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Review

# Positioning the preventive potential of microbiome treatments for cystic fibrosis in the context of current therapies

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## SUMMARY

Antibiotics and cystic fibrosis transmembrane conductance regulator (CFTR) modulators play a pivotal role in cystic fibrosis (CF) treatment, but both have limitations. Antibiotics are linked to antibiotic resistance and disruption of the airway microbiome, while CFTR modulators are not widely accessible, and structural lung damage and pathogen overgrowth still occur. Complementary strategies that can beneficially modulate the airway microbiome in a preventive way are highly needed. This could be mediated via oral probiotics, which have shown some improvement of lung function and reduction of airway infections and exacerbations, as a cost-effective approach. However, recent data suggest that specific and locally administered probiotics in the respiratory tract might be a more targeted approach to prevent pathogen outgrowth in the lower airways. This review aims to summarize the current knowledge on the CF airway microbiome and possibilities of microbiome treatments to prevent bacterial and/or viral infections and position them in the context of current CF therapies.

## INTRODUCTION

Cystic fibrosis (CF) is one of the most common hereditary life-threatening conditions, affecting approximately 160,000 individuals worldwide.[1] It is characterized by the production of viscous and adherent mucus due to mutations in the CF transmembrane conductance regulator (CFTR) gene.[2] Impaired airway clearance plays a crucial role in CF pathology. The sticky mucus entraps microorganisms, and cilia are unable to transport them for mechanical clearing (e.g., coughing).[3] Entrapped pathogens, such as *Pseudomonas aeruginosa, Burkholderia cepacia*, and *Achromobacter xylosoxidans* can establish biofilms, which are linked to chronic infection and exaggerated inflammation of the airways.[3] Progressive lung disease and subsequent respiratory failure used to be the primary causes of death in persons with CF.[4] Recently, CFTR modulators, which improve the function of the faulty CFTR protein, represent a major advancement in the treatment, resulting in a near-normal life expectancy of persons with CF with early initiation.[5] Currently, four modulator therapies are approved by the regulatory authorities for use in the US and Europe, but some countries lag behind due to reimbursement or access issues. This is especially the case for low- and middle-income countries, where access to these treatments is even worse due to the very high price and lack of proper insurance. Moreover, even in countries where CFTR modulators are available and reimbursed, approximately 10% persons with CF are not eligible for this treatment[6] because of the type of mutation and/or age restrictions (https://www.ema.europa.eu/en/medicines/national-registers-authorised-medicines). This age restriction increases the need for more early-on preventive strategies to slow down or prevent disease progression. Even with modulator therapy, structural lung damage seems to persist, and exacerbations still occur in these treated patients.[7]

Recent clinical evidence highlights that, in order to better understand and slow down the disease progression of CF, it is important to not only consider specific pathogens but also study the role of the entire airway microbial communities or airway microbiota, including their interactions and functions, and how they can impact respiratory health and disease. For example, *P. aeruginosa* is an important CF pathogen often associated with worse disease outcomes, but under specific conditions, mucoid *P. aeruginosa* can provide a protective function, such as decreased inflammation preventing chronic lung allograft dysfunction after lung transplantation in CF.[8] Next to an overgrowth of pathogens, data are accumulating that persons with CF lack specific microbiome members in the upper respiratory tract (URT) that can exert a protective function. For example, the culture-independent method 16S rRNA amplicon

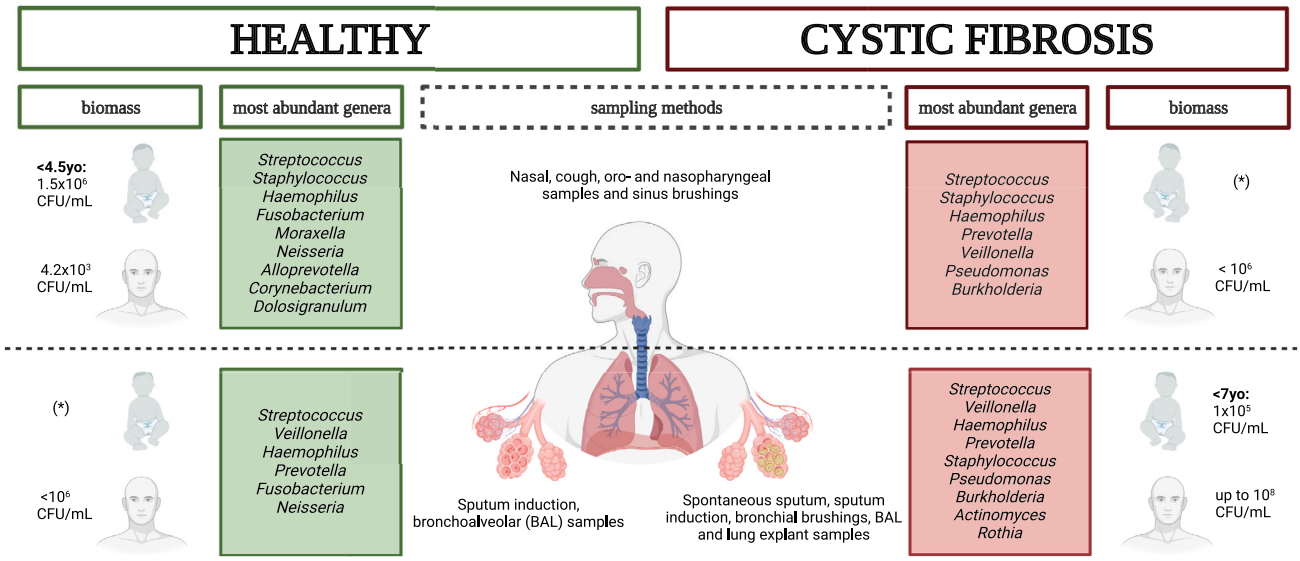


**Figure 1. Overview of the respiratory tract in healthy versus cystic fibrosis (CF) subjects**

Differences in biomass (in children and adults), sampling methods, and most abundant genera (microbial composition) between healthy and CF upper and lower respiratory tracts. (*) To the best of our knowledge, no information was found in the literature for newborns with CF. The figure is largely based on the following references: Feigelman et al.,[32] Marsh et al.,[33] De Boeck et al.,[34] Lucas et al.,[35] Pittman et al.,[36] Stearns et al.,[37] Watson et al.,[38] and Gangell et al.[39] Please note that the oral cavity, upper respiratory tract (URT), and lower respiratory tract (LRT) are anatomically linked, which has implications for diagnostics and (microbiome) therapeutics. Created with BioRender.com.

sequencing has shown a decreased abundance of potentially protective commensals such as *Dolosigranulum*, *Prevotella*, and *Veillonella* in 20 children with CF versus 45 controls.[9] A lower overall alpha diversity in the lungs of persons with CF compared to healthy controls using sequencing techniques was also observed when comparing sputum samples,[10] bronchoalveolar lavage (BAL) samples,[11] and bronchial brushings[12] of persons with CF to their healthy control group (n = 9–16 in each group). A lower alpha diversity and specific pathogen overgrowth were also linked to worse clinical status and lung function when analyzing sputum samples.[13–17] The most commonly detected pathogens detected are *Pseudomonas* spp., *Staphylococcus* spp., *Burkholderia* spp., *Stenotrophomonas* spp., and *Achromobacter* spp., in line with the CF patient registries based on standard culture methods.[18]

Pathogen overgrowth is linked to chronic respiratory infections, which are mainly treated with systemic and/or nebulized antibiotics. Yet, it is clear that also for persons with CF, caution is needed with antibiotic use because of the development of antibiotic-resistant bacteria and profound airway microbiome disruptions.[9] More targeted complementary and/or alternative treatments, such as phage therapy,[17] against specific CF pathogens are being widely explored but are, to date, still very experimental. In addition, phage therapy is often use in late stages of the disease.[17,19]

Taking all available treatment options for CF into account, there is a clear need for more preventive therapies that can be used in very early stages, for instance as soon as the CF mutation is detected at the neonatal screening. Here, we review the available literature on microbiome-focused therapies that could play an important role in this early stage, as there is an important link between bacterial communities and CF disease progress. We discuss the present knowledge on the CF lung microbiome, how it is linked to the URT, and how treatments such as antibiotics and CFTR modulators impact these microbial communities in persons with CF. We also describe the potential of topical probiotics or live biotherapeutic products (LBPs) as novel strategies to modulate the microbiome in CF and position such strategies to prevent respiratory infections within the context of current therapies. Expert opinions from microbiome scientists, microbiologists, and CF clinicians are combined with input from persons with CF and the community advisory board from CF Europe.

## THE RESPIRATORY MICROBIOME IN PERSONS WITH CF AND CHALLENGES ON HOW TO STUDY IT

Shifting our viewpoint, we have now come to understand that it is crucial to assess entire microbial communities in their ecological settings rather than just individual pathogens. This viewpoint is relevant to many respiratory diseases, including chronic infections in persons with CF, for which increasing evidence is collected that they result from disruptions in the overall microbial environment, which lead to pathogenic overgrowth.[20] A large part of the evidence is based on the increasing number of studies that implement next-generation sequencing techniques to describe diverse bacterial communities in healthy and diseased lungs (Figure 1). Based on studies in the US and Canada using 16S rRNA amplicon sequencing, *Streptococcus*, *Veillonella*, *Neisseria*, *Haemophilus*, *Prevotella*, and *Fusobacterium* are most abundant genera in the lungs of healthy individuals when using BAL samples (n = 6–29).[21–24] In the CF lung microbiome, *Pseudomonas*, *Staphylococcus*, *Haemophilus*, *Burkholderia*,

*Streptococcus*, *Prevotella*, *Veillonella*, *Actinomyces*, and *Rothia* appear to be the most commonly detected bacterial genera using 16S rRNA amplicon sequencing of BAL or sputum samples of all age groups (n = 17–299).[15,16,25–29] Persons with CF also show a decreased alpha diversity in their lungs compared to their healthy-matched cohort in children[11] and adults[10] (n = 13–16). Additionally, in a larger-scale study, the decreased microbial alpha diversity in sputum of 299 persons with CF was linked with decreased lung function based on the forced expiratory volume in 1 s (%FEV1).[16] Of note, 16S rRNA amplicon sequencing generally can only identify bacteria up to the genus level or the species level when full 16S has been sequenced, which is insufficient to predict the pathogenic nature of the microorganisms because the latter is often expressed at the strain level.[30,31]

Metagenomic shotgun sequencing—on the other hand—uses the entire genome and is therefore more suitable to functionally characterize the microbial communities in a culture-independent way, and it allows for better taxonomic resolution at species and even strain levels.[40] Using this method, Feigelman and colleagues confirmed a significantly lower bacterial alpha diversity (Shannon diversity index [SDI] of 1.08) compared to healthy individuals (SDI of 3.07) in sputum.[32] The microbial community of persons with CF had not only fewer genera but was particularly characterized by a dominance of only one or a few bacterial species such as *P. aeruginosa*, *Staphylococcus aureus*, *Stenotrophomonas maltophilia*, and *A. xylosoxidans*.[32] These detected taxa are also in line with the most commonly detected CF pathogens monitored in patient registries using culturing techniques.[18] A unique "CF-bacterial community" was also confirmed based on metagenomic shotgun sequencing of 4[41] and 65 sputum samples[42] of persons with CF. In addition to an increase in pathobiont taxa, these data also point at a decrease in potential protective commensal taxa, and this provides a new viewpoint on the development of infections in chronic CF. Shotgun sequencing has also provided more insight into the presence of fungi such as *Saccharomyces cerevisiae*[43] as well as into metabolic pathways[44,45] and antibiotic-resistance genes[32,46] in persons with CF. Nevertheless, the results from these shotgun sequencing approaches should still be interpreted with care due to the high levels of human DNA present in especially lower respiratory tract samples (>90%).[32] Optimization of the processing of these samples is still crucial to provide more in-depth microbiome and antibiotic resistance information[32,47] and allow for more personalized medicine.

Not only technical but also sampling challenges are a complication for research on the microbiome of the respiratory tract in persons with CF.[33] For instance, lower respiratory tract (LRT) samples are either invasive (BAL) or not always possible/desirable to collect (sputum) due to young age or lack of sputum production resulting from the recent CFTR modulator therapies.[48,49] These challenges make it difficult to compare results of different clinical studies on the microbiome composition, as they vary in sampling methods (BAL and sputum samples), extraction protocols, targeted 16S rRNA genetic regions, sequencing technologies, and bioinformatics analysis.

Due to the forenamed sampling challenges of LRT samples, clinicians often opt for the use of URT samples, such as nasal, nasopharyngeal, and oropharyngeal swabs (Figure 1). The URT and LRT appear to be rather well connected in terms of their microbial members (Figure 1), but some conflicting data exist. For example, one study highlighted that the predominant species identified in the lungs with pyrosequencing (V1–V3) did not match the dominant species identified in throat samples.[50] For example, while *P. aeruginosa* showed a very high relative abundance in the lungs (73%), it was present in much lower abundance in the throat samples from the same persons with CF (0.2%). A more recent study analyzed the metagenome of oropharyngeal swabs (URT) and induced sputum samples (LRT) from 65 persons with CF using metagenomic shotgun sequencing.[42] They observed that URT and LRT samples from the same persons with CF were more similar than, for example, LRT samples from two different persons with CF. These personalized metagenome signatures suggest that—although the relative abundances of taxa between URT and LRT can differ—there is a clear exchange of taxa between both sites.[42,51] Also, in the clinical practice, the use of oropharyngeal swabs during standard surveillance is the common method.[52] In addition, the association between the URT and LRT microbiome is not only relevant from a diagnostic point of view but has also implications for treatment. For example, Boutin and colleagues suggest that changes in the oropharyngeal microbiome could be the source of dysbiosis in the LRT microbial composition,[53] providing a window for beneficially modulating the lung microbiome by directly modifying the URT microbiome.

## LINK BETWEEN VIRAL AND FUNGAL INFECTIONS AND CF RESPIRATORY MICROBIOME DISTURBANCES

Alongside bacterial pathogens, respiratory viruses are generally associated with worsening of CF symptoms, possibly provoking pulmonary exacerbations. A large cohort study in the US (n = 21022) showed an association between influenza activity and pulmonary exacerbations in children and adult persons with CF, whereas respiratory syncytial virus (RSV) was significantly associated with disease severity in children, based on geographic influenza and RSV surveillance data.[54] It remains unclear whether higher viral titers are detected in CF lungs compared to healthy individuals. It is contradicted in different studies,[55–57] but the virus-related clinical morbidity (including LRT episode severity and hospitalization rates) in persons with CF appears to be higher compared to healthy individuals.[56,58] In addition, respiratory viral infections can contribute to bacterial dysbiosis and inflammation via several direct and indirect mechanisms, thereby making it difficult to interpret whether viral infections are associated with specific alterations of the respiratory microbiome composition in CF.[59] For example, infection with rhinoviruses in CF has been associated with an increase in *P. aeruginosa* live counts in sputum during exacerbation in adults (n = 17; Australia).[60] In addition, RSV has been shown to facilitate the adherence of *P. aeruginosa* to the healthy and CF respiratory epithelia *in vitro*.[61,62] However, another study showed that viral infection did not alter recovery rates of *P. aeruginosa* and *S. aureus* as key CF pathogens in children (n = 138; US), while the recovery of other common respiratory pathogens (*Haemophilus* spp., *Moraxella* spp., and *S. pneumoniae*) was more frequent.[63] In addition, a mechanism that has not yet been

studied in CF but which is highly relevant is the fact that epithelial cell damage caused by primary viral infection could cause increased epithelial permeability, resulting in decreased barrier function and, consequently, the onset of secondary bacterial infections, as, for example, observed in experimental RSV infections *in vitro*.[64] Viral respiratory infections also affect the beating frequency of cilia, thereby altering mucociliary clearance processes, which are important for the removal of invading pathogens.[65] Moreover, experimental data show that viral infections can alter immune responses creating inefficient immune effectiveness against bacterial pathogens, thereby indirectly increasing bacterial pathogenesis. For example, an impairment of the functionality of neutrophils and cytokine signaling, a decrease in reactive oxygen species production, and insufficient phagocyte recruitment after influenza infection have been proposed to result in inefficient clearance of bacterial pathogens based on results from mice and *in vitro* studies.[66,67] Consequently, these experimental observations suggest that viral respiratory infections could incite higher susceptibility to bacterial infections in both healthy individuals and persons with CF. These mechanisms have been predominantly studied in experimental settings, and validation of their relative importance in CF is still required. However, it is important to prevent excessive LRT inflammation and pulmonary exacerbations due to viral infection in persons with CF, as well as the potential negative effects of viral infections on the lung microbiome and onset of secondary bacterial infections.

In addition, fungi can also interplay with both bacteria and viruses.[68] *Aspergillus* and *Candida* species are most frequently detected in persons with CF. However, their exact role in the disease remains unclear. For example, a potential association of *C. albicans* and pulmonary exacerbation in CF was reported.[69] In contrast, another study found *C. albicans* to be more abundant in stable persons with CF, while the same study found *C. dubliniensis* to be associated with CF exacerbations.[70] Moreover, coinfection of important bacterial CF pathogens and fungi is reported. For instance, coinfection of the CF airways with *P. aeruginosa* and *Aspergillus* is associated with worse clinical outcomes.[71] In addition, fungal coinfection with respiratory viral disease often complicates disease outcome.[72] The entire interplay between bacteria, viruses, and fungi is thus of importance, and the prevention of bacterial, viral, and/or fungal infections using complementary treatments is needed.

## IMPACT OF ANTIBIOTICS ON THE RESPIRATORY TRACT MICROBIOME IN CF

As already introduced above, different strategies are used for controlling CF progression and symptom management, with lung transplantation being the last option in the case of advanced lung disease. Next to airway clearance techniques and mucus thinners, antibiotics play an important role in the treatment of persons with CF. They have significantly increased the life expectancy and will stay important in managing the disease progression, although they also have limitations. For instance, antibiotic treatments, rather than age and lung function, appeared to be the primary driver of a decrease in microbiome alpha diversity when analyzing sputum samples from 6 persons with CF.[13] This decrease was also linked to a progressive decline in lung function.[13] This is in line with other studies where a lower bacterial richness was linked to a worse clinical status.[16,73] However, it remains to be investigated whether this loss of alpha diversity is a direct consequence of the administered antibiotics, as they form a major part of the treatment path for persons with CF, which means that most microbiome studies in persons with CF are confounded by antibiotics. It is important to explore whether antibiotics—while they show clear short-term benefits for persons with CF—could worsen clinical outcome in the long term when they also decrease the natural protective function of the microbiome. For example, Bacci and colleagues observed that even though antibiotic treatment had a clear effect on pathogenic taxa in the short term, the bacterial community structure always recovered, with these pathogenic taxa emerging again.[46] In addition, it is well recognized that antibiotics lead to the development of multidrug-resistant bacteria, which form a serious threat to our healthcare system. For example, methicillin-resistant *S. aureus* (MRSA), which is linked to more hospitalizations and antibiotic treatments than methicillin-susceptible *S. aureus* (MSSA),[74] had a prevalence of 16.0% in persons with CF in 2022.[18] Moreover, antibiotic resistance genes were consistently detected in the lung microbiome of 22 persons with CF using metagenomic sequencing.[46] Of note, antibiotic resistance genes also have been detected in the lungs of healthy controls (sputum samples) but with a lower prevalence than in patients with chronic obstructive pulmonary disease.[75] More research on the meaning of the presence of this antibiotic resistome is required.

It has also been recognized that the lung microbiota composition itself influences the effect of the administered antibiotics in CF[76] and the persons with CF's responsiveness to therapies such as aztreonam for inhalation solution (AZLI), which is used to treat chronic *P. aeruginosa* infections.[77] For example, responsiveness to the AZLI therapy was shown to be decreased in persons with CF with a higher abundance of pathobionts such as *Staphylococcus* and anaerobic organisms including *Prevotella* and *Fusobacterium*.[77] Modulating the airway microbiome composition using microbiome therapy could potentially improve the working mechanism of antibiotics or could help stabilize the community after antibiotic treatment. The latter is already commonly used for restoring the gastrointestinal microbiome after antibiotic use in the non-CF population[78] as well as in the CF community.[79]

## NOVEL MODULATOR THERAPY AND ITS IMPACT ON THE RESPIRATORY TRACT MICROBIOME

Next to antibiotics, highly effective CFTR modulators that target the defective protein have been revolutionizing the treatment of persons with CF in the last years.[7] More specifically, for the majority of the CF population, triple combination therapy (elexacaftor, tezacaftor, and ivacaftor [ETI]) has shown to improve lung function, pulmonary exacerbations, BMI, and overall quality of life in an unprecedented way, as reviewed by Bacalhau et al.,[80] Kapouni et al.,[81] and Tümmler.[82] For example, Schaupp and colleagues showed that ETI treatment improved sputum viscoelastic properties as well as chronic airway infection and

inflammation in persons with CF over the first 12 months of therapy.[83] This was supported by the results of Sheikh et al., where an increase in %FEV1 and BMI, a decrease in pro-inflammatory cytokines, and a reduction in *Pseudomonas* and *Staphylococcus* positivity were observed after 6–12 months of treatment with ETI.[84] Furthermore, with recent evidence showing that people with rare mutations could also, in theory, benefit from ETI,[85] the number of persons with CF whose health could improve under triple combination modulators has never been higher. Nevertheless, it is important to note that in Europe, 10%–20% of the CF community is not eligible for this novel therapy, with large regional differences, mainly because of their genotype (ETI is only approved in Europe for persons with CF with at least one F508del mutation). Second, access to these modulators is limited due to the high cost of between $270,000 and $310,000 each year for a condition that requires lifelong treatment.[1] To this day, the lack of reimbursement and thus sustained accessibility of these modulators in low- and middle-income countries will lead to disparity of CF outcomes.[86] Moreover, structural lung damage seems to persist,[87] and exacerbations still occur in these treated persons with CF, indicating the need for additional research. For example, when the bacterial composition of 12 persons with CF was studied before and after treatment with a monotherapy of ivacaftor (only given to patients with one of the following mutations: R117H, G551D, G1244E, G1349D, G178R, G551S, S1251N, S1255P, S549N, or S549R), a decrease in the abundance of *P. aeruginosa* was observed during the first year, but this pathogen was not eradicated, and densities rebounded after the first year of treatment.[7] Similarly, no changes of the airway microbial community were observed after ivacaftor treatment based on 16S rRNA amplicon sequencing of sputum samples of 31 persons with CF.[88] Along the same line, Neerincx and colleagues only observed a temporary and moderate change in lung microbiome composition of 20 persons with CF using 16S rRNA amplicon sequencing.[89] In addition, although ivacaftor treatment was associated with less hospitalizations, pulmonary exacerbations, death incidence, and organ transplantation, a high prevalence of important pathogens such as *S. aureus* and MRSA was still observed.[90] The same results were observed by Nichols and colleagues, who analyzed sputum samples of 236 persons with CF during their first 6 months of ETI therapy. After 1 month, most persons with CF remained culture positive for their initial pathogen, and even when they became culture negative, sequencing methods could still detect the pathogen in sputum samples.[87] Even combining ivacaftor and an intensive antibiotic course was not able to clear chronic *P. aeruginosa* or *S. aureus* lung infection.[91] Another study analyzing cough swabs and sputum samples from 31 persons with CF after 14 and 50 weeks of treatment with ETI therapy using whole-genome metagenomic sequencing observed an overall decrease in bacterial load.[92] However, they observed a decrease in relative dominance of single species (such as pathogens *P. aeruginosa* and *S. aureus*) and an increase in the proportion of commensals such as *Streptococcus*, *Rothia*, *Veillonella*, and *Prevotella* spp. in the lungs, which could be beneficial to slow down CF progression but remains to be documented.[92] It is also important to note that at the moment, almost all studies focus on persons with CF older than 10 years. Only the study of Bessonova et al. included children younger than 6 years old, but they did not make any comparison between results of different age groups.[90] Earlier intervention with modulators before the onset of disease progression could potentially lead to better results regarding residing pathogens. Thus, although the available data illustrate the improvement in quality of life due to these modulator therapies, it is still unclear why the reappearance of certain pathogens cannot be avoided and what impact these modulators have on the airway physiology and microbiology.[93] Therefore, the search for complementary approaches that contribute to a more stable microbiome and normalize the mucus structure in the airways is still needed. In the paragraphs below, we summarize the available evidence on why targeted microbiome therapies could potentially improve the working mechanism of these modulators.

## The potential of probiotics and LBPs for CF airways

With the increased insights into the microbial communities colonizing the airways summarized above, it is becoming clear that the airway microbial community structure is shaped by the individual host[42] and that better knowledge of the beneficial functions and activity of the commensal and potential protective microbiota members could inspire novel treatment options for CF. These commensals could inhibit pathogen (bacteria, viruses, and fungi) outgrowth, inhibit the expression of their virulence genes, compete with pathogens for adhesion and nutrients, enhance the epithelial barrier function, or provide immunomodulation to the host (Figure 2).[94] Complementary to current treatments, they could prevent the chronic colonization of pathogens, positioning them early on in the treatment plan, while they could also enhance the working mechanism of therapies such as antibiotics and modulators by creating a more stable microbial ecosystem. The key question is which commensal taxa show the most potential as probiotics[95] or LBPs[96] for persons with CF and in which phase of the disease progression. Probiotics are defined as live microorganisms that, when administered in adequate amounts, confer a health benefit on the host.[95] This term mostly refers to beneficial bacteria that are used as food supplements (including use in infant formula), medical food, or as a drug, depending on the intended use.[97] Because of their long history of safe use, many currently available probiotics include often taxa belonging to lactic acid bacteria (LABs). More recently, increasing interest has been given to the use of a more diverse compendium of beneficial bacteria and with more focus on drug applications. Therefore, a novel definition came into place when the intended use is for a diseased population: LBPs. LBPs are defined by the FDA as a biological product that (1) contains live microorganisms, such as bacteria; (2) is applicable to the prevention, treatment, or cure of a disease or condition of human being; and (3) is not a vaccine.[96] LBPs are often species that do not belong to LABs. For example, it has been suggested that taxa such as *Veillonellaceae*, present in healthy lungs but almost absent in CF samples, could protect the pulmonary system.[10] Additionally, lower airway communities containing abundant *Streptococcus* were identified as more protective.[98] However, more analyses regarding the safety, efficacy, and beneficial functions are needed to state that *Veillonellaceae* or *Streptococcus* have LBP potential. Based on computer-based

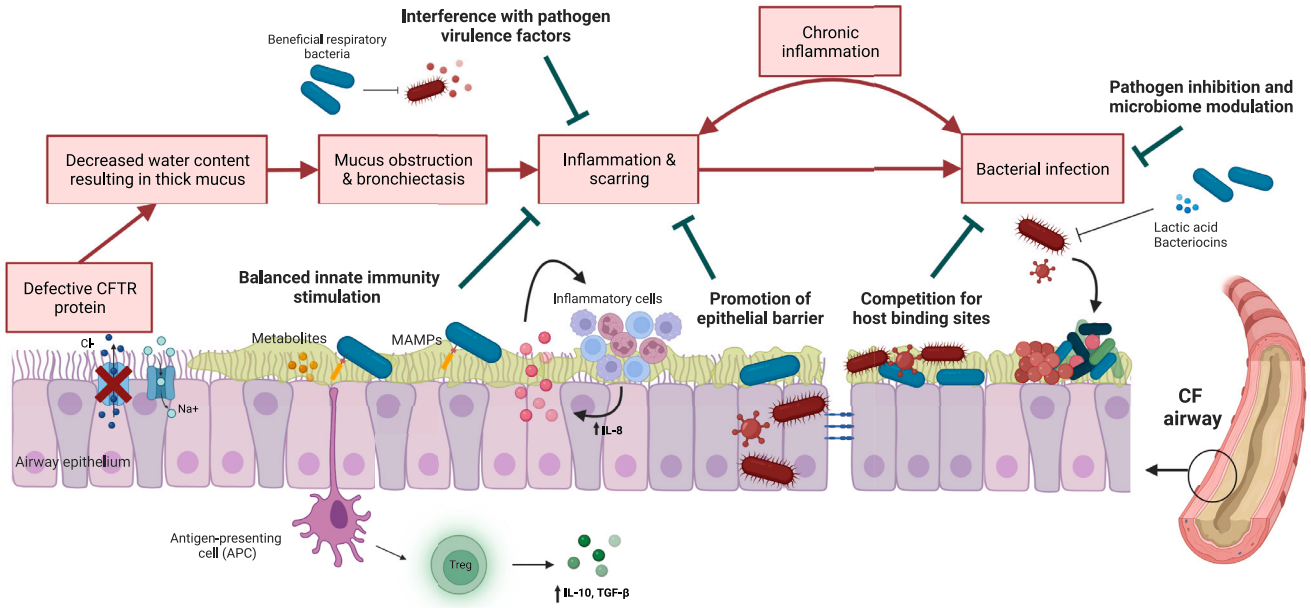


**Figure 2. Overview of the disease progression and different steps of the pathogenesis of patients with CF at the airway epithelium level**[101,102]
The potential multifactorial direct and indirect mechanisms of action of microbiome therapeutics or probiotics are highlighted with "inhibition arrows." More information on these mechanisms is described in the main text. Created with BioRender.com. This figure was largely based on Batoni et al.,[101] De Boeck et al.,[102] and Spacova et al.[103]

model simulations, transferring a key combination of bacterial taxa, *Rothia mucilaginosa* and *Streptococcus* spp. from the healthy airway community, among others, was shown to stabilize the CF microbial community.[99] Next to the choice of commensal taxa, the use of a single-strain probiotic or multistrain formulations should be considered. The latter could limit nonresponders and target multifactorial conditions, as each probiotic strain has its own unique set of properties.[100] These data highlight the importance of careful selection of commensal taxa and their combinations in the development of LBPs.

As yet indicated, LABs have a long history of safe use as food and probiotics.[104] An increasing number of microbiome studies of the URT also show that different LAB taxa such as *Dolosigranulum*[105] and *Lacticaseibacillus*[106] form a part of the healthy respiratory tract microbiome. Moreover, their prevalence and relative abundance appears to be reduced in specific patient cohorts such as those with asthma[107] and chronic rhinosinusitis.[106] These data indicate that specific LAB taxa could have important protective functions in the URT. However, a better understanding of host-microbiome interaction and well-designed clinical studies are necessary to implement such approaches in clinical settings in the more vulnerable CF populations.

## Oral probiotics

Since persons with CF often suffer from maldigestion and malnutrition due to dysfunction of the CFTR, which is also present in the gastrointestinal tract, the use of oral probiotics is already more widely considered to improve gastrointestinal health. In a recent survey study, 70% adults with CF self-reported to have used probiotics for gastrointestinal- or antibiotic-related reasons.[79] Although the initial focus is gastrointestinal health, some studies have also looked at the impact on the respiratory tract.[108–111] The focus here will be on studies that report pulmonary health effects after oral administration, as summarized in Table 1.

The available data (Table 1) highlight that the specific probiotic strains applied and the formulation and timing of administration could all impact the clinical efficacy in persons with CF. The clinical effects appear to be mainly mediated due to systemic effects, for instance by inducing systemic cytokine release via interaction with intestinal immune receptors, which signal through the body, resulting in an improved lung function,[122] reduction of pulmonary exacerbations,[115] and reduction of hospital visits.[109] The use of oral probiotics to improve pulmonary health via the gut-lung axis has been extensively reviewed by Batoni et al.[101] Here, we argue that direct effects could also apply due to the anatomical link between the oral cavity and the nasopharynx and the possible transmission of probiotics to the URT (Figure 1). However, it is important to highlight that this possible direct mode of action is largely underexplored or validated. Moreover, safety needs to be considered. A recent systemic review did not find proof of any harm when using probiotics in stable persons with CF who showed no acute exacerbations and severe lung disease.[123] However, some minimal adverse effects have been reported, such as vomiting in 1/38 persons with CF receiving the probiotic *Lacticaseibacillus rhamnosus* GG (LGG) in an oral rehydration solution[109] and mild flatulence in 3/10 persons with CF receiving a Bio-plus tablet containing *Lactobacillus acidophilus*, *Lactobacillus bulgaricus*, *Bifidobacterium bifidum*, and *Streptococcus thermophilus*.[114] It is thus necessary to further monitor both the safety and efficacy

**Table 1. Overview of clinical studies with oral probiotics in patients with cystic fibrosis that reported pulmonary health effects**

| Probiotic strain(s) | Vehicle | No. of subjects | Dose and duration of administration | Outcome | Reference |
|---|---|---|---|---|---|
| *L. rhamnosus* GG | oral rehydration solution (ORS) | randomized, single blind, placebo controlled; 24 children with CF | 6 months, 6 × $10^9$ CFU/day | reduction in infections requiring antibiotic treatment and reduction of abdominal pain; increased weight gain | Di Benedetto et al.[112] |
| *L. rhamnosus* GG | capsule | case-controlled, prospective, open study; 30 pwCF, n = 10 received probiotic | 4 weeks, 5 × $10^9$ CFU/capsule, 1 capsule/day | reduction of calprotectin levels, microbial richness, intestinal inflammation, and pulmonary exacerbations | Bruzzese et al.[113] |
| *L. rhamnosus* GG | ORS | randomized, single blind, placebo controlled, crossover (13 months); 43 pwCF (5 drop outs), n = 19 started with placebo | 6 months, 6 × $10^9$ CFU/day | reduction in pulmonary exacerbations and hospital admissions and an increase in %FEV1 and body weight | Bruzzese et al.[109] |
| Bio-plus (Supherb, Netanya, Israel): *L. acidophilus*, *L. bulgaricus*, *B. bifidum*, *S. thermophilus* | tablet | prospective pilot study; 10 pwCF, mild-moderate disease, *Pseudomonas aeruginosa* colonization | 6 months, 6 × $10^9$ CFU daily | reduction in exacerbation rate (from 1.3 pulmonary exacerbations in 6 months prior to probiotic treatment to 0.6 in 6 months post-probiotic treatment); no change in sputum cultures, neutrophil count, or interleukin-8 (IL-8) levels | Weiss et al.[114] |
| Protexin: *L. casei*, *L. rhamnosus*, *S. thermophilus*, *B. breve*, *L. acidophilus*, *B. infantis*, and *L. bulgaricus* | capsule | randomized, placebo controlled (7 months); 37 pwCF, n = 17 received placebo | 1 month, $10^9$ CFU/capsule, 2 capsules/day | improvement in quality of life compared to placebo group but not 6 months after treatment; rate of pulmonary exacerbations was reduced | Jafari et al.[115] |
| *L. rhamnosus*, *L. acidophilus*, *L. casei*, *L. bulgaricus*, *B. breve*, *B. infantis*, *S. thermophilus* | powder | randomized, double blind, placebo controlled; 47 pwCF, n = 23 received placebo | 1 month, $10^9$ CFU daily | decrease in calprotectin levels and intestinal inflammation | Fallahi et al.[111] |
| *L. reuteri* DSM17938 | chewable tablet | randomized, double blind, placebo controlled, crossover (6 months); 39 pwCF (9 dropouts) | 6 months, $10^8$ CFU/tablet, 1 tablet/day | improvement in gastrointestinal health; decrease in calprotectin levels; increase in microbial diversity; no change in %FEV1 | del Campo et al.[110] |
| *L. rhamnosus* GG | capsule | randomized, double blind, placebo controlled; 22 pwCF | 1 month, 6 × $10^9$ CFU/day | reduction of calprotectin levels, microbial richness, intestinal inflammation, and pulmonary exacerbations | Bruzzese et al.[116] |

(*Continued on next page*)

**Table 1.** ***Continued***

| Probiotic strain(s) | Vehicle | No. of subjects | Dose and duration of administration | Outcome | Reference |
|---|---|---|---|---|---|
| *Limosilactobacillus reuteri* ATCC55730 | drops | randomized, double blind, placebo controlled; 61 pwCF, n = 31 received placebo | 6 months, $10^{10}$ CFU/day | reduction in pulmonary exacerbations and number of URT infections | Di Nardo et al.[117] |
| *L. rhamnosus* SP1 (DSM21690) and *B. animalis* spp. *BLC1* (LGM23512) | capsule | double blind, placebo controlled, crossover (9 months); 31 pwCF included (6 dropouts), n = 14 started with placebo | 4 months, $10^{10}$ CFU/capsule, 1 capsule/day | normalization of gut permeability in 13% patients; no effect on pulmonary exacerbations, pulmonary function, or abdominal pain | Van Biervliet et al.[118] |
| Synbiotic supplementation containing fructo-oligosaccharides and 4 probiotic strains (*L. paracasei*, *L. rhamnosus*, *L. acidophilus*, and *B. lactis*) | powder | randomized, placebo controlled, double blind; 72 children and adolescents, n = 19 received placebo | 90 days, FOS: 5.5 g/day, probiotics: $10^{8}$–$10^{9}$ CFU/day each strain | diminished pro-inflammatory markers IL-6 and IL-8 in pwCF showing the presence of pathogenic microorganisms for CF | De Freitas et al.[119] |
| *L. rhamnosus* GG | capsule | randomized, double-blind, placebo-controlled clinical trial; 95 pwCF (18 drop outs), n = 40 started with placebo | 12 months, $6 \times 10^{9}$ CFU/day | no effect on exacerbations, odds of hospitalization, or nutritional outcomes | Bruzzese et al.[120] |
| *L. rhamnosus* GG | capsule | randomized, double-blind, placebo-controlled clinical trial; 95 pwCF (18 drop outs), n = 40 started with placebo | 12 months, $6 \times 10^{9}$ CFU/day | *Bifidobacteria*-dominated fecal microbiota more likely when LGG was supplemented; children with bifidobacteria-dominated gut microbiota showed a reduction in rate of pulmonary exacerbations and in lower intestinal inflammation | Ray et al.[121] samples collected from Bruzzese et al.[120] |

pwCF, persons with CF.

of oral probiotics in persons with CF and explore novel more targeted administration routes for probiotics.

### Topical airway probiotics

As already alluded to, in addition to oral probiotics, direct topical probiotic use in the respiratory tract might be a more targeted next treatment option for persons with CF.[10] Importantly, additional safety and efficacy considerations need to be taken into account when using such live probiotics directly in the airways as LBPs. Currently, to our knowledge, no clinical trials using topical probiotics have been reported with patients with CF, and no such trials are registered at ClinicalTrials.gov (as checked on October 24th, 2023). This contrasts with some exploratory trials that are currently being carried out in persons with CF with intravenous phages,[19] as well as individual case reports with nebulized phage therapy.[17] Results of clinical trials with nebulized phages are underway (ClinicalTrials.gov: NCT05010577 and NCT04684641). Although the use of phages seems very promising, they exclusively target specific pathogens and are thus not feasible to use at large scale. In addition, they are mostly used in end stages of lung disease and remain, to date, experimental since the optimal dose, route of administration, and duration of treatment remain to be determined.

Topical applications of medical therapies have been tested in patients with respiratory diseases or symptoms that are also common in patients with CF, such as sinusitis.[124] For example, the safety of topical application of live probiotic bacteria was shown in patients with chronic rhinosinusitis for a probiotic nasal spray containing a mixture of lactobacilli and bifidobacteria of honeybees.[125] Another trial with *Lactococcus lactis* W136 showed positive effects after the bacteria were administered by self-irrigation of the dissolved bacterial powder.[126] The latter study also indicated an improvement of sinus symptoms (including nasal congestion, post-nasal drip, and "need to blow nose"), quality of life, and mucosal scores associated with the probiotic treatment.[126] Based on recent research, using a strain isolated from the healthy respiratory tract would be more efficient because it is better adapted to the inherent conditions of the airways.[127] For example, *L. casei* AMBR2 was isolated from the nose of a healthy volunteer and showed unique adaptation potential to this niche due to the presence of catalase genes and fimbriae structures to adhere to the airways. Furthermore, it showed a multifactorial mode of action via, among others, the inhibition of CF-related pathogens such as *S. aureus*.[106] In addition, this strain has antiviral activity *in vitro*[128] and *in vivo* in patients with COVID-19 when administered via a throat spray together with two other LAB strains.[129] *L. casei* AMBR2 could potentially also have benefits for persons with CF to reduce their load of bacterial and viral pathogens, but this remains to be substantiated via clinical trials.[129] To the best of our knowledge, pulmonary administration of probiotics has not been studied in humans. One study of Le Noci et al. evaluated the immunostimulatory properties of safe bacteria such as LGG by aerosolization in mice. Their results showed that probiotic aerosolization is associated with a reduction in the immune suppression present in the lung environment.[130] Although these results stimulate further research on this LRT administration route, direct administration of probiotics to the LRT still comes with potential safety risks. Application of probiotics or LBPs to the URT would be more feasible, especially considering that this could also benefit the LRT via the link between the URT and LRT microbiome described above (see also Figure 1).

## POSITIONING TOPICAL LBPs IN THE CF LANDSCAPE AND TECHNICAL BARRIERS TO OVERCOME

Research on the potential of topical microbiome therapies in persons with CF is of interest for the CF community because "studying therapies that are effective in delaying or preventing progression of lung disease in real life" was defined as one of the priorities in CF research in 2018 and was further refined in 2023 as "studying the available options for those that are not able to take CFTR modulators."[131] However, there are still some bottlenecks to overcome. First of all, the optimal timing of administration should be considered. It has been described that the earlier days in life have a large impact on microbiome development and eventually their susceptibility to diseases.[132] Since changes within the airway microbiome occur already during the first years of life in young children with CF, these are associated with disease progression.[27] The purpose of standard neonatal screening for CF is to improve survival rates through timely follow-up care, which aims to slow down the disease progression and prevent serious CF-related complications. In this light, modulating the airway microbiome already in early life offers several advantages and could improve overall quality of life. Ideally, we could stabilize the airway microbiome early on as a more preventive therapy, which could potentially delay early-life respiratory tract infections in persons with CF. This preventive method of applying microbiome therapy early in life would benefit all persons with CF, even those who are not eligible or do not have (early) access to modulator therapies. For the youngest CF population, it can also serve as a bridge from birth up until they can receive modulator therapy.

Secondly, as mentioned before, the optimal administration method should be evaluated in further research. Is a URT application, using a nasal or throat spray, more effective compared to oral supplementation in persons with CF? Or would we need even more LRT-focused therapies such as inhalation methods to provide an even more targeted approach? Such topical applications would require the regulatory framework of medicinal/pharmaceutical products with strict guidelines on quality control, safety, and efficacy and, consequently, come with a higher cost. Cost prices of probiotic therapies can be reduced if they can be used as food supplements or infant formula, but the current regulation and quality control of food supplements is not well enough defined.[97] Moreover, patient preferences should be taken into account when designing an LBP.

Next to this, there is also insufficient knowledge on the quantity and duration of these microbiome therapies, which in turn also depends on the administration method. The long-term effect of microbiome therapies on the human microbial composition in different human body sites, including the airways, is still unclear. Moreover, there are no strict standards to monitor engraftment and/or persistence of applied strains in the human body. More preclinical and clinical data could elucidate the stability of the microbial community after topical probiotic administration.

## Conclusion

Both antibiotics and modulator therapies have immensely improved the quality of life of persons with CF, but the available data highlight that pulmonary exacerbations and pathogen overgrowth still occur even with treatment. Furthermore, modulator therapy is, to date, not yet available for the whole CF community that would benefit from it. In addition, viral and fungal infections, altered mucus properties, and mucociliary clearance are also involved in these microbiome shifts observed in CF. Restoring the respiratory tract microbial compositions using topical administration of beneficial microbiome members could improve the working mechanism of both treatments, target multiple aspects of CF pathogenesis, and preserve the protective airway microbiota. At the moment, mainly oral probiotic treatments are studied because of their easier application route and market availability. However, the topical application of beneficial microbiome members as LBPs directly in the URT or even in the LRT of persons with CF represents a promising complementary route for targeted treatment in persons with CF. Because of their well-established safety profile, current research is largely focused on LABs as probiotics or LBPs for CF. However, their abundances in the URT are relatively low, so other taxa could be even more potent as CF LBPs. Hereto, functional characterization and detailed safety assessment are needed beyond microbiome sequencing and culturing to provide rigorous scientific data for their use in CF.

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## AUTHOR CONTRIBUTIONS

E.C., I.D.B., I.S., and S.L. worked on the conceptualization of the manuscript. S.V. and K.V.H. provided the necessary clinical input. S.L. supervised the project. E.C., I.D.B., and I.S. wrote the original draft and reviewed and edited the manuscript together with I.V.T., J.B., S.V., K.V.H., and S.L. E.C. and J.B. worked on the visualization of the figures and table. E.L. provided information on the clinical relevance via the patient organization CF Europe.

## DECLARATION OF INTERESTS

I.D.B., I.S., and S.L. are inventors on patent applications related to this work on probiotic bacteria for the respiratory tract (BE2021/5643, WO2018172537, WO2022049244, WO2020128022). S.L. is a co-founder of Yun NV (www.yun.be) and a member of the academic board of ISAPP (https://isappscience.org).

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