nature portfolio

Peer Review File

Early nasal microbiota and subsequent respiratory tract infections in infants with cystic fibrosis

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

The authors present some interesting data, but it is not presented clearly and leaves the reader confused.

1. Were the children with CF on prophylactic antibiotics as is the case for many across Western Europe eg prophylactic flucloxacillin.

2. pg6 110-112 - this section does not make sense

3. Figure 1 - How did the authors make a diagnosis of LRTI? Chest xray changes? clinically?

4. Why would you diagnose a LRTI in CF and NOT give antibiotics? This is NOT standard practice so I am unsure what the authors mean by after first LRTI but before antibiotics.

5. pg 7 "Antibiotic treatment at nasal swab" - what does this mean?

6. Table 1 - 2 children in control looks as though they received antibiotics but are not included further down

7. The authors state the potential role for probiotics but the evidence of enteral effects on the nasal microbiome is scarce - they do acknowledge this fact but fail to mention the new modulator medications which will undoubtedly reduce exacerbation frequency and need for antibiotic intervention which will ultimately allow more normal development of airway, lung and gut microbiomes. I appreciate they are not all licenced from birth, but over next few years this will happen.

Reviewer #2 (Remarks to the Author):

The submission entitled “Early nasal microbiota and subsequent respiratory tract infections 1 in infants with cystic fibrosis” is a very detailed and interesting multicenter national observational analysis comparing the nasal microbial communities of two groups of newborns/infants, one diagnosed with cystic fibrosis (N=50) and the other unaffected (N=30). The authors provide clear descriptions of their methodology. I personally feel that this type of study is extremely important in understanding the beginning of the natural history of CF airway infection and natural history and I applaud the investigators and the Swiss government for taking on this important project,

Major Comments

1. I understand the nature of the microbiomics analyses conducted but am not sufficiently versed to critique technical approaches or methodologies, so I will defer to other reviewers for this (important) feedback. I will limit my comments to the larger conclusions drawn from these very interesting data.

2. With respect to the diagnosis/occurrence of LRTI (lower respiratory tract infection), I suspect that this is a term of convenience used by the authors to describe infants who presented during observation with elevated respiratory sign/symptom scores. It’s not clear from the information provided if there were any additional tests/observations to confirm that these events included lower respiratory tract involvement. In other words, were there instances where infants presented with an elevated respiratory score but were found by imaging, etc. to be experiencing events that did not appear to involve the lower respiratory tract, and if so, were these excluded from LRTI counts? Were all events evaluated in the same manner? To be clear, I am not concerned about medical practice here, I am concerned about terminology… unless methodology was systematically employed to confirm lower airway involvement for respiratory events, perhaps just calling them respiratory events is more precise and less prone to misinterpretation. Further, it is now generally accepted that there are lower airway microbial communities in infants (and particularly in CF infants). If a microbial population resides in the lower airways before observed sign and symptom increases, is “infection” really the right term to use for a change in symptoms (and by extension, if a second event is observed, is this best described as a “recurrence of infection”)? I understand that this is legacy terminology, but isn’t “airway infection” now recognized as a chronic CF condition that transcends clinical state?

3. I’m particularly interested in differences in management of infants with and without CF with respect to respiratory events. Could the authors provide greater detail on a) the total numbers of respiratory events recorded in each group during the observation period, b) the proportion of events in each group that were treated with antibiotics, and c) whether the average or median event severity for treated events differed between groups? Does it appear that a CF diagnosis is associated with a greater likelihood of antibiotic intervention for a given level of event severity? Was prior antibiotic treatment associated with a higher probability of treatment of a subsequent event? Can the authors elaborate on any differences in primary care providers between the two groups? Were CF infants more likely to have primary care providers following other CF patients?

4. The authors suggest “the possible preventive and therapeutic potential of targeting the nasal microbiota in CF-related LRTI management,” a reasonable conclusion in keeping with a certain

orthodoxy of CF care developed over the past three decades. However, don't these data also suggest a need to reconsider reflexive use of antibiotics in CF infants as opposed to more deliberate and selective antibiotic use, with the intention of delaying/slowing (inevitable) changes in microbial community structure, with potential for better long-term outcomes? The demonstration that the very first antibiotic intervention starts CF infants down a road of airway microbial community change is sobering. One has to ask, is the administration of antibiotics to CF infants presenting with respiratory symptoms (pressuring their microbial communities in a seemingly undesirable direction) associated with better immediate outcomes? Do the benefits of antibiotic treatment early in life outweigh the possible long-term consequences? It would seem that once a CF infant is treated with antibiotics for a respiratory event, they are at increased risk for being treated with antibiotics for nearly all subsequent events. Don't these data suggest the possibility that the orthodoxy of treating bacteria for every respiratory event drives the very airway dysbiosis that is used to later to justify antibiotic use? CF orthodoxy interprets associations between respiratory event rates and different microbial genera as an indication of causality. The authors echo this orthodoxy when they suggest that "Early nasal microbiota alterations may contribute to an increased susceptibility to LRTIs in CF infants and may further increase after LRTIs and antibiotic treatment." But isn't it also possible that "susceptibility to respiratory events" in infants is driven primarily by underlying airway biology and that consistent, aggressive antibiotic response to each event drives further dysbiosis, creating this association between event rate and degree of dysbiosis? Would our patients be better served if we recognized dysbiosis as a marker of intervention? It would appear that we drive enrichment for opportunists that can survive greater antibiotic pressure and then blame the selected opportunists for causing our need to treat. Aren't we responsible for identifying the incremental benefit of antibiotic treatment in CF infants? I think these are interesting questions raised by these data.

Minor Comments

Page 4, line 67: "Pathogenic bacteria begin to colonize the airways of children with CF in early life, leading to recurrent, mostly polymicrobial lower respiratory tract infections (LRTIs)…" Although it is true that some bacterial genera and species found in the CF airways have been shown to be pathogens in other contexts, these are more correctly identified as "opportunists" as opposed to "pathogens". (this term is found in more than one place in the submission). I have already noted my concern with the description of respiratory events in children with CF as being LRTIs or that a second event is a "recurrence of infection". These children have chronic bacterial communities in their lower respiratory tracts (a consequence of an atypical host environment secondary to CFTR dysfunction) regardless of their clinical state. Infections do not come and go.

Page 4, Line 68: "These may lead to pulmonary exacerbations that require antibiotic treatment and hospitalizations…" There is no question that respiratory events result in antibiotic treatment, but there are no objective data that antibiotics are required for event resolution.

Page 10, Line 203: "Thus, certain microbiota profiles in CF infants might predispose to respiratory disease." As noted above, "certain microbiota profiles" are likely markers of more intensive past antibiotic use secondary to greater event rates and/or more aggressive treatment. Are the profiles a cause or a post-treatment consequence of more aggressive CF airway disease?

Page 10, Line 207: “In the respiratory tract, acute infections like otitis media or chronic rhinosinusitis…” Is otitis a respiratory event? Is chronic sinus inflammation associated with acute infection?

Page 10, Line 217: “Rare and transient (potentially environmental) species may increase…” It’s not clear what constitutes a rare species (perhaps that it is not commonly observed in the CF airway?). Aren’t all bacteria derived from the host environment?

D.R. VanDevanter

Reviewer #3 (Remarks to the Author):

Here the authors present a longitudinal study which allows powerful comparisons between the nasal microbiota of infants with CF and healthy infants. In this study the authors show that the nasal microbiota in CF infants is altered before first lung infection or antibiotic intervention. Indicating that targeting nasal microbiota could be a sensible and desirable option for managing infection in the lower airways in CF. The study benefits greatly from the underpinning multi-centre cohort studies (Swiss CF Infant Lung Development study and the Basel Bern Infant Lung Development study). The paper is clearly written and well presented. I have some minor comments below:

Line 93: Change to Table 1

Line 98: ‘… β-diversity … ’. It took some digging through the manuscript to find that the Bray-Curtis index of similarity was used. That was mentioned once and is way back in the materials and methods section at the ned of the manuscript. Please also state here (Line 98) B-diversity as measure by Bray-Curtis was used.

Lines 98-99: ‘… (PERMANOVA R2=0.016, $p<0.001$)…’ I note that although significant the R2 coefficient is very low / weak. This appear to be the case for the majority of PERMANOVA results throughout the manuscript. Is this because of the large variation within CF or healthy group nasal microbiota compositions? I would assume this is the case with enough differences between groups to be significantly different. May be worth mentioning at a pertinent part of the manuscript why these R2 values are so low.

Line 110: '… α-diversity … ' Same as above for Bray-Curtis. Please mention here that you used the Shannon index of diversity.

Lines 263-264: 'Parents collected biweekly anterior nasal swabs and sent them to the coordinating study center in Bern.' How where the sample sent? By post, by courier, by what? Please state.

Line 266: 'For transport and storage of nasal swabs UTM® system from Copan was used.' Please state which specific type of Copan UTM swabs that were used.

L271: '… or assigned to genus Burkholderia, …' Why were Burkholderia ASVs removed? What was the rational? Please state why.

No	Reviewer suggestion
	Reviewer #1
C1	Were the children with CF on prophylactic antibiotics as is the case for many across Western Europe eg prophylactic flucloxacillin
R1	Infants with CF were not on prophylactic antibiotics. As this information was not explicitly stated, we have now added it to the cohort description.
Lines 295-296	*Infants with CF received only therapeutic but not prophylactic antibiotic therapies.*
C2	pg6 110-112 - this section does not make sense
R2	We apologize if this is not clearly stated in the manuscript and rephrased the section, aiming to clarify the results.
Lines 114-117	*~~Infants~~Differences in α-diversity between infants with CF ~~had a higher α-diversity compared to~~ and healthy individuals could only be reported after first antibiotic treatment (higher α-diversity in CF measured by Shannon-diversity index) (Table 3). ~~However, differences in~~"Baseline" α-diversity ~~occurred first after~~at the ~~initial antibiotic treatment (with~~beginning of life or α-diversity after first RTI (without ~~LRTI reported), and~~ antibiotic treatment) did not ~~after~~ differ between the ~~first LRTI alone~~two groups (Table 3).*
C3	Figure 1 - How did the authors make a diagnosis of LRTI? Chest xray changes? clinically?
R3	We appreciate the reviewer's question. This is a very important aspect. We did not use chest x-rays or other imaging or laboratory tests to confirm the diagnosis of LRTI. The (clinically) assessed symptom score has been evaluated previously as a sensitive marker for LRTIs (Silverman et al. 2003, PMID: 12728166; Latzin et al. 2007, PMID: 17123315; Korten et al. 2014, PMID: 28778921). It has been used by others and us in several published studies before. However, the reviewer is correct that we are not able to differentiate between LRTI and URTI based on more objective measures if using only clinical modalities. We thus changed the terminology and state respiratory tract infection (RTI) instead of LRTI. The definition is still suitable and might be – as remarked by the reviewer – even more precise and more commonly used (Stern et al. 2013, PMID: 23594341). Using the term RTI for the respiratory symptoms assessed in our study is in line with recently published studies, also by leading microbiota and clinical CF experts (Bosch et al. 2017, PMID: 28665684, Jorth et al. 2019, PMID: 31018133). In addition, we added the following statement to the limitations section:
Lines 239-244	*~~In addition, LRTIs~~ RTI diagnoses were based on clinical assessment utilizing a previously validated respiratory symptom score (details are reported in the supplementary materials) [36,37]. Additional information (e.g. further diagnostics or treatment modalities during respiratory diseases in the infants CF study center) is not assessed systematically in our study. Fortunately, infants with CF rarely present with very severe respiratory tract symptoms (e.g. leading to hospitalizations) in Switzerland.* Entire manuscript: RTI instead of LRTI
C4	Why would you diagnose a LRTI in CF and NOT give antibiotics? This is NOT standard practice so I am unsure what the authors mean by after first LRTI but before antibiotics.
R4	We appreciate the inquiry. Perhaps using the term "RTI" instead of "LRTI" (see C3/R3) clarifies the matter, particularly from a clinical perspective. Please note that "severe" respiratory tract symptoms without the immediate application of antibiotics could also have the following reasons: First, study nurses might have assessed symptoms and nasal samples taken shortly before a doctor's visit, followed by antibiotics initiation thereafter. Second, in stable infants where a viral infection is highly probable (e.g., positive environmental history), some

	CF centers opt for cautious observation rather than immediate antibiotic administration, under close monitoring by physicians. Finally, in rare cases, parents may choose to observe symptom progression before initiating antibiotic therapy or delay contacting their treating physician upon symptom onset. We trust this explanation addresses the reviewer's concern. If preferred, we can include a similar statement in the manuscript or supplement.
C5	pg 7 "Antibiotic treatment at nasal swab" - what does this mean?
R5	The phrase "Antibiotic treatment at nasal swab" means that antibiotic administration occurred concurrently with the nasal swab. To clarify, we revised the wording.
Lines 154-155	~~*Antibiotic*~~*Ongoing antibiotic treatment at the time of nasal swab collection was associated with ~~a~~ higher α-diversity (…)*
C6	Table 1 - 2 children in control looks as though they received antibiotics but are not included further down
R6	We agree with the reviewer's statement and have now included the two healthy infants with antibiotic treatment in the lower part of the table. We performed the detailed antibiotic analysis only among infants with CF due to the low number of antibiotic therapies among healthy controls. Additionally, we have added a supplementary table and supplementary figure to provide further detail (see also Reviewer #2, comment 3).
Tables OLS	Table 1 New supplementary figure 1 (see below) New supplementary table 1 (see below)
C7	The authors state the potential role for probiotics but the evidence of enteral effects on the nasal microbiome is scarce - they do acknowledge this fact but fail to mention the new modulator medications, which will undoubtedly reduce exacerbation frequency and need for antibiotic intervention which will ultimately allow more normal development of airway, lung and gut microbiomes. I appreciate they are not all licenced from birth, but over next few years this will happen.
R7	We agree, and followed the reviewer's suggestion. We added an additional paragraph to the discussion.
Lines 260-267	*In the future, novel modulator therapies if started in infancy or early childhood are very likely to substantially decrease the frequency of exacerbations [38-40] and thus reduce antibiotic treatment. However, CFTR modulators are not (yet) available for infants and still not applicable for 10-15% of pwCF [41]. In light of potential improvements through early triple modulator therapy initiation, it is even more important to understand early microbiota development to prevent early lung damage. It becomes crucial to exercise caution in treatment strategies and prioritize antimicrobial stewardship. It might be promising to treat infants with CF with probiotics to prevent destabilization of the upper airway microbiota…*
	Reviewer #2
C1	I understand the nature of the microbiomics analyses conducted but am not sufficiently versed to critique technical approaches or methodologies, so I will defer to other reviewers for this (important) feedback. I will limit my comments to the larger conclusions drawn from these very interesting data.
R1	We thank the reviewer for the very thoughtful and constructive comments to our manuscript.
C2	With respect to the diagnosis/occurrence of LRTI (lower respiratory tract infection), I suspect that this is a term of convenience used by the authors to describe infants who presented during observation with elevated respiratory sign/symptom scores. It's not clear from the information provided if there were any additional tests/observations to confirm that these events included lower respiratory tract involvement. In other words, were there instances where

	infants presented with an elevated respiratory score but were found by imaging, etc. to be experiencing events that did not appear to involve the lower respiratory tract, and if so, were these excluded from LRTI counts? Were all events evaluated in the same manner? To be clear, I am not concerned about medical practice here, I am concerned about terminology… unless methodology was systematically employed to confirm lower airway involvement for respiratory events, perhaps just calling them respiratory events is more precise and less prone to misinterpretation. Further, it is now generally accepted that there are lower airway microbial communities in infants (and particularly in CF infants). If a microbial population resides in the lower airways before observed sign and symptom increases, is "infection" really the right term to use for a change in symptoms (and by extension, if a second event is observed, is this best described as a "recurrence of infection")? I understand that this is legacy terminology, but isn't "airway infection" now recognized as a chronic CF condition that transcends clinical state?
R2	This is a very important aspect. We did not use chest x-rays or other imaging or laboratory tests to confirm the diagnosis of LRTI. All events were evaluated in the same manner - via a (clinically) assessed symptom score via a standardized interview. We do not systematically ask if the infants have additional diagnostics (e.g. in their CF study center) and thus cannot further report or investigate respiratory symptoms in more detail (e.g. severity or a more specific diagnosis). Fortunately, CF infants rarely present with severe respiratory tract symptoms (e.g. leading to hospitalizations) in Switzerland. Thus, we believe this missing information does no not hamper our results (as numbers (if any) of very severe respiratory episodes are very low). We included this issue in the limitation section (details s. below). The reviewer is also right that the term "infection" could be discussed. However, we believe it is likely the most commonly used definition for respiratory events, especially in early CF disease/infancy (the terminology is also used in numerous studies in the CF population: Jorth et al. 2019, PMID: 31018133, Thornton CS et al. 2022, PMID: 36069903). As also remarked by reviewer 1 (C3), we cannot differentiate clearly between LRTI and URTI using only clinical modalities. We thus decided to change the terminology and state respiratory tract infection (RTI) instead of LRTI (see also reviewer #1, C3). However, if the reviewer prefers, we are happy to change the terminology to "respiratory event" in a further revision.
Lines 239-244	~~*In addition, LRTIs*~~ *RTI diagnoses were based on clinical assessment utilizing a previously validated respiratory symptom score (details are reported in the supplementary materials)* [36,37]*. Additional information (e.g. further diagnostics or treatment modalities during respiratory diseases in the infants CF study center) is not assessed systematically in our study. Fortunately, infants with CF rarely present with very severe respiratory tract symptoms (e.g. leading to hospitalizations) in Switzerland.* Entire manuscript: RTI instead of LRTI
C3	I'm particularly interested in differences in management of infants with and without CF with respect to respiratory events. Could the authors provide greater detail on a) the total numbers of respiratory events recorded in each group during the observation period, b) the proportion of events in each group that were treated with antibiotics, and c) whether the average or median event severity for treated events differed between groups? Does it appear that a CF diagnosis is associated with a greater likelihood of antibiotic intervention for a given level of event severity? Was prior antibiotic treatment associated with a higher probability of treatment of a subsequent event? Can the authors elaborate on any differences in primary care providers between the two

	groups? Were CF infants more likely to have primary care providers following other CF patients?
R3	Thank you for the valuable input. In addition to the information in table 1, we added a supplementary table with an overview of the detected symptomatic episodes / RTIs and a supplementary figure displaying longitudinal courses of each infant In summary, parents of the 50 infants with CF reported 145 weeks with RTIs and 55.2% of RTIs were treated with antibiotics. Parents of the 30 healthy infants reported 64 weeks with RTIs and around 4.7% of these RTIs were treated with antibiotics. Thus, our data clearly shows that a CF diagnosis is associated with a greater likelihood of antibiotic intervention for a given level of event severity. The likelihood of prior treatment was not clearly associated with subsequent treatment. The reported RTIs had a comparable severity as assessed by our symptom score. Regarding primary care provision: In Switzerland, infants with CF typically receive specialized care from pediatricians at CF centers. Routine appointments, scheduled every three months, focus on monitoring disease progression and infant development. Should respiratory symptoms arise or parents report concerns, oropharyngeal swabs are collected for microbiological analysis. The detection of typical CF opportunistic bacteria often prompts antibiotic therapy, commonly involving aminopenicillins during infancy. If the reviewer prefers to have this more detailed (next to the new figure in the OLS) information displayed in the main manuscript, we are happy to include an additional paragraph.
OLS	New supplementary figure 1 (see below) New supplementary table 1 (see below)
C4	The authors suggest “the possible preventive and therapeutic potential of targeting the nasal microbiota in CF-related LRTI management,” a reasonable conclusion in keeping with a certain orthodoxy of CF care developed over the past three decades. However, don’t these data also suggest a need to reconsider reflexive use of antibiotics in CF infants as opposed to more deliberate and selective antibiotic use, with the intention of delaying/slowing (inevitable) changes in microbial community structure, with potential for better long-term outcomes? The demonstration that the very first antibiotic intervention starts CF infants down a road of airway microbial community change is sobering. One has to ask, is the administration of antibiotics to CF infants presenting with respiratory symptoms (pressuring their microbial communities in a seemingly undesirable direction) associated with better immediate outcomes? Do the benefits of antibiotic treatment early in life outweigh the possible long-term consequences? It would seem that once a CF infant is treated with antibiotics for a respiratory event, they are at increased risk for being treated with antibiotics for nearly all subsequent events. Don’t these data suggest the possibility that the orthodoxy of treating bacteria for every respiratory event drives the very airway dysbiosis that is used to later to justify antibiotic use? CF orthodoxy interprets associations between respiratory event rates and different microbial genera as an indication of causality. The authors echo this orthodoxy when they suggest that “Early nasal microbiota alterations may contribute to an increased susceptibility to LRTIs in CF infants and may further increase after LRTIs and antibiotic treatment.” But isn’t it also possible that “susceptibility to respiratory events” in infants is driven primarily by underlying airway biology and that consistent, aggressive antibiotic response to each event drives further dysbiosis, creating this association between event rate and degree of dysbiosis? Would our patients be better served if we recognized dysbiosis as a marker of intervention? It would appear that we drive enrichment for opportunists that can survive greater antibiotic pressure and then blame the selected opportunists for causing our need to

	treat. Aren't we responsible for identifying the incremental benefit of antibiotic treatment in CF infants? I think these are interesting questions raised by these data.
R4	We thank the reviewer for this comment and this a very important thought. While our data do not directly address these questions, we have incorporated these considerations into our manuscript for further reflection by readers, including CF treating physicians.
Lines 249-259	*~~In the future, it~~Our data raise several questions concerning the "reflexive" use of antibiotics in infants with CF. Each respiratory event in infants with CF contributes to airway dysbiosis, which is subsequently used to justify antibiotic treatment. Perhaps a more deliberate and selective approach to antibiotic use could help in delaying or slowing the inevitable changes in microbial community structure, leading to potentially better long-term outcomes. We propose that early alterations in nasal microbiota may contribute to increased susceptibility towards RTIs in infants with CF and may exacerbate following RTIs and antibiotic treatment. However, it is also plausible that susceptibility to respiratory events in infants is primarily driven by underlying airway biology, with consistent and aggressive antibiotic responses exacerbating dysbiosis. Could we consider "dysbiosis" as a marker for intervention? These questions gain growing importance in times of CFTR modulator use.*
C5	Page 4, line 67: "Pathogenic bacteria begin to colonize the airways of children with CF in early life, leading to recurrent, mostly polymicrobial lower respiratory tract infections (LRTIs)…" Although it is true that some bacterial genera and species found in the CF airways have been shown to be pathogens in other contexts, these are more correctly identified as "opportunists" as opposed to "pathogens". (this term is found in more than one place in the submission). I have already noted my concern with the description of respiratory events in children with CF as being LRTIs or that a second event is a "recurrence of infection". These children have chronic bacterial communities in their lower respiratory tracts (a consequence of an atypical host environment secondary to CFTR dysfunction) regardless of their clinical state. Infections do not come and go.
R5	We agree with the reviewer's opinion and changed the wording accordingly throughout the manuscript.
Line 67	*Opportunistic ~~Pathogenic~~ bacteria begin to colonize the airways of children with CF in early life*
C6	Page 4, Line 68: "These may lead to pulmonary exacerbations that require antibiotic treatment and hospitalizations…" There is no question that respiratory events result in antibiotic treatment, but there are no objective data that antibiotics are required for event resolution.
R6	We agree with the reviewer's opinion and changed the wording.
Lines 68- 69	*These may lead to pulmonary exacerbations that are followed by~~require~~ antibiotic treatment and hospitalizations*
C7	Page 10, Line 203: "Thus, certain microbiota profiles in CF infants might predispose to respiratory disease." As noted above, "certain microbiota profiles" are likely markers of more intensive past antibiotic use secondary to greater event rates and/or more aggressive treatment. Are the profiles a cause or a post-treatment consequence of more aggressive CF airway disease?
R7	In our study, we could show that infants with a lower diversity *before* first application of antibiotics and *before* first RTI presented with more symptoms later on. Thus, we believe our data supports the hypothesis (statistically proven) that it is (at least also) a cause and not only the consequence of treatment. However, we acknowledge that many factors do contribute to the findings and rephrased the sentence accordingly.

Lines 209-213	*~~Importantly, next~~Next to the (expected) microbial alterations through respiratory infections and/or antibiotics, in our study, a lower α-diversity (importantly: prior first RTI and antibiotic application) is associated with a higher number of subsequent ~~LRTIs~~RTIs in infants with CF independent from antibiotic therapy. Thus, although different additional factors contribute to disease development, certain microbiota profiles in CF infants ~~might~~likely predispose to respiratory disease.*
C8	Page 10, Line 207: "In the respiratory tract, acute infections like otitis media or chronic rhinosinusitis…" Is otitis a respiratory event? Is chronic sinus inflammation associated with acute infection?
R8	The sentence might have been misleading. We changed it accordingly.
Lines 217-218	~~In the respiratory tract, acute infections like otitis~~Otitis media [30,31] or chronic rhinosinusitis [32] are associated with decreased α-diversity.
C9	Page 10, Line 217: "Rare and transient (potentially environmental) species may increase…" It's not clear what constitutes a rare species (perhaps that it is not commonly observed in the CF airway?). Aren't all bacteria derived from the host environment?
R9	We agree with the reviewer that the term "rare" is not precise and could be misleading, as we have not defined it well enough. We have revised the text for clarity.
Lines 225-228	*An opening of microbial niche spaces with elimination of single ~~pathogens~~opportunistic bacteria by antibiotics might explain our findings, e.g. a rise in gram-negative bacteria after antibiotics in CF infants has been reported [18]~~.~~, or transient environmental species may increase after initial antibiotic treatment.*
	Reviewer #3
C1	Line 93: Change to Table 1
R1	We are sorry for this mistake and followed the reviewer's suggestion.
Line 93	*T~~t~~able 1*
C2	Line 98: '… β-diversity … '. It took some digging through the manuscript to find that the Bray-Curtis index of similarity was used. That was mentioned once and is way back in the materials and methods section at the end of the manuscript. Please also state here (Line 98) B-diversity as measure by Bray-Curtis was used.
R2	Thank you for bringing this to our attention. We followed the reviewer's suggestion and changed the manuscript accordingly.
Line 100	*β-diversity, measured by Bray-Curtis dissimilarity, was higher in infants with CF*
C3	Lines 98-99: '… (PERMANOVA R2=0.016, p<0.001)…' I note that although significant the R2 coefficient is very low / weak. This appear to be the case for the majority of PERMANOVA results throughout the manuscript. Is this because of the large variation within CF or healthy group nasal microbiota compositions? I would assume this is the case with enough differences between groups to be significantly different. May be worth mentioning at a pertinent part of the manuscript why these R2 values are so low.
R3	Thank you for pointing this out. We acknowledge that the R^2 values from the PERMANOVA analyses are indeed low, despite being statistically significant. This is indeed likely due to the large variation within the nasal microbiota compositions of both groups, which becomes more pronounced in the CF group over time. Such intra-group variability can result in low R^2 values even when differences between the groups are present. Additionally, the (pseudo-)F-values of the models were quite large, indicating strong effects. We have included a statement in the manuscript to explain this observation.

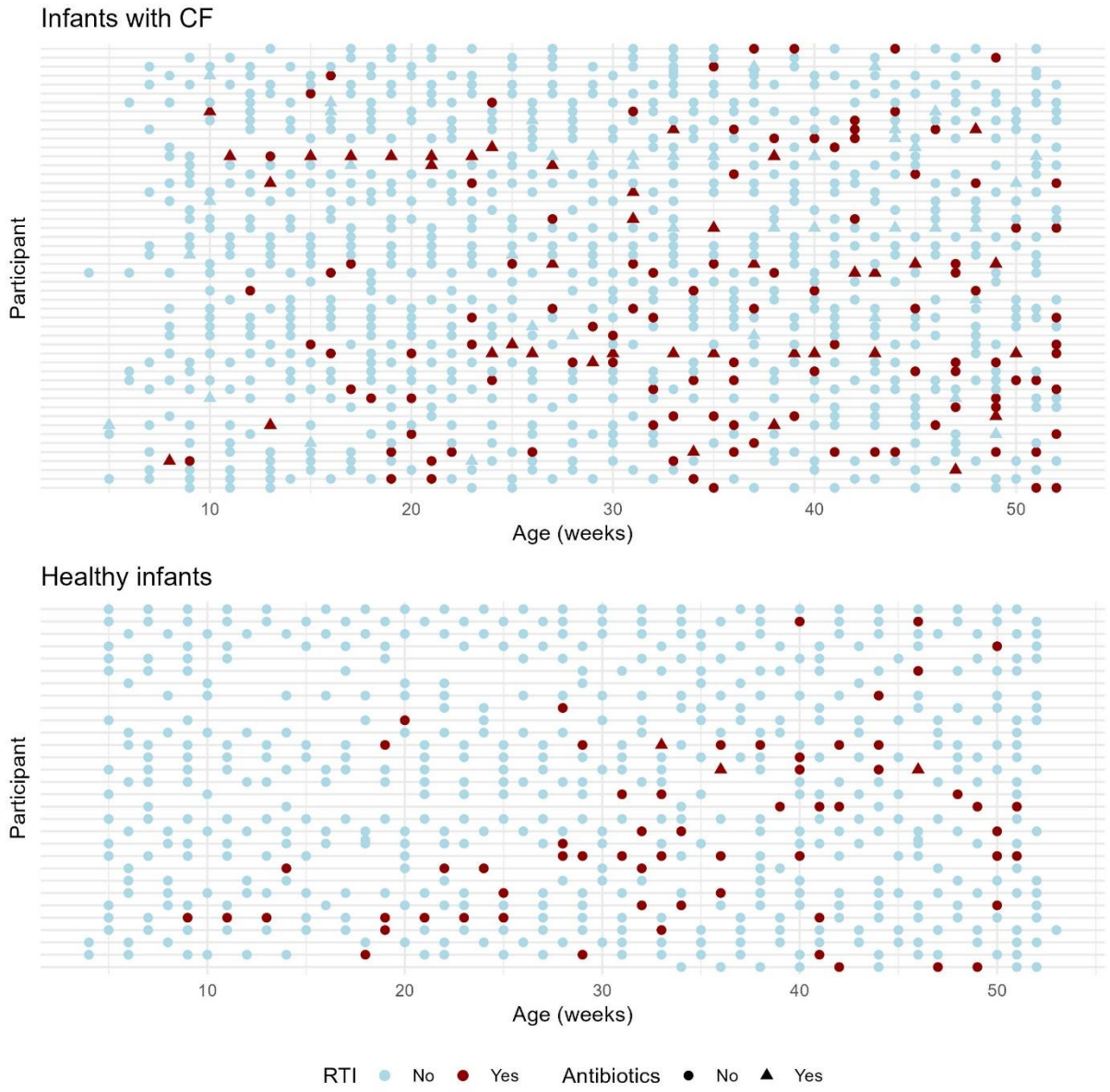
Lines 105-107	*The R^2-values from the PERMANOVA analyses were low overall despite being statistically significant, likely due to the large intra-group variation within the nasal microbiota compositions of both CF and healthy groups.*
C4	Line 110: '… α-diversity … ' Same as above for Bray-Curtis. Please mention here that you used the Shannon index of diversity.
R4	We agree and followed the reviewer's suggestion and changed the manuscript accordingly.
Lines 115-116	*…higher α-diversity in CF measured by Shannon-diversity index…*
C5	Lines 263-264: 'Parents collected biweekly anterior nasal swabs and sent them to the coordinating study center in Bern.' How where the sample sent? By post, by courier, by what? Please state.
R5	We followed the suggestion and added that nasal swabs were sent by post.
Lines 294-295	*Parents collected biweekly anterior nasal swabs and sent them by post to the coordinating study center in Bern.*
C6	Line 266: 'For transport and storage of nasal swabs UTM® system from Copan was used.' Please state which specific type of Copan UTM swabs that were used.
R6	The used nasal swabs are called Cat N 330C. 16x100mm tube filled with 3ml UTM® medium.
Line 298	*For transport and storage of nasal swabs UTM® system Cat N 330C from Copan was used.*
C7	L271: '… or assigned to genus Burkholderia, …' Why were Burkholderia ASVs removed? What was the rational? Please state why.
R7	Thank you for spotting this relevant mistake, it is a typo in the Methods description. We removed ASVs that were not assigned to Kingdom *Bacteria* (either *Mitochondria* or *Chloroplast*) or that were identified as background contamination by the R package *decontam*.
Lines 302-304	*We removed ASVs not assigned to kingdom Bacteria ~~or assigned to genus Burkholderia,~~(family "Mitochondria" or class "Chloroplast") or identified as contaminating ASV with the decontam package in R [41, 47].*

New supplementary table and figure:

Supplementary table 1: Summary of weeks with RTI and antibiotic treatments

	CF (n=50)	Healthy (n=30)
Weeks with RTI, all infants (sum (%))	145 (15.5)	64 (11.1)
- Of those with AB treatment (%)	55.2	4.7
Weeks with AB, all infants (sum (%))	80 (8.6)	3 (0.5)
Weeks with RTI per infant (mean (range))	2.9 (0, 12)	2.1 (0, 8)
Weeks with AB per infant (mean (range))	1.6 (0, 14)	0.1 (0, 2)

Abbreviations: RTI = respiratory tract infection; CF = Cystic Fibrosis; AB= antibiotic treatment



Supplementary figure 1: Longitudinal display of study cohort. Each row of the y-axes displays a study participant. The x-axes show the age in weeks. Each symbol shows a data point (nasal swab and interview). Red colours show that RTIs were reported and triangles show antibiotic treatment.

REVIEWERS' COMMENTS:

Reviewer #1 (Remarks to the Author):

The authors addressed the comments appropriately and in full

Reviewer #2 (Remarks to the Author):

I am impressed with the authors' careful attention to detail in their responses to reviewer comments, and I believe that their revised submission is markedly improved for this reason. I have two minor comments that I'd like the authors to consider:

1. Throughout the document, the authors have employed the term “healthy” (e.g., healthy infants, healthy individuals) to identify the infants included in the study who were unaffected by CF. Although this is common legacy terminology, it is imprecise (and perhaps even a bit offensive to families with members with CF). Given that inclusion in the study required presentation with elevated respiratory signs and symptoms sufficient to result in physician consultation, is it correct that unaffected infants were “healthy”? The construct that people with CF are “unhealthy” (which follows from describing those without CF as “healthy”) may have been more appropriate when CF diagnoses were driven primarily by symptomology. I urge the authors to consider a more objective term for infants not diagnosed with CF, such as “unaffected” or “without CF”.

Page 10, lines 212-213. The authors suggestion that “ …although different additional factors contribute to disease, certain microbiota profiles in CF infants *likely* predispose to respiratory disease” lacks equipoise, in my opinion. The authors have demonstrated the association between certain microbiota profiles and more concerning airway disease, but they certainly have not demonstrated causality (i.e., that the potential for disease was equivalent across the population until certain microbiota profiles “arose”, predisposing infants to disease). Is it any *less likely* that these microbiota profiles are markers of a more problematic underlying airway biology (which, combined with a diagnosis of CF, result in massive antimicrobial use driving further change in microbial communities)?

Reviewer #3 (Remarks to the Author):

The authors have fully adressed all of my comments on the original review.

No	Reviewer suggestion
	Reviewer #1
C1	The authors addressed the comments appropriately and in full.
R1	We are pleased that we were able to satisfactorily address all the reviewer's comments.
	Reviewer #2
C1	I am impressed with the authors' careful attention to detail in their responses to reviewer comments, and I believe that their revised submission is markedly improved for this reason. Throughout the document, the authors have employed the term “healthy” (e.g., healthy infants, healthy individuals) to identify the infants included in the study who were unaffected by CF. Although this is common legacy terminology, it is imprecise (and perhaps even a bit offensive to families with members with CF). Given that inclusion in the study required presentation with elevated respiratory signs and symptoms sufficient to result in physician consultation, is it correct that unaffected infants were “healthy”? The construct that people with CF are “unhealthy” (which follows from describing those without CF as “healthy”) may have been more appropriate when CF diagnoses were driven primarily by symptomology. I urge the authors to consider a more objective term for infants not diagnosed with CF, such as “unaffected” or “without CF”.
R1	We thank the reviewer for appreciating the revised version. We substituted the term “healthy” after introducing it in the Methods section with “controls”.
C2	Page 10, lines 212-213. The authors suggestion that “ …although different additional factors contribute to disease, certain microbiota profiles in CF infants *likely* predispose to respiratory disease” lacks equipoise, in my opinion. The authors have demonstrated the association between certain microbiota profiles and more concerning airway disease, but they certainly have not demonstrated causality (i.e., that the potential for disease was equivalent across the population until certain microbiota profiles “arose”, predisposing infants to disease). Is it any *less likely* that these microbiota profiles are markers of a more problematic underlying airway biology (which, combined with a diagnosis of CF, result in massive antimicrobial use driving further change in microbial communities)?
R2	We agree with the reviewer that our results are not suitable to establish causality and changed the wording following the reviewer’s suggestion.
	Thus, although different additional factors contribute to disease development, certain microbiota profiles in CF infants might predispose to respiratory disease or reflect an underlying airway biology that is followed by higher number of RTIs and antibiotic treatments.
	Reviewer #3
C1	The authors have fully adressed all of my comments on the original review.
R1	We are pleased that we were able to satisfactorily address all the reviewer's comments.