exposed to tobramycin and T(+). The data generated by spectrophotometry shows how we determined the MIC to be 2ug/mL for all samples.

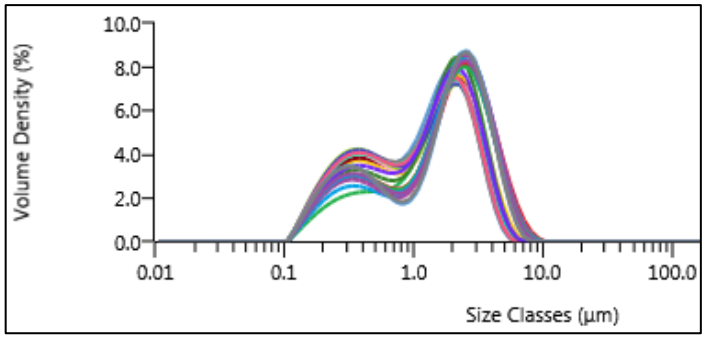
Supplemental Table 3: For determination of bacterial MIC, this table includes the spectrophotometric readings of the various tests. These absorbance readings are why MIC was determined to be 2mg/mL for all studies in our experiment.

[Tobramycin] ug/mL		**1024 ug/mL**	**->**	**2 ug/mL**	**1 ug/mL**
E. coli	Tobramycin	0.048	Serial dilutions	0.045	0.14
	T(+)	0.052		0.044	0.13
MRSA	Tobramycin	0.036		0.044	0.12
	T(+)	0.056		0.044	0.22

Supplementary figure 2, 3 show the PSD distribution following prolonged storage of our SD powders at 4 °C and 40 °C for 6 months. While these assessments were performed to prove the stability of the SD samples over time at two temperatures, the results offered minimal added information. They were insufficient (without other additional tests) to make claims about the longevity of the formulation. I.e., to state whether the stored powder retained efficacy over this period, bacterial and cell testing must have been repeated, as the PSD data only highlights that no changes in particle size occurred through storage. It is not uncommon for SD particles to clump or aggregate during storage, thus altering their PSD or APSD characteristics. Given the insignificance to the final study conclusions, we kept this data in the supplementary section, not the primary manuscript.



Supplemental Figure 2: PSD data for T(+) and T(-) samples following storage at 40 °C for 6 months. A legend is not included given the similarity of curves.



Supplemental Figure 3: Stability data overlaid with the initial PSD to highlight that distribution was maintained over the storage period.

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