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Distinct Nasal Airway Bacterial Microbiota Differentially Relate to Exacerbation in Pediatric Asthma

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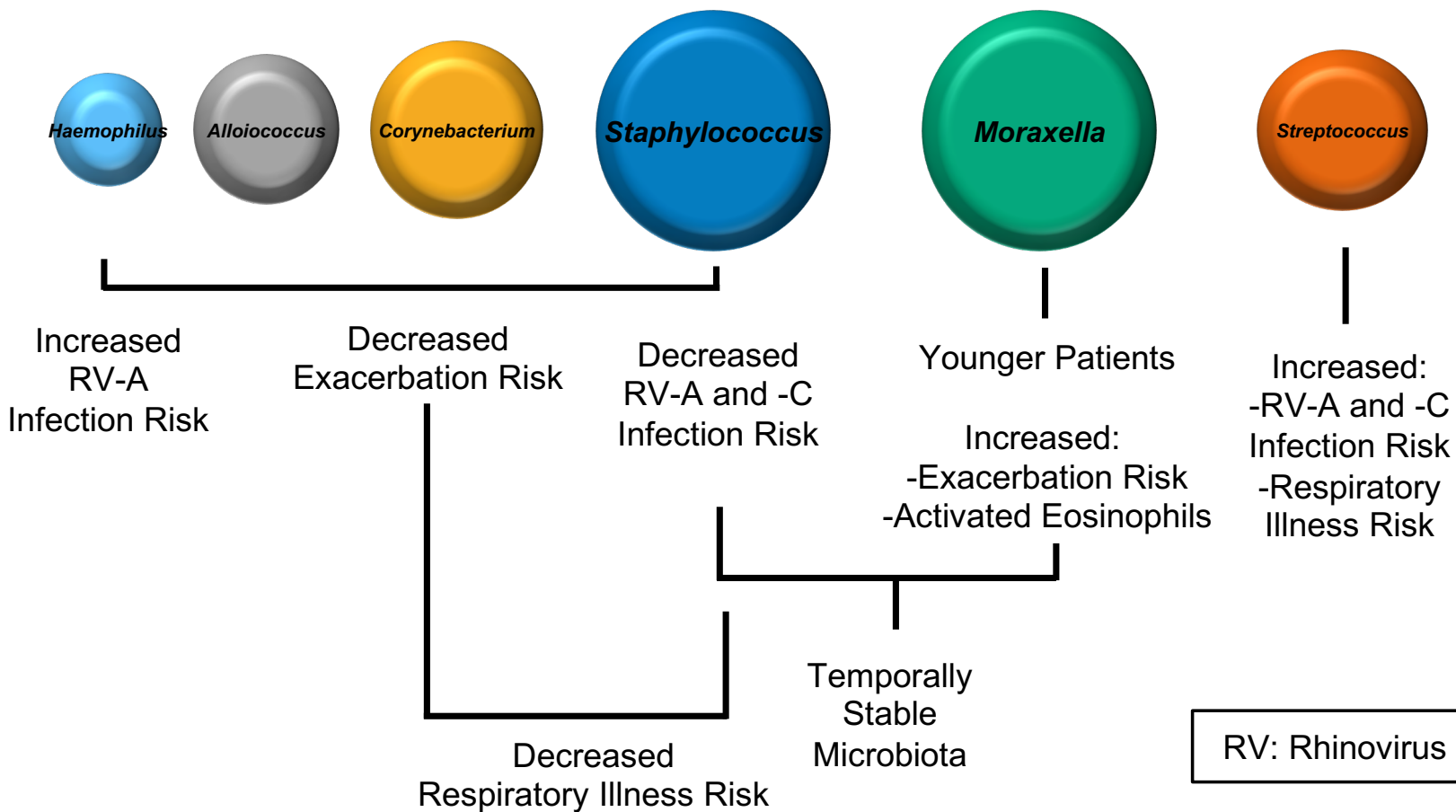
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6 YEARS 17

N=413 subjects
N=3,122 nasal airway samples



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Conflicts of Interest Statement:

All authors, with the exception of A. Togias, report grants from NIH during the conduct of study. R. Valladares reports personal fees for employment with Siolta Therapeutics outside the submitted work. H. Tran reports personal fees from Glaxo SmithKline outside the submitted work. J. Pongracic reports provision of study drug for other asthma and allergy studies from Glaxo SmithKline, Boehringer-Ingelheim, and Genentech/Novartis outside the submitted work. C.M. Kerckmar reports personal fees from Glaxo SmithKline for service on a DSMB and

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antibiotic resistance (WO2012027302A3)” issued, a patent “Nitroreductase enzymes (US7687474B2)” issued, a patent “Sinusitis diagnostics and treatments (WO2013155370A1)” licensed by Reflourish, LLC, and a patent “Methods and systems for phylogenetic analysis (US20120264637A1)” issued. S.V. Lynch is a co-founder of Siolta Therapeutics, a startup developing a mixed-species microbial oral therapeutic for induction of immune tolerance. A. Calatroni, G.K.K. Hershey, D. W. Fadrosch, H. Kim, B. LaMere, P. Lebeau, K. Lynch, J. Durack, D.L. Lin, G. Merana, R.T. Cohen and K. McCauley have nothing to disclose outside the submitted work.

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Background: In infants, distinct nasopharyngeal bacterial microbiota differentially associate with incidence and severity of acute respiratory infection and childhood asthma development.

Objective: We hypothesized that distinct nasal airway microbiota structures also exist in children with asthma and relate to clinical outcomes.

Methods: Nasal secretion samples (n=3,122) collected post-randomization during the fall season from children with asthma (6-17 years, n=413) enrolled in a trial of omalizumab (anti-IgE) underwent 16S rRNA profiling. Statistical analyses with exacerbation as the primary outcome and RV infection and respiratory illnesses as secondary outcomes were performed. Using A549 epithelial cells, we assessed nasal isolates of *Moraxella*, *Staphylococcus* and *Corynebacterium* for their capacity to induce epithelial damage and inflammatory responses.

Results: Six nasal airway microbiota assemblages, each dominated by *Moraxella*, *Staphylococcus*, *Corynebacterium*, *Streptococcus*, *Alloiococcus* or *Haemophilus* were observed. *Moraxella* and *Staphylococcus*-dominated microbiota were most frequently detected and exhibited temporal stability. Nasal microbiota dominated by *Moraxella* associated with increased exacerbation risk and eosinophil activation. *Staphylococcus*- or *Corynebacterium*-dominated microbiota associated with reduced respiratory illness and exacerbation events, while *Streptococcus*-dominated assemblages increased the risk of RV infection. Nasal microbiota composition remained relatively stable despite viral infection or exacerbation; only a few taxa belonging to the dominant genera exhibited relative abundance fluctuations during these events. *In vitro*, *M. catarrhalis* induced significantly greater epithelial damage and inflammatory cytokine expression (IL33 and IL8), compared with other dominant nasal bacterial isolates tested.

Conclusion: Nasal airway microbiota of children with asthma relate to the likelihood of exacerbation, RV infection, and respiratory illnesses during the fall season.

Key Messages:

Children with asthma, ages 6 to 17 years, who possess a *Moraxella*-dominated nasal airway microbiota are typically younger and at increased risk of exacerbation.

Nasal airway microbiota dominated by *Staphylococcus*- or *Corynebacterium* associated with reduced risk of exacerbation and respiratory illness events, while *Streptococcus*-dominated microbiota increased the risk of upper respiratory illnesses.

Moraxella- and *Staphylococcus*-dominated microbiota are stably maintained in the upper airways of children with asthma.

Capsule Summary:

Distinct nasal airway microbiota exist in children with severe asthma and relate to exacerbation risk, RV infection and respiratory illnesses. Understanding the mechanisms of such microbiome-epithelial interactions may offer insights into clinical heterogeneity.

Key Words: Microbiota, *Moraxella*, *Staphylococcus*, 16S rRNA, Airway, Asthma, Exacerbation, Rhinovirus

Abbreviations: Eosinophil Cationic Protein (ECP), Rhinovirus (RV), Preventive Omalizumab or Step-Up Therapy for Severe Fall Exacerbations (PROSE), Ribosomal Ribonucleic Acid (rRNA), Quantitative Polymerase Chain Reaction (qPCR)

Introduction

Viral respiratory infections have long been recognized as an important contributor to wheezing illnesses and asthma exacerbations; however, there is growing interest in the role of the airway microbiome and its potential to modulate these events in children with asthma(1). Bisgaard and colleagues demonstrated that detection of *S. pneumoniae*, *M. catarrhalis* or *H. influenzae* in nasopharyngeal samples obtained at 1 month of age was linked to increased risk for asthma at 5 years of age(2). The Childhood Asthma Study (CAS), a prospective cohort of infants (n=234), examined nasopharyngeal bacterial communities over the first year of life, including samples collected during periods with and without acute respiratory illness(3). Within this cohort, six compositionally distinct microbiota, each dominated by *Moraxella*, *Staphylococcus*, *Corynebacterium*, *Streptococcus*, *Alloiococcus* or *Haemophilus* were identified. Microbiota dominated by *Moraxella*, *Streptococcus* or *Haemophilus* were associated with significantly elevated risk of virus-associated acute respiratory illness(3) while *Streptococcus*-dominated microbiota co-associated with increased risk of lower airway infection and subsequent childhood asthma development.

Prior acute sinus infection has been associated with significant relative enrichment of upper airway *Moraxella* in children, and expansion of this genus in healthy (non-URI) samples predicted subsequent acute sinusitis(4) and relates to airway eosinophilia and bronchial inflammation in adults with asthma(5). Independently, upper airway detection of *S. pneumoniae* has been associated with increased respiratory illness symptoms and moderate asthma exacerbations, particularly when co-detected with rhinovirus (RV), while *M. catarrhalis* and RV in combination increased the likelihood of respiratory illness or asthma symptoms, or both compared with infection with RV alone(6). Thus, the presence of specific bacterial genera and

their co-associated microbiota assemblages strongly and reproducibly relate to respiratory illness, particularly in relation to viral infection. Given that viral respiratory infection is a risk factor for asthma exacerbation(7), and that bacterial lipopolysaccharide has recently been shown to modulate viral stability(8), we hypothesized that compositionally distinct microbiota exist in the nasal airways of children with asthma, and are related to risk of viral infection and asthma exacerbation.

Methods

Study Design and Clinical Outcomes

This study followed 478 children with asthma (aged 6-17 years) from eight urban clinics (Boston, Chicago, Cincinnati, Dallas, Denver, Detroit, New York, Washington D.C.) participating in the “Preventative Omalizumab or Step-up Therapy for Severe Fall Exacerbations” (PROSE; NCT01430403) randomized controlled trial (Table E1)(9). Participants provided home-collected nasal secretion samples every two weeks throughout the 90-day Fall outcome periods from one of two study years (September-December 2012 or 2013) for analysis in this study (Figure 1). Sample collection and storage was standardized across all clinical sites. Participants with fewer than three high-quality microbiota profiles available for analyses were removed. See Supplementary Methods for details of sample acquisition, processing and analyses. Asthma exacerbations were defined *a priori* as physician-prescribed use of systemic corticosteroids for asthma symptoms and/or hospitalization for asthma(10,11). RV infection (detection of RV), was assessed by qPCR and partial sequencing to identify viral strain type(12).

Microbiota Analyses

DNA from nasal secretion samples was extracted using a modified cetyltrimethylammonium bromide (CTAB)–polyethylene glycol (PEG) protocol, and the

variable region 4 (V4) of the 16S rRNA gene was amplified, quantified, and sequenced on a NextSeq 500 (Illumina, San Diego, CA); details in Supplemental Methods. Statistical analyses were conducted using R(13) and QIIME(14). Relationships between community composition in initially-collected independent samples and a range of clinical and viral infection variables were assessed using *adonis* in the *vegan* package(15) in R. Respiratory illnesses were defined as an increased symptom score compared to pre-treatment baseline symptoms(16). Respiratory symptom scores were based on a 0-3 severity score (absent, mild, moderate, severe) for five different symptoms (runny nose, stuffy nose, sore throat, sneezing and cough); scores ranged from a minimum of 0 to a maximum of 15. Significant differences in taxon relative abundance were assessed using a three-model approach as previously described(17). Samples were stratified based on dominant taxon i.e. taxon that exhibited the largest proportion of sequence reads in each sample. Co-occurrence networks of OTUs were constructed using SparCC(18) and WGCNA(19) packages in R. Repeated measures analyses (Linear Mixed Effects and Generalized Estimating Equations) used participant as the random effect. P-values were adjusted for false discovery using the Benjamini-Hochberg method; a q-value of 0.15 was considered significant.

In vitro *Epithelial Response Studies*

Human alveolar epithelial cells (A549) were exposed to the sterile products of 72-hour biofilm cultures of nasal *Moraxella catarrhalis*, *Staphylococcus epidermidis*, *Staphylococcus aureus* and *Corynebacterium propinquum* isolates. Epithelial cell damage was assessed by lactate dehydrogenase (LDH) release and inflammatory gene expression by qPCR. Further information is available in Supplemental Methods.

Results

Clinical and Viral Factors Are Related to Nasal Airway Microbiota Composition

Of the 3,840 samples received, 3,122 samples from 413 children provided a high-quality microbiota profile for analysis. An average of 7.5 samples per person were available and samples were collected an average of 10.7 days apart (Figure 1). Clinical, demographic and microbiological factors (Table E2) measured during the 3-month post-randomization observation period were assessed for relationships with nasal airway microbiota composition (beta-diversity). Using the first post-randomization sample (“baseline” sample) available from each study participant for analysis (Figure 1, teal bars), we determined that variance in nasal airway microbial composition (beta diversity) was significantly associated with the dominant bacterial genus present, study site, age, eosinophil cationic protein (ECP) concentration in nasal airway secretions at randomization, total number of RV infections per participant, and samples in which RV was detected (RV infection samples; PERMANOVA; $p < 0.05$ for all; Table E2). In contrast, microbiota composition at baseline was not significantly related to respiratory illness symptoms at the time of sample collection ($R^2 = 0.048$, $P = 0.067$), treatment group (placebo, omalizumab or inhaled corticosteroid boost; $R^2 = 0.008$; $p = 0.079$; Table E2) or future exacerbations in the outcome period ($R^2 = 0.0043$, $p = 0.101$). These observations were supported by bootstrapped analysis i.e. repeatedly sub-sampling a single randomly selected sample for each participant (500 times; Table E2; additional information in supplemental methods).

While overall nasal microbiota composition at baseline was not related to exacerbation in the outcome period, this did not preclude the possibility that specific taxa may predict future exacerbations. To examine this possibility, we performed comparative and machine learning analyses to determine relationships between baseline bacterial taxon relative abundance and

asthma exacerbations in the outcome period. A number of distinct *Corynebacterium* and *Acinetobacter* taxa were depleted at baseline in children who went on to experience an exacerbation (Figure E1A). Elastic Nets analysis identified distinct *Moraxella* and *Staphylococcus* taxa that were predictive of exacerbation or non-exacerbation outcomes (Figure E1B), indicating that these species may relate to subsequent exacerbation susceptibility.

Taxa associated with exacerbation and RV-infection co-associate in bacterial networks

The bacterial genus most abundant in each sample (the dominant taxon) explained a large proportion of variability in microbiota composition (Table E2; Figure 2A), indicating that a gradient of microbiota compositions, punctuated by the presence of distinct dominant bacterial genera, exist in the nasal airways of children with asthma. The majority of samples in our study were dominated by either *Moraxella* (n=1,058; 33.9 %) or *Staphylococcus* (n=953; 30.5 %); remaining samples were dominated by *Streptococcus* (n=263; 8.4 %), *Alloiococcus* (n=261; 8.4 %), *Corynebacterium* (n=153; 4.9 %), *Haemophilus* (n=135; 4.3 %), or other genera (n=299; 9.6 %; Figure 2B). The specific taxa characteristically dominating each of the six most common microbiotas were also amongst those found to be significantly enriched or depleted in samples with RV infection (versus no infection) or respiratory illness (versus none) or children who experienced asthma exacerbations (versus no exacerbations) during the outcome period (Figure E2-E3). This suggested that distinct nasal airway microbiota dominated by different bacterial genera exist in children with asthma and that taxa within these microbiota relate to the primary (asthma exacerbation) and secondary (RV infection, respiratory illness) clinical outcomes in this cohort.

To identify specific bacterial taxa that consistently co-associate within nasal airway microbiota, we next applied network analysis to microbiota profiles generated from all samples (Figure 3; Table E3). Notably, dominant *Moraxella* taxa consistently co-associated almost exclusively with other *Moraxella* taxa, including several that were enriched in participants who experienced exacerbations (Figure 3, Table E4). In contrast, the dominant taxa in each of the other nasal airway microbiota formed bacterial networks consisting of both phylogenetically similar and distinct genera, often including the same specific taxa enriched in RV negative and non-exacerbation samples.

Nasal microbiota are associated with clinical outcomes.

We next tested whether the six most prevalent nasal airway microbiota assemblages co-varied with primary (asthma exacerbation) and secondary (RV infection, respiratory illness events) clinical outcomes. Using all longitudinal samples (n=3,122) and generalized estimating equations we noted that asthma exacerbation, RV infection and respiratory illness events varied significantly across the six nasal airway microbiota. Children who experienced one or more exacerbations in the outcome period were more likely to possess *Moraxella*-dominated microbiota (RR=1.66, q=0.04) in their longitudinally collected samples. This observation was no longer significant following adjustment for participant's age (RR_{adj}=1.41, q=0.22). However, it should be noted that age was related to microbiota composition in our cohort (Table E2), and that younger children were more likely to possess *Moraxella*-dominated microbiota (Table 1; Figure E4). Though exacerbation was more likely to be associated with *Moraxella*-dominated microbiota, RV infection was not, suggesting that *Moraxella*-dominated microbiota may promote asthma exacerbation in the presence or absence of RV infection. This is supported by our

observation that eosinophil cationic protein (ECP) concentrations in nasal secretions (a marker of activated eosinophils) measured at randomization were significantly higher in *Moraxella*-dominated samples collected closest to the ECP measurement (Figure E4; Table 1), and that ECP concentrations did not significantly differ in RV-infected or uninfected *Moraxella*-dominated samples ($\beta=0.12$, $p=0.40$). In longitudinally collected samples, children who experienced one or more exacerbations were less likely to have nasal airway microbiota dominated by *Haemophilus* (RR=0.41, $q=0.04$), a finding that remained consistent following adjustment for age (RR_{adj}=0.39, $q=0.07$; Table E5). Similarly, microbiota dominated by *Alloiococcus*, *Corynebacterium* or *Staphylococcus* were also less likely to be observed in longitudinally collected samples from children who experienced one or more exacerbations in the outcome period (RR=0.61, $q=0.14$; RR=0.44, $q=0.12$; RR=0.70, $q=0.14$ respectively).

Distinct nasal microbiota relate to viral infection outcomes.

Using all available samples ($n=3,122$), we found that RV-infection samples, specifically those samples with RV-A detected, were less likely to exhibit *Staphylococcus*-dominated microbiota (RR=0.84, $q=0.05$; RR=0.75, $q=0.05$ respectively). In addition, participants with a greater number of virus-positive samples were also less likely to possess a *Staphylococcus*-dominated nasal airway microbiota (RR = 0.89, $q=0.03$). Conversely, RV infection, specifically RV-A, or respiratory illness events were more likely to occur in children with a *Streptococcus*-dominated microbiota (RR=1.54, $q<0.01$; RR=1.70, $q=0.03$; RR=1.78, $q<0.01$). However, asthma exacerbations were not significantly associated with the frequency of *Streptococcus*-dominated microbiota in this cohort.

We hypothesized that interactions between RV and the upper airway microbiota relate to clinical outcomes, specifically to asthma exacerbation and respiratory illness events. To test this, we determined the distribution of microbiota states in RV infected samples (n=969), RV-infected samples with a concomitant respiratory illness event (n=245) or RV-infected samples with a concomitant asthma exacerbation event (n=54; see supplemental methods for details; n=1,268 total samples). RV-infection with asthma exacerbation samples exhibited a distinct distribution of microbiota states (GEE ANOVA; $P < 0.001$; Figure E5). Consistent with our earlier taxonomic analyses, *Corynebacterium* and *Alloiococcus*-dominated microbiota were less likely to be detected in RV-infection with exacerbation samples. However, comparisons across the six individual microbiota states did not achieve statistical significance (Figure E5), likely due to the limited number of RV-infection with asthma exacerbation samples available for analysis (n=54). RV infections with respiratory illness (compared with RV-infection without asthma exacerbation or respiratory illness symptoms) also trended towards differences in microbiota distributions (GEE ANOVA $P = 0.087$). Specific comparisons between microbiota states indicated that *Streptococcus*-dominated microbiota and RV infection significantly increased relative risk of respiratory illness (RR=1.82, $P = 0.014$; Figure E5).

Staphylococcus- and Moraxella-Dominated Microbiota exhibit Temporal Stability

Leveraging the longitudinal nature of sample collection in this trial, we next examined microbiota stability and determined that *Moraxella*- and *Staphylococcus*-dominated microbiota were most frequently maintained in the nasal airways over time (Figure 4). These observations were consistent irrespective of whether analysis was performed on samples collected within a defined time period (7-13 days between sample acquisition), or only the first three samples per

participant were considered (to account for possible sample collection timing biases; Figure E6). Multiple factors differentiated individuals who were stably colonized by a *Moraxella*- or *Staphylococcus*-dominated nasal microbiota (defined as >50% of an individual's longitudinal samples dominated by *Moraxella* or *Staphylococcus*). Children with stable *Moraxella* colonization were more likely to have viral asthma exacerbations, and a higher number of RV infections and respiratory illnesses during the study period. Children with stable *Staphylococcus* colonization were typically older, had higher lung function values (likely co-linear with age), a longer duration of asthma and higher body mass index (Table E6).

We next used longitudinal samples from participants experiencing an asthma exacerbation in the outcome period (n=54 participants; n=498 samples) to examine the effect of exacerbation on both microbiota composition stability and bacterial taxon dynamics. Many participants (50%) exhibited compositionally stable microbiota (defined as a standard deviation of PC1 less than 0.25) dominated primarily (>50% of samples within an individual) by *Moraxella* (15/54; 28%) or *Staphylococcus* (6/54; 11%), despite exacerbation or RV infection (Figure E7A). At the taxon level, the majority of taxa exhibited consistent relative abundance over the period of observation; however, a small number of specific taxa, primarily belonging to the six dominant genera, exhibited temporal fluctuations in relative abundance during RV infection or exacerbation events (Figure E7B).

Differential impact of dominant nasal bacteria on in vitro airway epithelial responses

Spurred by the observation that nasal airway microbiota dominated by distinct opportunistic pathogenic bacterial genera differentially associate with clinical outcomes in our cohort, we hypothesized that the bacterial species dominating these microbiota have effects on

the airway epithelium. Using enrichment media, we isolated strains of *Moraxella catarrhalis*, *Staphylococcus epidermidis*, *Staphylococcus aureus* and *Corynebacterium propinquum* from nasal secretions of children with asthma and identified those exhibiting >97% sequence identity to dominant bacterial taxa identified in this study (Supplemental Methods). Cell-free sterile products of biofilm cultures (which mimic physiological features of mucosal-adherent bacteria) of each of these isolates, and *Lactobacillus sakei* [previously found to protect against nasal airway infection(20)] were used to stimulate airway epithelial cell-line cultures *in vitro*, prior to assessment of epithelial damage (LDH levels in supernatant) and inflammatory gene expression. Compared with *S. epidermidis* or *C. propinquum*, the cell-free products of *M. catarrhalis* isolates consistently increased epithelial damage (Wilcoxon $p < 0.01$) and gene expression of the pro-inflammatory cytokines IL-8 and IL-33 (Wilcoxon $p < 0.03$; Figure 5), as well as MUC5AC, CXCL10 and the epithelial tight junction protein Occludin (Figure E8). Of note, in comparison with *S. epidermidis* or *C. propinquum*, *S. aureus* also increased expression of IL-8 (Wilcoxon $p = 0.003$ and $p = 0.002$, respectively; Figure 5), in addition to CXCL10 and occludin (Wilcoxon $p < 0.05$; Figure E8). Hence, *M. catarrhalis* and *S. aureus*, but not *S. epidermidis* or *C. propinquum*, appear capable of promoting relatively increased epithelial damage and inflammation *in vitro*, offering a potential mechanism by which nasal microbiota dominated by these distinct species may differentially relate to clinical outcomes in this cohort.

Discussion

Our longitudinal analyses of the nasal airway bacterial microbiota of >3,000 samples from over four hundred children with asthma in the PROSE trial identified distinct microbiota associated with risk of asthma exacerbation, RV infection and respiratory illness. Omalizumab

therapy, which modulates IgE responses, successfully reduced fall exacerbations in children with asthma (10), however it did not significantly alter nasal airway microbiota composition, raising the possibility that nasal microbiota remain largely unaffected by treatments targeting specific features of asthma-associated immune dysfunction. The persistence of pathogenic nasal airway microbiota, particularly those dominated by *Moraxella*, which more frequently occurred in younger children, were associated with increased eosinophil activation and with asthma exacerbations plausibly helps explain several key questions in the field; namely why some children with asthma are “exacerbation prone”, some experience exacerbations in the absence of viral infection and why asthma symptoms frequently recur following cessation of anti-inflammatory treatments.

Perturbations to the upper respiratory microbiota have been described in children (2,3,6,21) and adults (5,22–24) with asthma, and recent evidence indicates that upper airway *Moraxella* are also detected in the lower airway of patients with asthma (5,25). Consistent with our observations, the relative abundance of upper respiratory *Moraxella* has been positively correlated with markers of eosinophilic inflammation (in both BAL fluid and blood) and negatively correlated with *Corynebacterium* (5). Although specific mechanisms by which microbiota of the upper airways contribute to lower airway inflammation and asthma exacerbations remain unclear, a growing body of evidence suggests that the upper airway microbiota may serve as an inflammatory trigger and/or the source of pathogenic microbes in the lower airway in those with chronic respiratory disease. Indeed, while many taxa detected in the nasal airway microbiota contribute to the relative temporal stability of these assemblages, the relative abundance of a small number of discrete taxa dynamically shift in parallel with asthma

exacerbation and RV infection events, suggesting that these organisms may be related to clinical outcomes.

Approximately 20-30% of asthma exacerbations in children are not associated with viral infection and their cause remains enigmatic (26). Our observations provide evidence that some asthma exacerbations may not be related to viral infection but rather to pathogenic bacterial activities, specifically, *Moraxella*-dominated microbiota. Our identification of *M. catarrhalis* as the dominant species in nasal airway microbiota of children who experience a greater frequency of exacerbation, and the subsequent *in vitro* evidence for the capacity of strains of this species to induce epithelial damage and increase IL-8 and IL-33 expression even in the absence of RV co-infection, support a role for this species in asthma pathogenesis. *M. catarrhalis* is an opportunistic human respiratory pathogen that encodes a range of proteins and ligands that promote its adherence and invasion of epithelial cells and can, via an induction of innate immune responses and complement evasion, result in tissue destruction and promote long-term persistence on the mucosal surface(27). Future studies employing shotgun metagenomic and paired RNAseq analyses will be required to determine the extent of virulence genes and their expression in airway microbiota. Such information could lead to novel treatment or vaccination strategies to prevent nasal airway colonization by pathogenic genera and reduce exacerbation risk in children with asthma.

The microbiota of children who did not experience asthma exacerbations were more likely to be *Alloiococcus*-, *Haemophilus*-, *Corynebacterium*- or *Staphylococcus*-dominated; reduced risk of respiratory illness was also associated with the latter two microbiota. These observations are largely consistent with the findings of Teo and colleagues in the CAS study(3) who noted that infants whose nasal airways were colonized by bacterial communities dominated

by *Alloiococcus*, *Corynebacterium* or *Staphylococcus* were at significantly lower risk of acute respiratory infection. Our observation that the biofilm products of *S. epidermidis* and *C. propinquum* (identified as dominant members of these protective microbiota) induced less epithelial damage and IL-8 and IL-33 expression compared to *M. catarrhalis* suggests that distinct nasal airway microbiota dominated by these species, may differentially modulate mucosal integrity and immunity in a manner that alters susceptibility and severity of respiratory illness in children with asthma.

It should be noted that the CAS study reported that while *Haemophilus*-dominated communities were infrequently detected in the infant nasopharynx (1.2% of population studied), these infants were at extremely high risk of acute respiratory illness(3). In our study of older children with established asthma, *Haemophilus*-dominated communities were also less frequent, but their presence was associated with reduced risk of exacerbation, plausibly explained by differences in age and airway development that exists between infants and children, bacterial strain differences, or increased *Haemophilus influenza* vaccination in this older population. Related to this, we did note a significant relationship between nasal airway microbiota and participant age, with *Moraxella*-dominated microbiota more frequently detected in younger participants, indicating that younger children with asthma who possess a *Moraxella*-dominated nasal airway microbiota may represent a particular subset of patients at heightened risk for exacerbation. Consistent with previous reports (6,28), RV infection or respiratory illness samples were more likely to be associated with *Streptococcus*-dominated microbiota, but contrary to those reports, not with asthma exacerbations in our cohort. When focusing on RV infections, we noted an elevated risk of asthma exacerbation in the presence of *Streptococcus*, but this finding did not reach significance.

Despite our large sample size there are a few study limitations to consider. Our study did not include pre-randomization samples or nasal airway samples from children without asthma, limiting our ability to determine whether the microbiota we describe characterize children without asthma at baseline. Also, the findings presented in this study derive from children selected on the basis of specific entry criteria and may be generalizable primarily to children with severe asthma living in low-income, urban environments. Further, fungal communities may also play a significant role in the nasal airway microbiota of children with asthma, however they have yet to be characterized in this population. We also note that other factors such as host genetics, epigenetics, pre-term birth (29), non-rhinovirus viral infections, allergens or microbial products may also be related to the observations made in this study. In addition, the V4 region of the 16S rRNA gene used to determine bacterial taxonomy, is frequently unable to discriminate between bacterial species or strains whose V4 region is identical. This is exemplified by our finding that both *S. epidermidis* and *S. aureus* belong to the same dominant *Staphylococcus* taxon; however, each elicits a distinct and opposing effect on epithelial integrity and inflammatory gene expression. This could explain the apparent contradiction that while *Staphylococcus*-dominated communities (possibly *S. epidermidis*-dominated) were overall protective against asthma exacerbation in our overall cohort, many of the children who provided acute asthma exacerbation samples also exhibited *Staphylococcus*-dominated (possibly *S. aureus*-dominated) microbiota. Indeed, a recent report demonstrated that *S. aureus* directly induces type-2 cytokine expression in nasal polyp tissue from older patients with chronic rhinosinusitis, which was not recapitulated upon infection with *S. epidermidis*(30), suggesting these distinct *Staphylococcus* species induce differential inflammatory responses.

In conclusion, this study identifies distinct nasal airway microbiota which are differentially related to risk for asthma exacerbation, RV infection, and respiratory illnesses. Our data form a foundation for more in-depth investigations to determine how distinct nasal airway microbiomes, and more specifically, active members of these assemblages interact with the host mucosa to promote or protect from exacerbations in children with asthma. Moreover, microbiome-based identification of children with asthma at heightened risk for exacerbation could lead to targeted strategies to promote appropriate nasal airway mucosal colonization and potentially reduce exacerbation risk. Additional studies are necessary before such strategies could be considered for clinical implementation.

Author contributions: S.V.L., D.J.J., J.E.G. designed the study; R.V., D.F., K.E.F., B.L. processed samples and generated microbiota data; J.D., G.M., K.L. undertook bacterial isolation and culture experiments; J.D. and D.L.L. performed *in-vitro* assays; S.V.L., D.J.J., K.M., J.E.G, J.D., and P.L. wrote the manuscript; S.V.L., K.M., P.L., H.T., A.C. performed the statistical analyses; R.T.C., J.P., G.K.K.H., C.M.K., M.G., A.H.L., H.K., M.K., S.T., P.J.G., A.T. collected the samples. All authors have approved the final version of this manuscript.

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Data and materials availability: Sequence data is available on the European Nucleotide Archive under accession number PRJEB25616.

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Figure Legends

Figure 1A. Study design and distribution of microbiota profiles from children in the Preventive Omalizumab and Step-Up Therapy for Severe Fall Exacerbations (PROSE) study (10) used in the current study. **B.** Frequency and timing of sample collection; blue bars depict the first post-randomization (baseline) sample collected from participants, all subsequent longitudinal samples collected are indicated by red bars. Green, blue and red dots indicate exacerbation, RV infection and respiratory illness events, respectively.

Figure 2. Compositionally distinct nasal airway microbiota exist in children with asthma.

A. The majority of asthmatic nasal airway microbiota are dominated by taxa belonging to either *Moraxella* or *Staphylococcus*, with smaller proportions of samples dominated by *Streptococcus*, *Alloiococcus*, *Corynebacterium*, *Haemophilus* or other genera. **B.** Six compositionally distinct nasal airway microbiota are evident in the nasal airways of children with asthma (Bootstrapped PERMANOVA; $P=0.001$).

Figure 3. Network analyses identifies three distinct modules of co-associated nasal airway

bacterial taxa. Taxa identified as differentially enriched in taxon comparisons of exacerbation versus non-exacerbation and RV-infection versus non-RV infection comparisons are indicated and color coded according to the dominant microbiota colors defined in Figure 2. Hub OTUs (triangles) exhibit greater inter-module connectivity while connector OTUs (circles) exhibit a higher frequency of intra-module connectivity. The size of the node (triangles or circles) scales

with the total number of connections with other OTUs. Genus classification and OTU number indicated.

Figure 4. *Staphylococcus* and *Moraxella*-dominated nasal airway microbiota exhibit

temporal stability in children with asthma. Heat map indicates the frequency with which a specific microbiota at a given time point (y-axis) remains the same or transitions to a distinct microbiota assemblage in the subsequent patient sample (Time point 2; x-axis). Frequency of these events is provided within each square and the proportion indicated by the color intensity e.g. 74% of *Moraxella*-dominated microbiota remain *Moraxella*-dominated in the subsequent sample. The diagonal represents transitions that resulted in maintenance of the same microbiota assemblage over time. Data were generated from longitudinally collected sample transitions (n=2,709) from all participants (n=413).

Figure 5. Biofilm-derived products of bacterial isolates dominating nasal airway

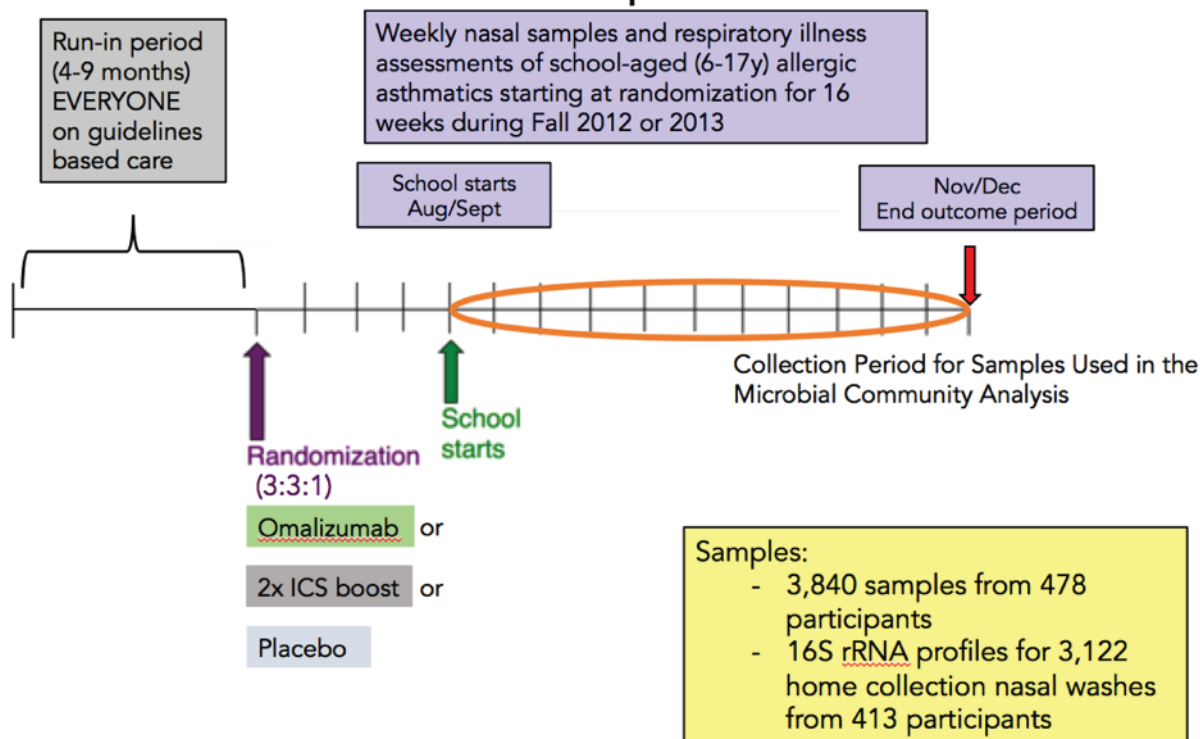
microbiota differentially influence epithelial responses *in vitro*. Comparative analysis of airway epithelial immune gene expression (relative to PBS) and lactate dehydrogenase (LDH) release [relative to lipopolysaccharide (LPS) stimulation] following exposure to sterile biofilm supernatants of *M. catarrhalis* [two strains, see supplemental material for more information], *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Corynebacterium propinquum*. *M. catarrhalis* strains consistently induce increased epithelial damage (LDH) and inflammation (IL-8 and IL-33) compared with *S. epidermidis*, *C. propinquum*, and *L. sakei*. Statistical significance was determined using Kruskal-Wallis (KW) tests. Results were obtained from two or more independent experiments using three biological replicates.

Table 1. Distinct Nasal Airway Microbiota Differentially Associate with Exacerbation, Viral Outcomes and Clinical Events.

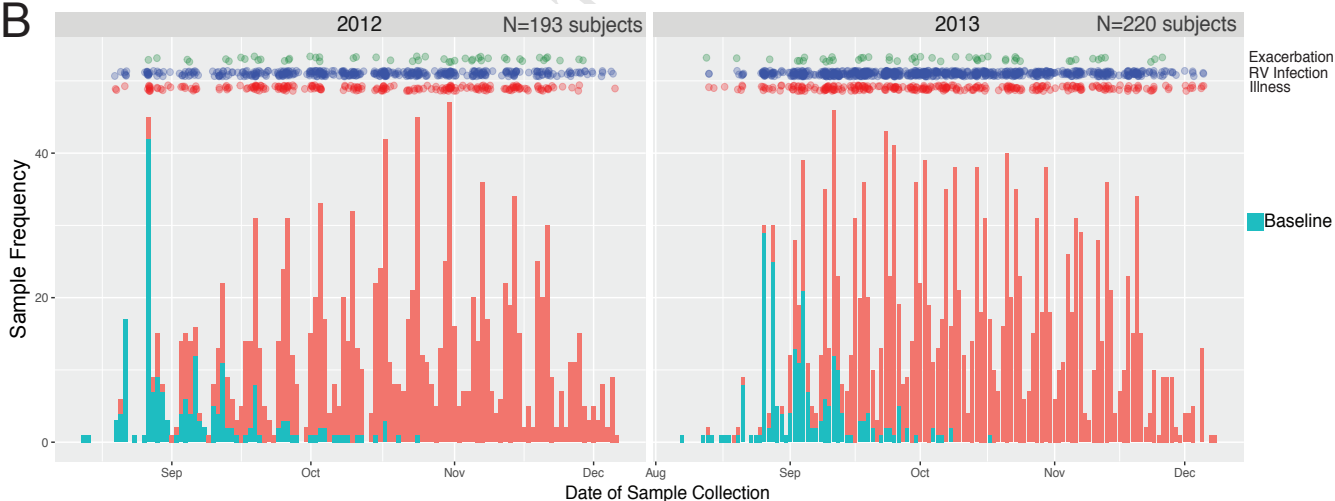
	<i>Alloiococcus</i>		<i>Corynebacterium</i>		<i>Haemophilus</i>		<i>Moraxella</i>		<i>Staphylococcus</i>		<i>Streptococcus</i>		Other	
	RR [*]	Q [†]	RR	Q	RR	Q	RR	Q	RR	Q	RR	Q	RR	Q
Per Participant Outcomes														
Child's Age at Randomization [‡]	1.01	0.90	1.35	<0.01	1.07	0.69	0.84	<0.01	1.05	0.32	0.96	0.69	0.97	0.69
FEV ₁ /FVC at Randomization [‡]	1.01	0.68	0.97	0.68	1.02	0.69	0.99	0.69	1.00	0.69	1.02	0.68	0.98	0.68
Log(ECP) at Randomization [‡]	0.94	0.78	0.58	0.21	0.65	0.58	1.75	0.03**	0.86	0.58	0.90	0.78	1.09	0.78
Exacerbation (Participant) [§]	0.61	0.14	0.44	0.12	0.41	0.04**	1.66	0.04**	0.70	0.14	1.22	0.42	1.23	0.40
Number of Viral Infections [§]	1.05	0.63	0.88	0.32	1.01	0.96	1.10	0.10	0.89	0.03**	1.06	0.48	1.00	0.96
Number of Respiratory Illnesses [§]	1.05	0.92	0.97	0.92	1.03	0.92	1.00	0.99	0.90	0.29	1.15	0.29	1.07	0.51
Per Sample Outcomes[§]														
Any Rhinovirus Infection	0.96	0.75	0.94	0.75	1.26	0.38	1.09	0.38	0.84	0.05	1.54	<0.01	0.80	0.22
Rhinovirus-A Infection (RV-A) vs. RV-	0.89	0.65	0.87	0.65	1.80	0.05	1.14	0.35	0.75	0.05	1.70	0.03	0.68	0.17
Rhinovirus-B Infection (RV-B) vs. RV-	1.29	0.39	0.85	0.50	0.87	0.67	1.13	0.39	0.89	0.39	1.34	0.39	0.65	0.26
Rhinovirus-C Infection (RV-C) vs. RV-	0.96	0.85	1.22	0.80	1.26	0.80	0.94	0.80	0.79	0.14	1.63	0.14	1.08	0.80
Respiratory Illness Sample ^{***}	0.84	0.49	0.56	0.05	1.35	0.33	0.95	0.63	0.76	0.05	1.78	<0.01	1.40	0.05

^{*}Relative Risks; [†]Q-values are p-values adjusted for multiple comparisons using a Benjamini-Hochberg False Discovery Rate across distinct microbiota for each covariate of interest; [‡]All variables measured at randomization were associated solely with first-collected microbiota sample, Generalized Linear Models (GLM) using the initial sample were used with a binomial outcome; [§]Results use all data from samples collected from an individual throughout the outcome period, Generalized Estimating Equations (GEE) using all longitudinal samples were used with a binomial outcome, and an exchangeable correlation structure; Estimate changed by more than 10% after adjustment for age; ^{**}P-value no longer significant after adjustment for age. Significant differences are emphasized q<0.15 in bold text; ^{***}Respiratory illness occurred concurrently with sample collection.

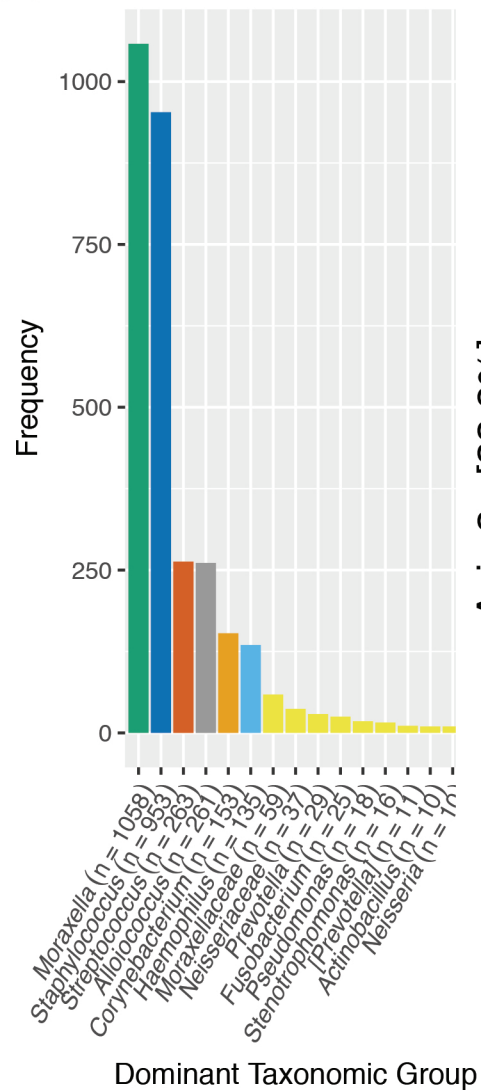
A



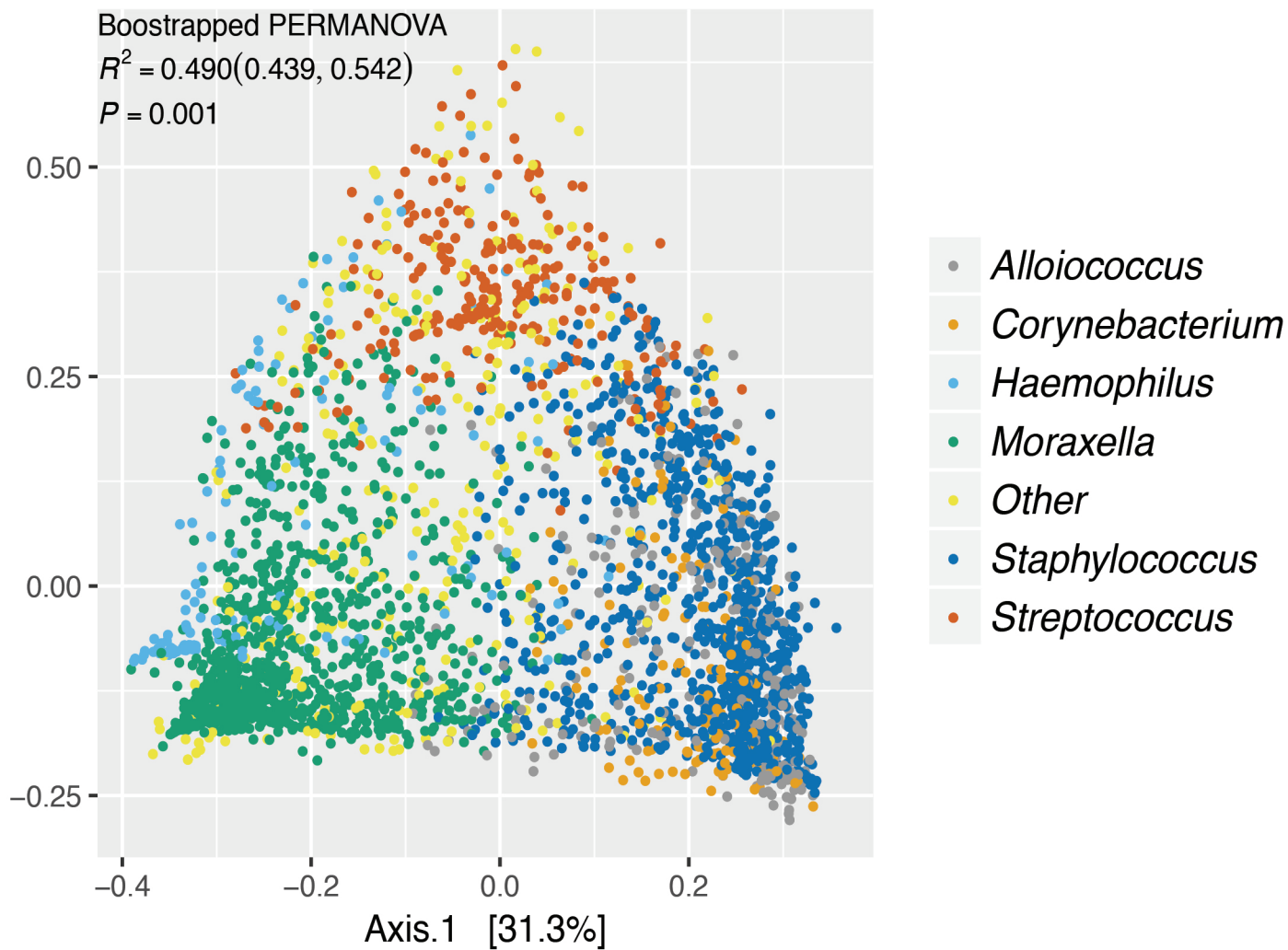
B

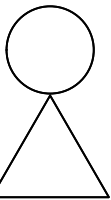


A

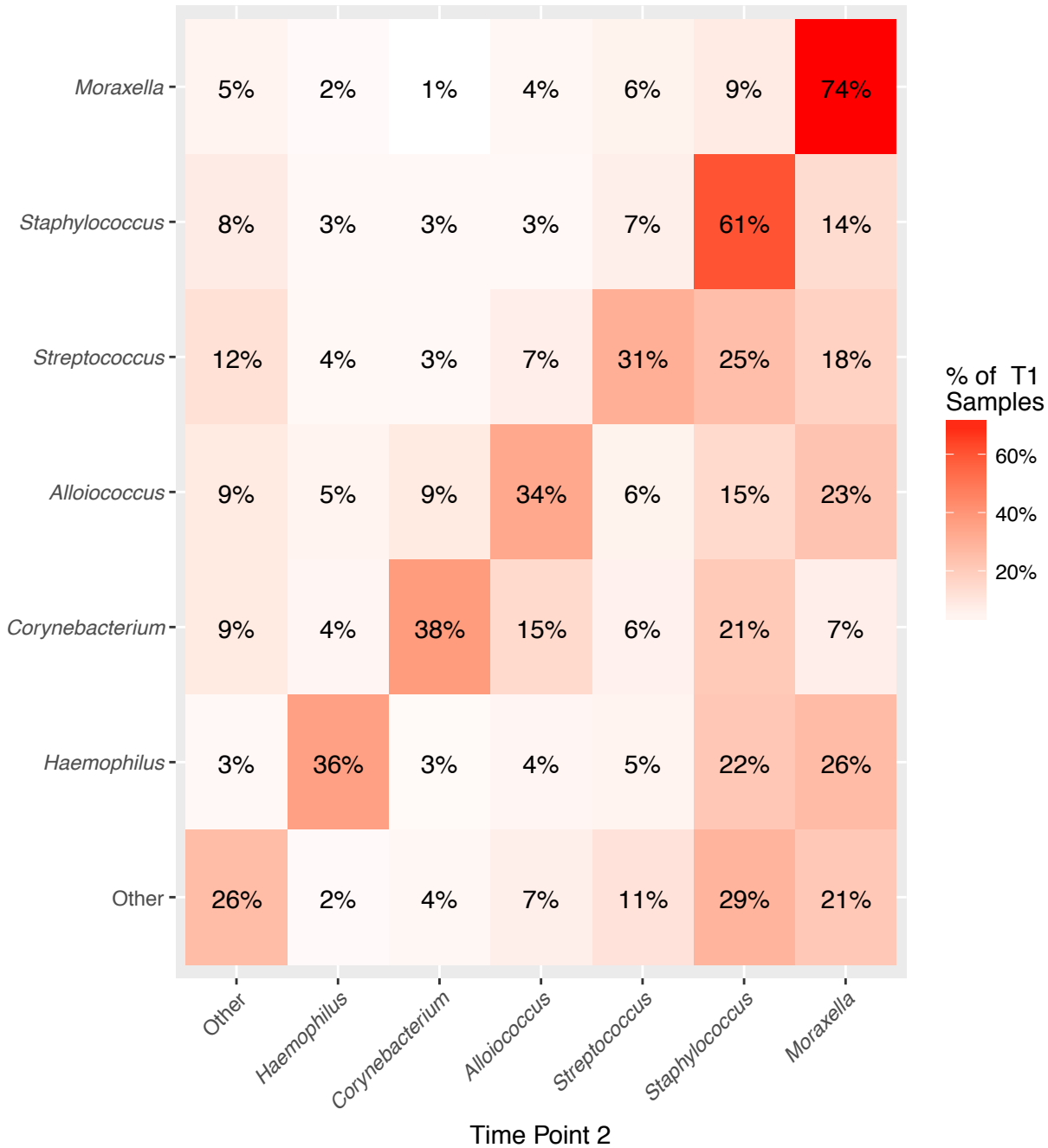


B





Time Point 1



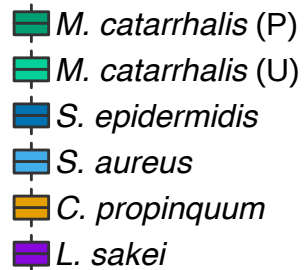
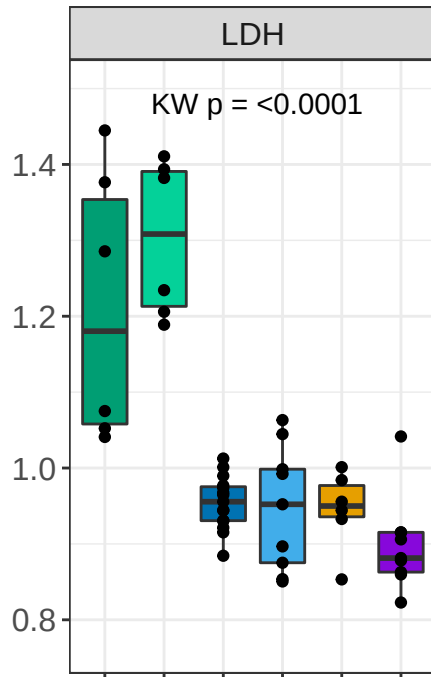
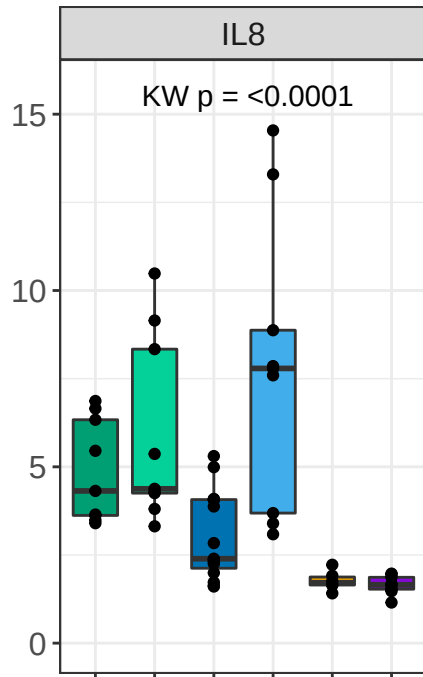
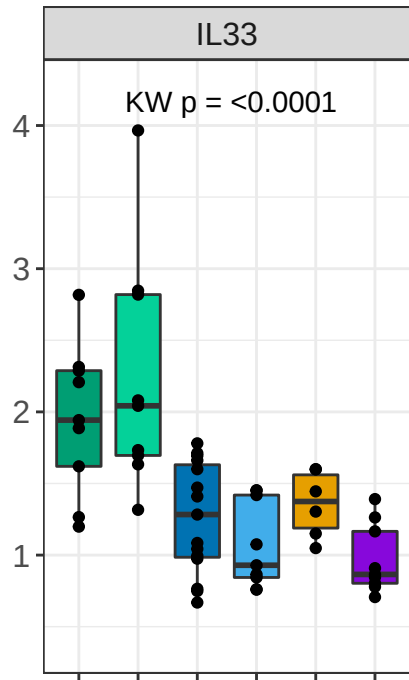


Table E1. Participant and Sample Characteristics for the Preventive Omalizumab or Step-Up Therapy for Severe Fall Exacerbations (PROSE) study.

Variable	<u>Individuals</u>		<u>Samples</u>	
	N (Mean)	% (SD)	n	%
Treatment Group				
ICS	113	0.27	863	0.28
Placebo	79	0.19	596	0.19
Xolair	221	0.54	1663	0.53
Treatment Step at Randomization				
Step 2-4	252	0.61	1928	0.62
Step 5	161	0.39	1194	0.38
Study Site				
Boston	51	0.12	401	0.13
Chicago	43	0.10	353	0.11
Cincinnati	49	0.12	365	0.12
Dallas	40	0.10	329	0.11
Denver	68	0.16	489	0.16
Detroit	41	0.10	281	0.09
New York	47	0.11	364	0.12
Washington D.C.	74	0.18	540	0.17
Exacerbation During the Outcome Period				
None	346	0.84	2624	0.84
One or More	67	0.16	498	0.16
Number of Viral Infections	(3.48)	(1.75)		
Rhinovirus Infection				
No			1883	0.60
Yes			1239	0.40

Rhinovirus Species				
None			1883	0.60
A			386	0.12
B			449	0.14
C			335	0.11
M			69	0.02
Sex				
Female	155	0.38	1158	0.37
Male	258	0.62	1964	0.63
Race				
Black (non-Hispanic)	239	0.58	1749	0.56
Hispanic	141	0.34	1126	0.36
Other/Mixed	26	0.06	201	0.06
White (non-Hispanic)	7	0.02	46	0.01
Age at Randomization				
6-10 years	242	0.59	1852	0.59
11-14 years	128	0.31	957	0.31
15-18 years	43	0.1	313	0.1
Age at Randomization, Overall	(10.25)	(2.97)		
Income above 15 th Percentile				
No	186	0.45	1398	0.45
Yes	223	0.55	1690	0.55

Table E2. Relationships Between Microbiological, Clinical and Viral Events and Nasal Microbiota Composition

Variable	R ² (baseline)	P-value (baseline)	R ² (bootstrapped ^a)	R ² (min, max)	P-value (bootstrapped)	P-value (min, max)
Dominant Genus	0.4680	0.001	0.4897	0.439, 0.542	0.001	0.001, 0.001
Study Site	0.0387	0.001	0.0259	0.015, 0.039	0.067	0.001, 0.658
Age at randomization ^b	0.0283	0.001	NA ^f	NA	NA	NA
Treatment Group	0.0080	0.079	0.0065	0.002, 0.015	0.282	0.002, 0.967
Log(ECP ^c), randomization ^b	0.0074	0.013	NA	NA	NA	NA
Rhinovirus Infected Sample	0.0067	0.010	0.0059	0.001, 0.018	0.121	0.001, 0.858
Total RV Infections, post-randomization	0.0051	0.053	0.0068	0.002, 0.016	0.053	0.001, 0.583
Sex	0.0039	0.121	0.0023	0, 0.008	0.503	0.011, 0.994
Exacerbation, outcome period	0.0043	0.101	0.0075	0.002, 0.016	0.041	0.001, 0.464
Treatment Step	0.0031	0.241	0.0017	0, 0.005	0.668	0.035, 1.000
Exacerbation Sample	0.0028	0.327	0.0025	0, 0.009	0.472	0.004, 0.995
Total Number of Respiratory Illnesses, post-randomization	0.0024	0.413	0.0035	0, 0.010	0.310	0.001, 0.980
FEV1 ^d /FVC ^e , randomization ^b	0.0011	0.859	NA	NA	NA	NA

^aBootstrapped: A single sample per individual is used to calculate PERMANOVA R² and p-value, repeating this process 500 times; resulting mean R² and p-values, as well as range of values are provided. ^bBootstrapped analyses are not presented as this measure occurs only at randomization.

^cECP: Eosinophil Cationic Protein. ^dFEV1: Forced Expiratory Volume 1. ^eFVC: Forced Vital Capacity. ^fNA: Not applicable.

Table E5. Distinct Nasal Airway Microbiota Differential Association with Exacerbation, Viral Outcomes and Clinical Events after adjustment for age.

	<i>Alloiococcus</i>		<i>Corynebacterium</i>		<i>Haemophilus</i>		<i>Moraxella</i>		<i>Staphylococcus</i>		<i>Streptococcus</i>		Other	
	RR	Q	RR	Q	RR	Q	RR	Q	RR	Q	RR	Q	RR	Q
Per Participant Measurements														
FEV1/FVC at Randomization	1.02	0.58	0.98	0.58	1.02	0.59	0.99	0.58	1.01	0.59	1.02	0.58	0.98	0.58
Log(ECP) at Randomization	0.95	0.82	0.72	0.71	0.68	0.71	1.68	0.07	0.88	0.71	0.87	0.82	1.06	0.82
Exacerbation (Participant)	0.62	0.25	0.63	0.38	0.39	0.08	1.41	0.22	0.79	0.38	1.16	0.56	1.22	0.46
Number of Viral Infections	1.06	0.82	1.00	0.97	1.00	0.97	1.04	0.82	0.92	0.39	1.04	0.82	0.99	0.97
Number of Colds	1.05	0.77	1.04	0.77	1.03	0.77	0.98	0.77	0.92	0.46	1.14	0.38	1.07	0.53
Per Sample Events														
Any Rhinovirus Infection	0.96	0.89	1.00	0.99	1.26	0.50	1.08	0.50	0.85	0.09	1.51	0.01	0.79	0.20
Rhinovirus-A (RV-A) vs. RV-	0.90	0.71	0.95	0.88	1.78	0.06	1.12	0.47	0.75	0.06	1.68	0.04	0.67	0.16
Rhinovirus-B (RV-B) vs. RV-	1.30	0.43	0.95	0.80	0.87	0.77	1.12	0.49	0.90	0.49	1.32	0.43	0.65	0.25
Rhinovirus-C (RV-C) vs. RV-	0.97	0.88	1.41	0.43	1.25	0.71	0.91	0.71	0.80	0.25	1.55	0.25	1.07	0.86
Cold Sample	0.84	0.49	0.57	0.07	1.35	0.33	0.94	0.57	0.76	0.06	1.78	<0.01	1.40	0.06

Table E6. Clinical factors Associated with Stable Colonization (>50% of samples) of *Moraxella* vs. *Staphylococcus* Microbiota

Variable	Stable Community	Estimate	P-value	ANOVA P-value	FDR P- value
Age at Screening	<i>Staphylococcus</i>	-0.061	<0.001		<0.001
Age at Randomization	<i>Staphylococcus</i>	-0.058	<0.001		<0.001
FEV1 at Screening	<i>Staphylococcus</i>	-0.205	<0.001		<0.001
FVC at Screening	<i>Staphylococcus</i>	-0.148	<0.001		0.001
FEV1 at Randomization	<i>Staphylococcus</i>	-0.194	<0.001		0.001
FVC at Randomization	<i>Staphylococcus</i>	-0.143	<0.001		0.001
FEV25/75 at Screening	<i>Staphylococcus</i>	-0.154	<0.001		0.002
BMI	<i>Staphylococcus</i>	-0.020	<0.001		0.006
FEV25/75 at Randomization	<i>Staphylococcus</i>	-0.128	0.001		0.013
Number of HRV-A Infections	<i>Moraxella</i>	0.102	0.002		0.021
Duration of Asthma at Randomization	<i>Staphylococcus</i>	-0.027	0.005		0.038
Number of Infections Post-Randomization	<i>Moraxella</i>	0.053	0.005		0.038
Adherence in the Run-in Period	<i>Moraxella</i>	0.006	0.006		0.038
Number of HRV-A,B,C Infections	<i>Moraxella</i>	0.055	0.008		0.038
Number of Infections After School Start	<i>Moraxella</i>	0.054	0.008		0.038
Number of Infections	<i>Moraxella</i>	0.050	0.008		0.038
Rhinovirus Exacerbations	<i>Moraxella</i>	0.265	0.008		0.038
Study Site	Boston	Ref.		0.009	0.038
	Chicago	<i>Staphylococcus</i>	-0.234	0.114	
	Cincinnati	<i>Staphylococcus</i>	-0.199	0.140	
	Dallas	<i>Staphylococcus</i>	-0.123	0.405	
	Denver	<i>Staphylococcus</i>	-0.005	0.968	
	Detroit	<i>Staphylococcus</i>	-0.179	0.190	
	New York	<i>Staphylococcus</i>	-0.464	<0.001	
	Washington D.C.	<i>Staphylococcus</i>	-0.190	0.108	
Any Viral Exacerbation	<i>Moraxella</i>	0.234	0.014		0.059
Proportion of Viral Infected Samples	<i>Moraxella</i>	0.326	0.016		0.068
Rhinovirus and Enterovirus Exacerbations	<i>Moraxella</i>	0.237	0.017		0.068
Participant in Mechanistic Study	<i>Moraxella</i>	0.170	0.020		0.074
Visit Occurred	<i>Moraxella</i>	0.277	0.021		0.074
Visits Missed	<i>Staphylococcus</i>	-0.277	0.021		0.074
Blood Eosinophil Count	<i>Moraxella</i>	0.296	0.024		0.083
Per-Protocol Deviation	<i>Staphylococcus</i>	-0.493	0.029		0.097
Number of HRV Infections	<i>Moraxella</i>	0.042	0.030		0.097
Number of Colds Post-School Start	<i>Moraxella</i>	0.064	0.031		0.097

Stable communities were defined as microbiota dominant in more than 50% of samples among all samples with a profile available. All individual-level variables available were tested; factors with a Q-value less than 0.1 are presented. "Stable Community" identifies whether the variable was increased in *Moraxella*-stable or *Staphylococcus*-stable communities (identified by the direction of the estimate).

Table E7: Summary of sequence alignment results for variable region V4 of 16S rRNA gene of nasal isolates compared to dominant organisms identified in PROSE children.

Isolate	Dominant Taxon	Align	Max score	Total score	E-value	% Identity
<i>Moraxella catarrhalis</i> (PROSE)	<i>Moraxella</i> OTU9408	251/253	457	457	4.00E ⁻¹³³	99
<i>Moraxella catarrhalis</i> (URECA)	<i>Moraxella</i> OTU9408	253/253	468	468	2.00E ⁻¹³⁶	100
<i>Staphylococcus epidermidis</i>	<i>Staphylococcus</i> OTU13177	253/253	468	468	2.00E ⁻¹³⁶	100
<i>Staphylococcus aureus</i>	<i>Staphylococcus</i> OTU13177	253/253	468	468	2.00E ⁻¹³⁶	100
<i>Corynebacterium propinquum</i>	<i>Corynebacterium</i> OTU12355	243/254	407	407	4.00E ⁻¹¹⁸	96

Table E8. Primers used for PCR reactions to determine relative expression of epithelial immune genes.

Gene	Forward primer	Reverse primer
IL-4	ACTTTGAACAGCCTCACAGAG	TTGGAGGCAGCAAAGATGTC
IL-8	ACTGAGAGTGATTGAGAGTGGAC	AACCCTCTGCACCCAGTTTTTC
IL-33	TCAGGTGACGGTGTTGATGG	GGAGCTCCACAGAGTGTTCC
Eotaxin	ACACCTTCAGCCTCCAACAT	GGTCTTGAAGATCACAGCTT
CXCL10	CCAGAATCGAAGGCCATCAA	CATTTCTTGCTAACTGCTTTCAG
IFN γ	ATCCAGTTACTGCCGGTTTG	GAAGCACCAGGCATGAAATC
MUC5AC	TACTCCACAGACTGCACCAACTG	CGTGTATTGCTTCCCGTCAA
Occludin	GATGAGCAGCCCCCAAT	GGTGAAGGCACGTCCTGTGT
β -actin	AAGATGACCCAGATCATGTTTGAGACC	AGCCAGTCCAGACGCAGGAT

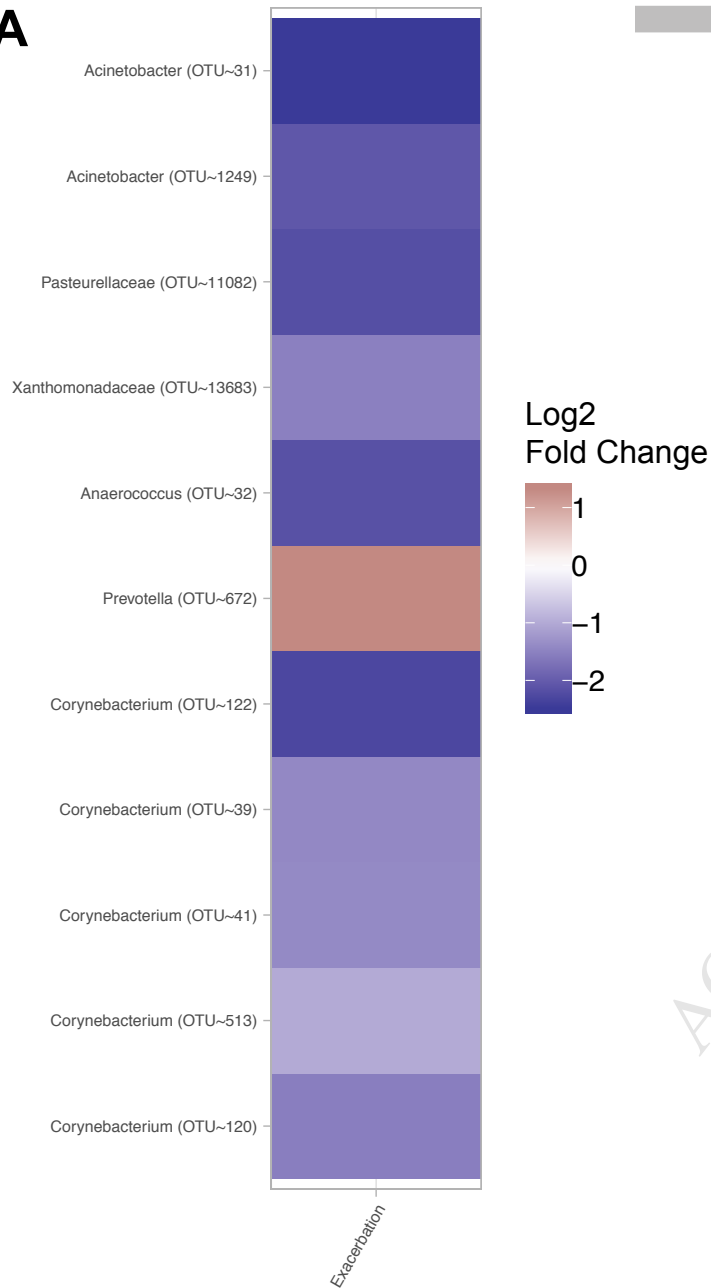
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	101	102	103	104	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120	121	122	123	124	125	126	127	128	129	130	131	132	133	134	135	136	137	138	139	140	141	142	143	144	145	146	147	148	149	150	151	152	153	154	155	156	157	158	159	160	161	162	163	164	165	166	167	168	169	170	171	172	173	174	175	176	177	178	179	180	181	182	183	184	185	186	187	188	189	190	191	192	193	194	195	196	197	198	199	200	201	202	203	204	205	206	207	208	209	210	211	212	213	214	215	216	217	218	219	220	221	222	223	224	225	226	227	228	229	230	231	232	233	234	235	236	237	238	239	240	241	242	243	244	245	246	247	248	249	250	251	252	253	254	255	256	257	258	259	260	261	262	263	264	265	266	267	268	269	270	271	272	273	274	275	276	277	278	279	280	281	282	283	284	285	286	287	288	289	290	291	292	293	294	295	296	297	298	299	300	301	302	303	304	305	306	307	308	309	310	311	312	313	314	315	316	317	318	319	320	321	322	323	324	325	326	327	328	329	330	331	332	333	334	335	336	337	338	339	340	341	342	343	344	345	346	347	348	349	350	351	352	353	354	355	356	357	358	359	360	361	362	363	364	365	366	367	368	369	370	371	372	373	374	375	376	377	378	379	380	381	382	383	384	385	386	387	388	389	390	391	392	393	394	395	396	397	398	399	400	401	402	403	404	405	406	407	408	409	410	411	412	413	414	415	416	417	418	419	420	421	422	423	424	425	426	427	428	429	430	431	432	433	434	435	436	437	438	439	440	441	442	443	444	445	446	447	448	449	450	451	452	453	454	455	456	457	458	459	460	461	462	463	464	465	466	467	468	469	470	471	472	473	474	475	476	477	478	479	480	481	482	483	484	485	486	487	488	489	490	491	492	493	494	495	496	497	498	499	500	501	502	503	504	505	506	507	508	509	510	511	512	513	514	515	516	517	518	519	520	521	522	523	524	525	526	527	528	529	530	531	532	533	534	535	536	537	538	539	540	541	542	543	544	545	546	547	548	549	550	551	552	553	554	555	556	557	558	559	560	561	562	563	564	565	566	567	568	569	570	571	572	573	574	575	576	577	578	579	580	581	582	583	584	585	586	587	588	589	590	591	592	593	594	595	596	597	598	599	600	601	602	603	604	605	606	607	608	609	610	611	612	613	614	615	616	617	618	619	620	621	622	623	624	625	626	627	628	629	630	631	632	633	634	635	636	637	638	639	640	641	642	643	644	645	646	647	648	649	650	651	652	653	654	655	656	657	658	659	660	661	662	663	664	665	666	667	668	669	670	671	672	673	674	675	676	677	678	679	680	681	682	683	684	685	686	687	688	689	690	691	692	693	694	695	696	697	698	699	700	701	702	703	704	705	706	707	708	709	710	711	712	713	714	715	716	717	718	719	720	721	722	723	724	725	726	727	728	729	730	731	732	733	734	735	736	737	738	739	740	741	742	743	744	745	746	747	748	749	750	751	752	753	754	755	756	757	758	759	760	761	762	763	764	765	766	767	768	769	770	771	772	773	774	775	776	777	778	779	780	781	782	783	784	785	786	787	788	789	790	791	792	793	794	795	796	797	798	799	800	801	802	803	804	805	806	807	808	809	810	811	812	813	814	815	816	817	818	819	820	821	822	823	824	825	826	827	828	829	830	831	832	833	834	835	836	837	838	839	840	841	842	843	844	845	846	847	848	849	850	851	852	853	854	855	856	857	858	859	860	861	862	863	864	865	866	867	868	869	870	871	872	873	874	875	876	877	878	879	880	881	882	883	884	885	886	887	888	889	890	891	892	893	894	895	896	897	898	899	900	901	902	903	904	905	906	907	908	909	910	911	912	913	914	915	916	917	918	919	920	921	922	923	924	925	926	927	928	929	930	931	932	933	934	935	936	937	938	939	940	941	942	943	944	945	946	947	948	949	950	951	952	953	954	955	956	957	958	959	960	961	962	963	964	965	966	967	968	969	970	971	972	973	974	975	976	977	978	979	980	981	982	983	984	985	986	987	988	989	990	991	992	993	994	995	996	997	998	999	1000	1001	1002	1003	1004	1005	1006	1007	1008	1009	1010	1011	1012	1013	1014	1015	1016	1017	1018	1019	1020	1021	1022	1023	1024	1025	1026	1027	1028	1029	1030	1031	1032	1033	1034	1035	1036	1037	1038	1039	1040	1041	1042	1043	1044	1045	1046	1047	1048	1049	1050	1051	1052	1053	1054	1055	1056	1057	1058	1059	1060	1061	1062	1063	1064	1065	1066	1067	1068	1069	1070	1071	1072	1073	1074	1075	1076	1077	1078	1079	1080	1081	1082	1083	1084	1085	1086	1087	1088	1089	1090	1091	1092	1093	1094	1095	1096	1097	1098	1099	1100	1101	1102	1103	1104	1105	1106	1107	1108	1109	1110	1111	1112	1113	1114	1115	1116	1117	1118	1119	1120	1121	1122	1123	1124	1125	1126	1127	1128	1129	1130	1131	1132	1133	1134	1135	1136	1137	1138	1139	1140	1141	1142	1143	1144	1145	1146	1147	1148	1149	1150	1151	1152	1153	1154	1155	1156	1157	1158	1159	1160	1161	1162	1163	1164	1165	1166	1167	1168	1169	1170	1171	1172	1173	1174	1175	1176	1177	1178	1179	1180	1181	1182	1183	1184	1185	1186	1187	1188	1189	1190	1191	1192	1193	1194	1195	1196	1197	1198	1199	1200	1201	1202	1203	1204	1205	1206	1207	1208	1209	1210	1211	1212	1213	1214	1215	1216	1217	1218	1219	1220	1221	12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ACCEPTED MANUSCRIPT

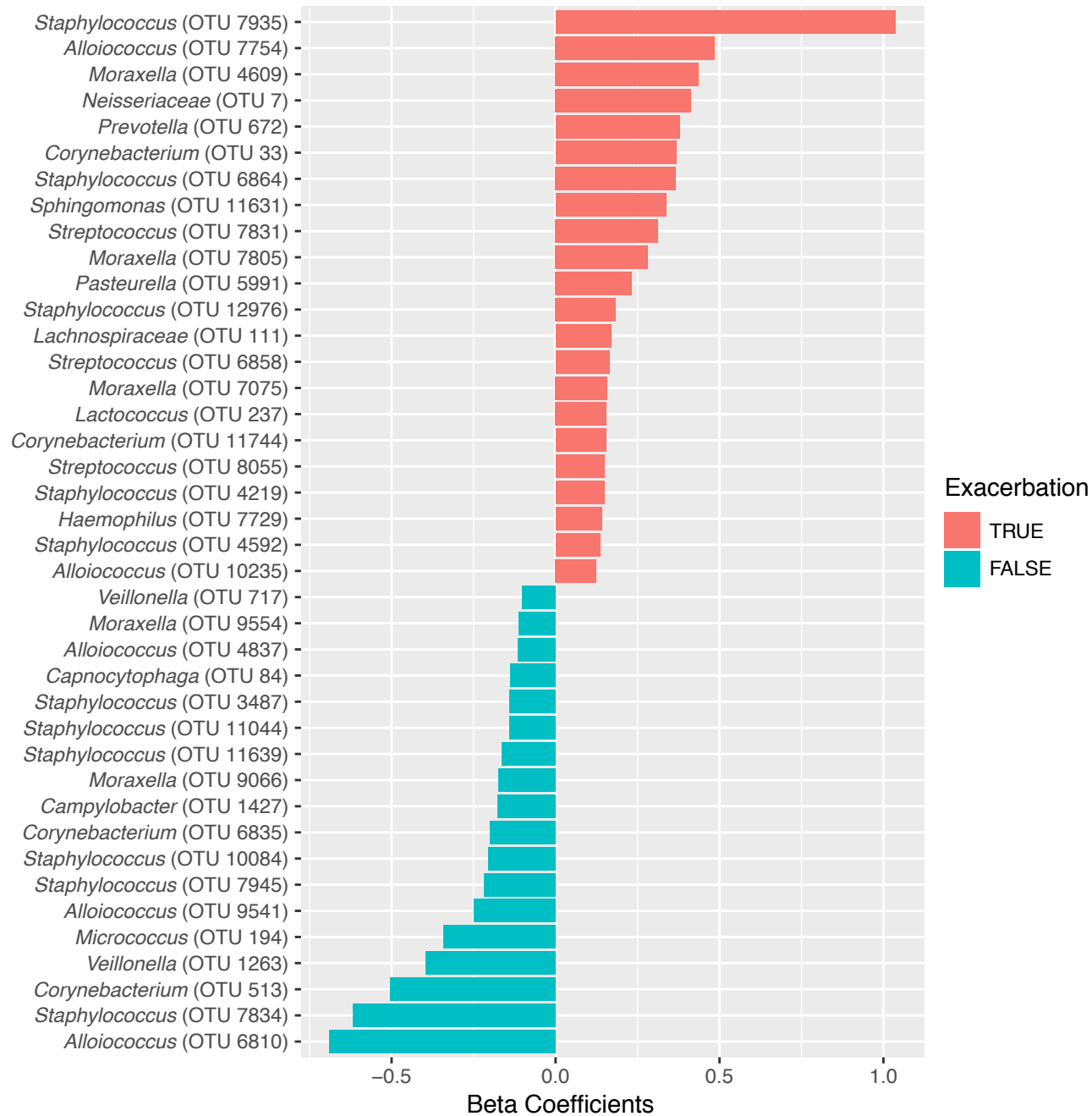
Table S2. Comparative Analysis of Taxon Relative Abundance.

[illegible]

A



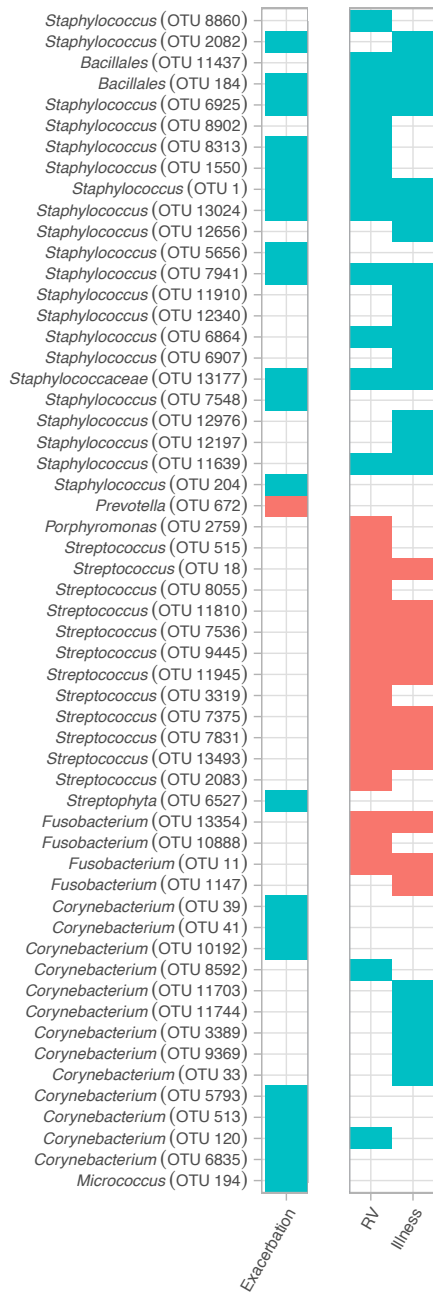
B



A

B C

ACCEPTED MANUSCRIPT



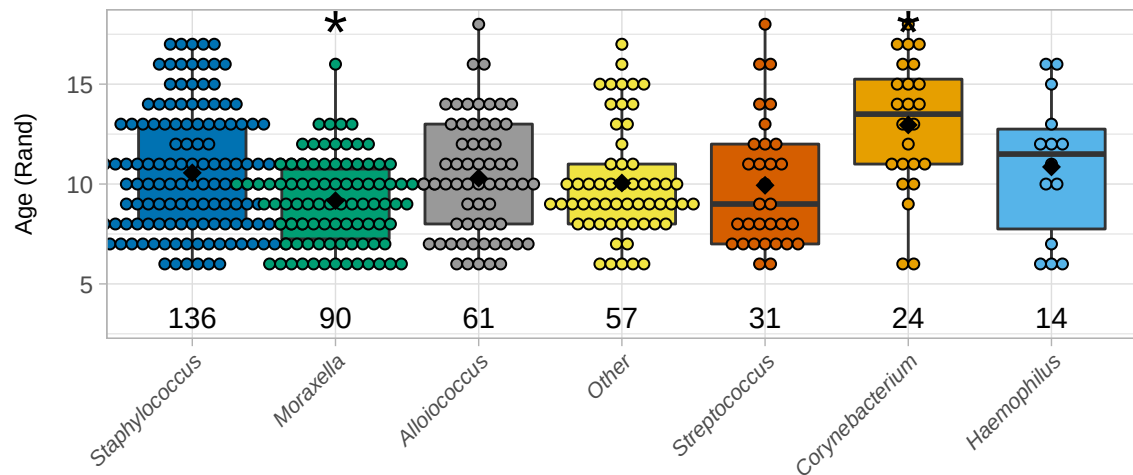
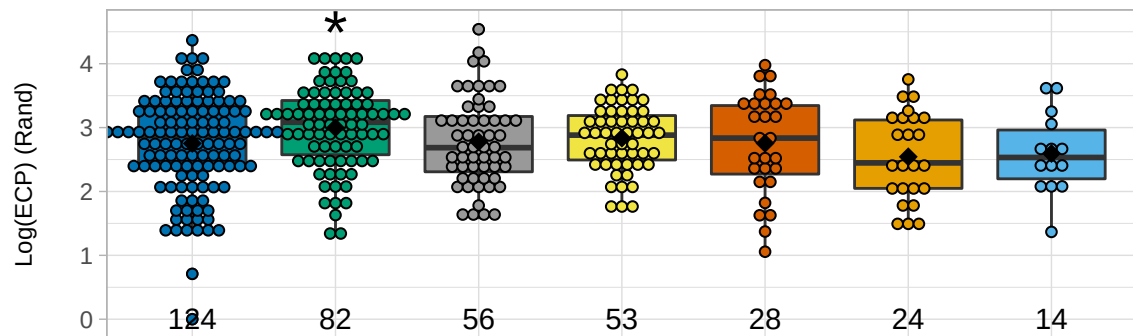
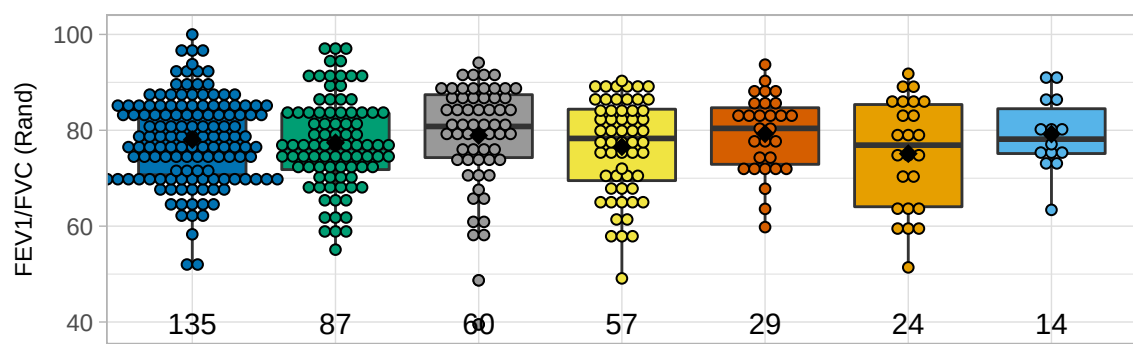
A

B C



Enriched
Depleted





Dominant Taxonomic Group at Baseline

Proportion of Samples

GEE ANOVA P=0.087

GEE ANOVA P<0.001

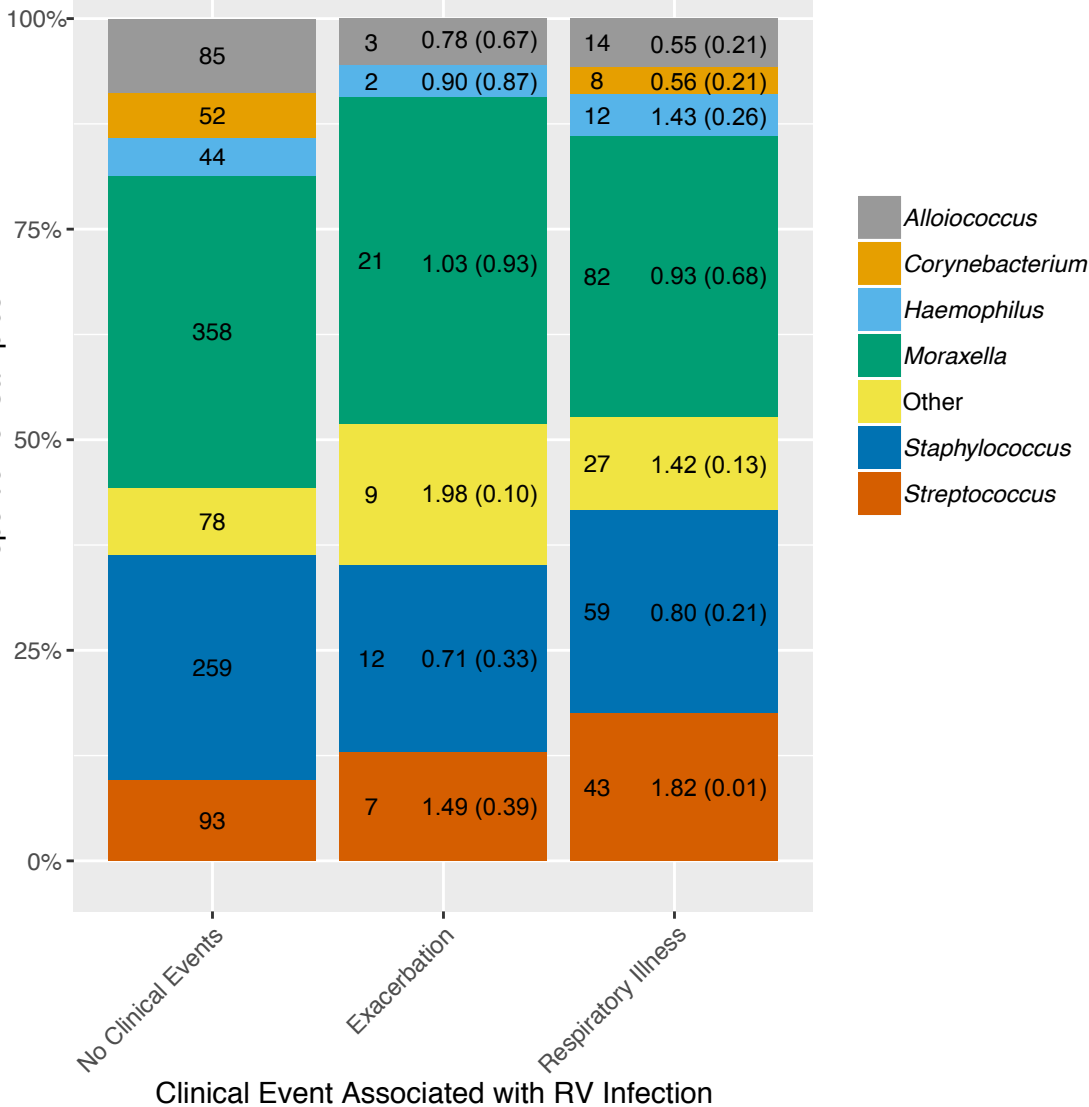
N

N

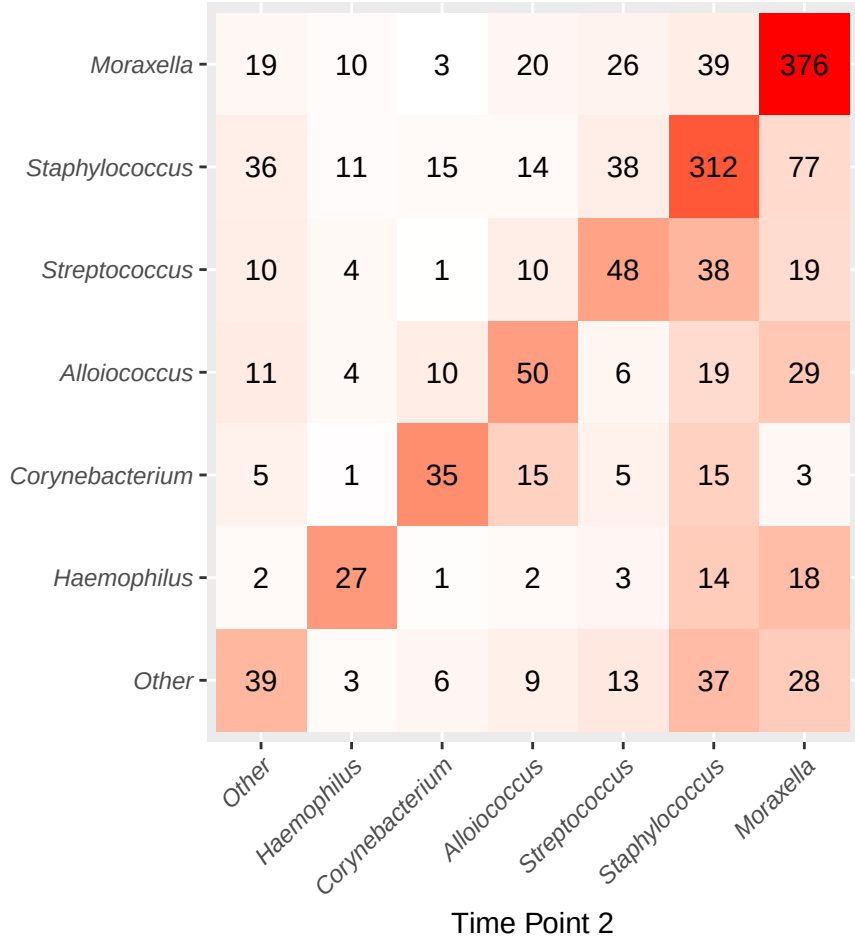
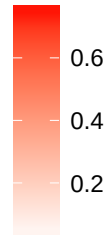
RR*(P)

N

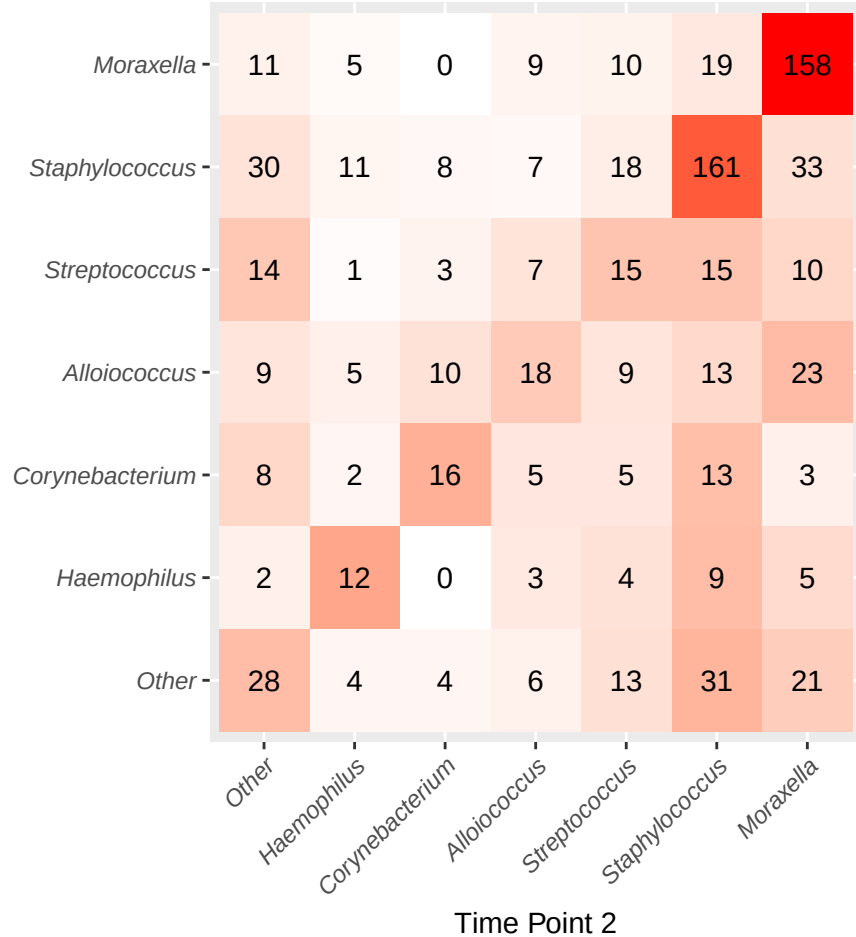
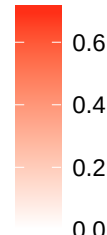
RR*(P)



Time Point 1

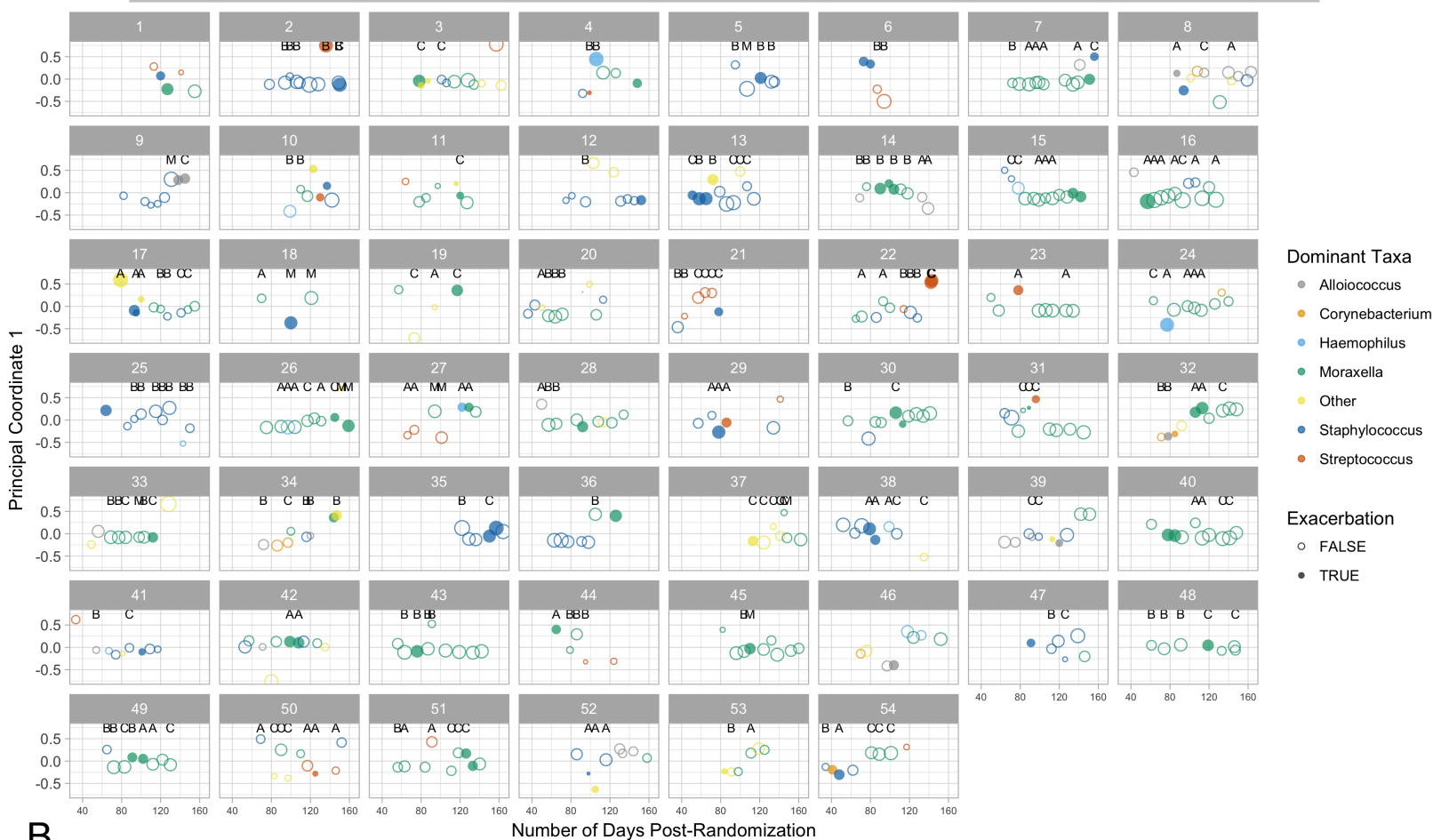
Proportion of
T1 Samples

Time Point 1

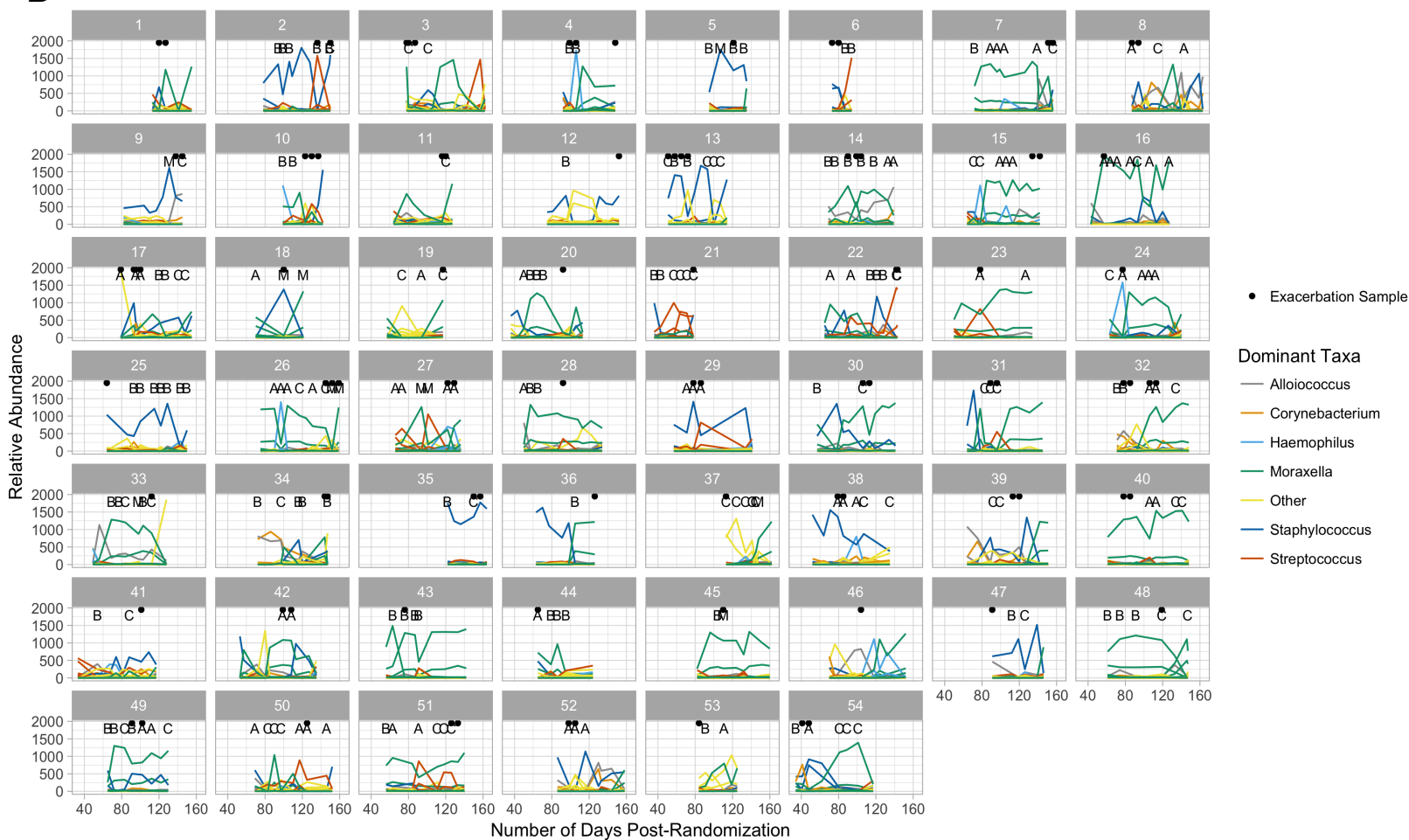
Proportion of
T1 Samples

Time Point 2

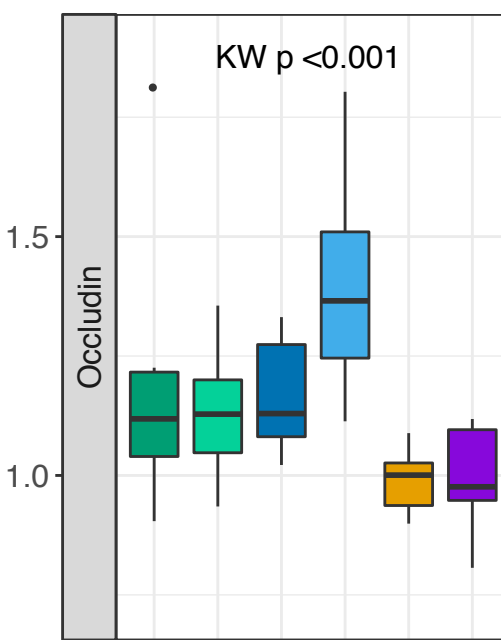
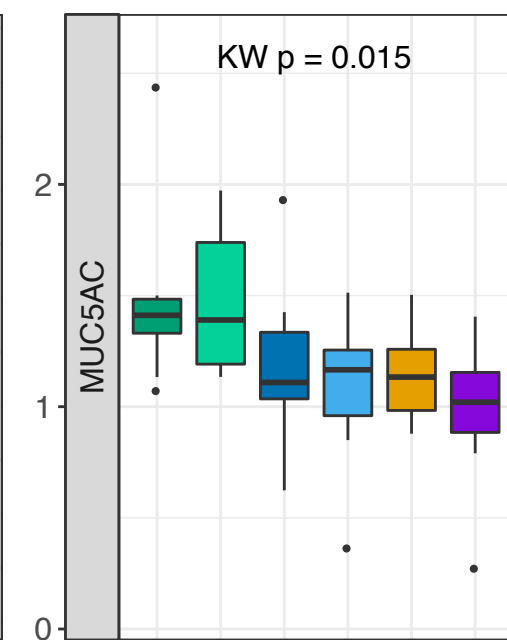
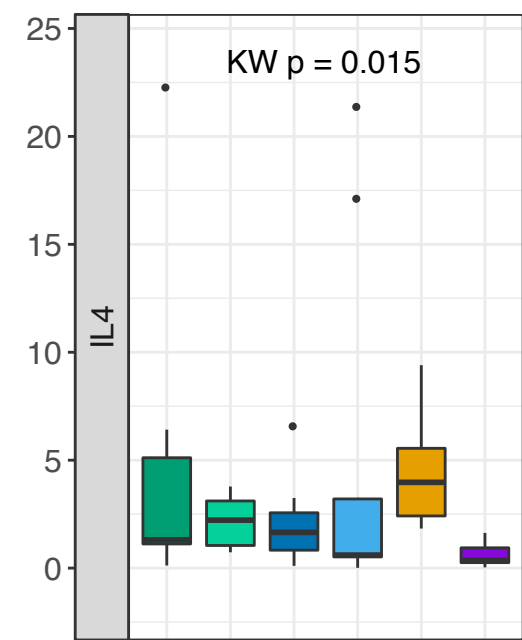
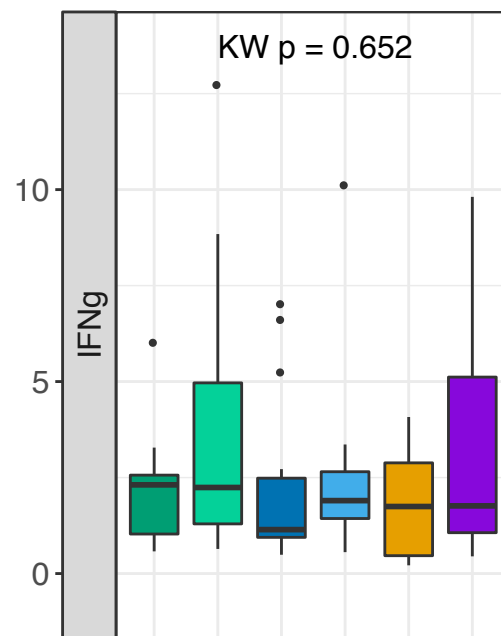
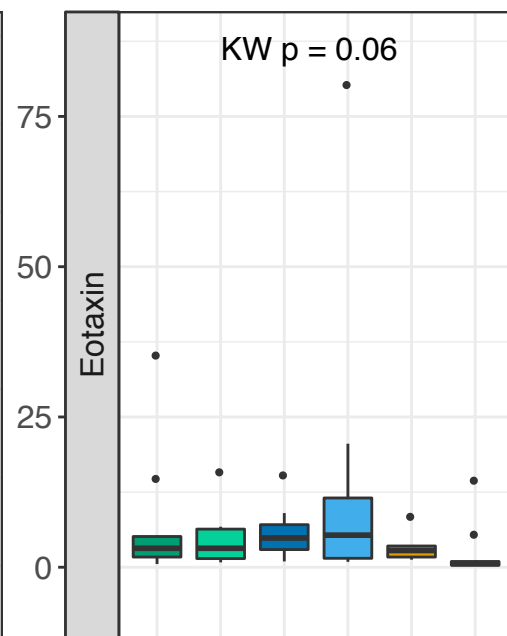
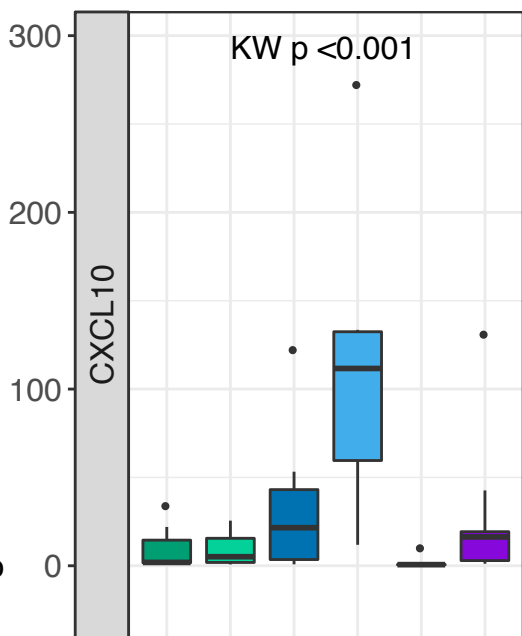
A



B



Fold Change



- M. catarrhalis* (P)
- M. catarrhalis* (U)
- S. epidermidis*
- S. aureus*
- C. propinquum*
- L. sakei*

Online Materials and Methods.

Study Design

This study leverages nasal secretion samples collected post-randomization from children with asthma (ages 6-17 years) participating in the Preventative Omalizumab (Xolair[®] anti-IgE antibody) or Step-up Therapy for Severe Fall Exacerbations (PROSE) randomized controlled trial(1). In brief, children with asthma from inner city communities were enrolled and followed during a run-in period of four to nine months prior to randomization. During this time a participant-specific standard of care was established based on asthma severity, defined by each participant's level of corticosteroid use, and clinical history of symptoms and exacerbations(2). At randomization, those at treatment steps two to four were randomized into (a) their current treatment ("Placebo"), (b) a doubled dose of ICS ("ICS"), or (c) subcutaneous omalizumab. Those with the most severe asthma (step five) were randomized into (a) placebo, or (b) omalizumab. Treatment began four to six weeks prior to participants return to school, and they were followed for 90 days following the start of their school year (outcome period). Asthma exacerbations in the outcome period were defined by the use of systemic corticosteroids and/or hospitalization for asthma(2,3). Nasal samples examined in this study were collected during weeks without a clinic visit throughout the outcome period. To collect the samples, participants sprayed nasal saline into one nostril, held the saline for five seconds, and blew all fluid into a sterile bag; this process was repeated for the second nostril. The sample was then preserved in M4RT transport media (Thermo Fisher Scientific). Participants provided between one and 13 nasal samples, with a mean of 7.15 samples per participant. Participants with fewer than three microbiota profiles available for analysis were removed from all downstream analyses (n=30

participants and 44 samples), resulting in 3,122 samples and 413 participants for analyses (Table S1). We note that a very small proportion of subjects (18/413 or 4.3%) had received antibiotics during the observation period of the trial.

Bacterial Microbiota Profiling

DNA from nasal secretion samples was extracted using a modified cetyltrimethylammonium bromide (CTAB)–polyethylene glycol (PEG) protocol as previously described(4), adjusted for high-throughput. Briefly, 0.6 mL of extraction buffer [4% CTAB, 1M NaCl, 200 mM phosphate buffer pH=8) were added to 0.2 mL of nasal secretion sample. Cells were lysed in a 96-well bead beating plate (MO BIO) at 20 m/s for 10 min followed by two extractions using 800 μ L of phenol:chloroform:isoamyl alcohol (25:24:1) and centrifugation at 4,000 $\times g$ for 30 min. Following phenol extraction, one volume of chloroform was added to each aqueous supernatant and centrifuged at 4,000 $\times g$ for 30 min at 4 °C. The aqueous layer was transferred to a new 96-well plate and DNA precipitated using two volumes of 20% PEG in 1.5M NaCl. Following 2 h incubation at room temperature, samples were centrifuged at 4,000 $\times g$ for 60 min. Pelleted DNA was washed twice with ice-cold 70% ethanol and resuspended in 50 μ L of molecular-grade H₂O.

The variable region 4 (V4) of the 16S rRNA gene was amplified from purified DNA using the 515F/806R primer combination as previously described(5,6). Triplicate 25 μ L PCR reactions were performed using a final concentration of 0.025 U of Takara ExTaq (Takara Clontech), 1 $\times$ Takara buffer with MgCl₂, 400 nM 515F and barcoded 806R primers, 200 μ M dNTP, 0.56 mg.mL⁻¹ BSA (Roche Applied Science) and 10 μ L of DNA template. Reaction conditions were as follows: initial denaturation at 98 °C for 3 min followed by 30 cycles of 98

°C for 20 s, annealing at 50 °C for 30 s, and extension at 72°C for 45 s, and a final extension of 72°C for 10 min. Amplicons were purified using SPRI beads (Beckman Coulter) per the manufacturer's protocol and quantified using the Qubit HS dsDNA kit (Invitrogen). Sample pools consisted of 2 ng of purified amplicon from each sample and 400-500 unique samples per library. A total of n=3,499 samples produced sufficient 16S rRNA amplicon to proceed to sequencing. Libraries were quantified using the KAPA QPCR Illumina Library Quantification kit (KAPA Biosystems), diluted to 2 nM and denatured. Each library was combined with PhiX control at equal molarity and diluted to 1.5 pM for sequencing on the NextSeq 500 (Illumina, San Diego, CA) using a high output cartridge. Of a total of n=3,840 available nasal secretion samples n=3,122 produced high-quality sequence data.

Sequence Analysis

Paired-end sequences were merged using FLASH 38 v 1.2.7(7) and processed to produce an Operational Taxonomic Unit (OTU) table using Quantitative Insights into Microbial Ecology (QIIME) pipeline(8) and USEARCH(9). Raw sequences were de-multiplexed and quality filtered to remove low quality sequences. Sequences with three or more consecutive bases with a Q score less than 30 were truncated and discarded if their length was less than 75% of the original 250 bp read length. Reads were then de-replicated, removing singletons; OTUs were defined by UPARSE-OTU using 97% OTU clustering in USEARCH and chimeras were simultaneously removed(9). Samples (n=3,122) possessing a minimum number (n=2,000) of high quality 16S rRNA reads were included in downstream analysis. All quality-filtered reads were then mapped back to the OTU sequences at 97% identity using UCLUST. A phylogenetic tree was constructed using sequences that aligned using PYNAST(10), and any OTUs that failed to align were

removed. Taxonomy was assigned using UCHIME(11) and the GreenGenes database (13_5)(12). OTUs were additionally filtered to include only those whose total read count over all samples was greater than 0.001% of the total read number of all samples, to remove spurious taxa from the dataset.

Alpha rarefaction curves of observed species determined the appropriate rarefying depth; at 2,000 sequence reads per sample, sufficient community coverage and retention of the greatest number of samples for downstream analysis was achieved. To produce a representative microbiota profile, sequence data from each sample was rarefied (sub-sampled) at 2000 reads, 100 times and the most representative bacterial community composition i.e. that which exhibited the shortest distance to all others generated for that sample, was used for downstream analyses.

De-identified data for participant characteristics were received for analyses following 16S rRNA sequencing and generation of the OTU table to be used for microbiota analyses. Participants provided between one and 13 nasal samples that produced a microbiota profile, with a median of seven samples per participant. Participants with fewer than three samples were removed from all analyses (n=30 participants and 44 samples), resulting in an analysis data set of 3,122 samples from 413 participants. Participants were equally distributed amongst study sites, were typically non-white and had incomes below the poverty line (Table S4).

Viral Detection

All nasal secretion samples were tested for the presence and quantity of RV by quantitative RT-PCR at the University of Wisconsin using a previously published protocol(13). Samples with detectable RV were considered as an RV infection, regardless of whether participants exhibited respiratory symptoms. Samples designated as RV-A, RV-B or RV-C, only

possessed the specific RV strain indicated. RV infection was defined as detection of the virus, in cases where RV was detected in consecutive samples, these were considered as one RV infection event. RV typing was performed by partial sequencing as previously described(13).

Symptomatic Cold Definition

Participants or their caregivers provided weekly reports of symptoms using a modified Jackson Score(14), employed in several independent studies since publication(15–17). Baseline scores were determined by the minimum weekly report of symptoms across all assessments. Illnesses were defined as an increase in symptom scores by at least four points compared to baseline, while those with chronic upper respiratory symptoms, illness was defined as an increase in symptom score by three points(18). Respiratory symptom scores were based on a 0-3 severity score (absent, mild, moderate, severe) for five different symptoms (runny nose, stuffy nose, sore throat, sneezing and cough); scores could range from a minimum of 0 to a maximum of 15.

Eosinophil cationic protein (ECP) measurement

Eosinophil cationic protein concentration in nasal secretions was measured by immunoassay (UniCAP, Phadia US Inc, Portage MI). ECP was used in the PROSE trial to determine whether children treated with omalizumab, an anti-IgE biologic, exhibited decreased concentrations of ECP, a marker of eosinophil activation in their nasal secretions(2).

Bacterial isolation and identification

Bacterial strains were isolated from 10uL of nasal secretions from five children enrolled in the Urban Environment and Childhood Asthma (URECA) study (briefly described below) and one participant from the PROSE study. Samples used for isolation were selected based on the 16S rRNA-based bacterial genus dominating each sample (*Moraxella*, *Staphylococcus* and *Corynebacterium*). The Urban Environment and Childhood Asthma study is a birth cohort based in four urban-based study centers in the United States(19).

Samples were plated on 5% Horse Blood agar plates (SIGMA) incubated aerobically for 72 hours at 37°C. Species identification was determined by full-length Sanger sequencing (QuintaraBio, San Francisco, CA) of the 16S rRNA gene which was amplified using 27F/1492R primers(20). The resulting sequences were classified using the ribosomal rRNA SILVA (<https://www.arb-silva.de/>) database(21). To identify the specific species that dominated the *Moraxella*, *Staphylococcus* or *Corynebacterium* nasal microbiota, the 16S rRNA V4 region of the dominant taxon in each of these microbiota was aligned to the V4 region of bacterial strains isolated from nasal secretions. *M. catarrhalis* isolates exhibited >97% V4 sequence identity with the dominant *Moraxella* taxa, *C. propinquum* was highly homologous (96% identity) with the dominant *Corynebacterium* taxon and both *S. epidermidis* and *S. aureus* exhibited >97% sequence identity with the dominant *Staphylococcus* taxon. This latter observation indicates that V4 16S rRNA analysis cannot discriminate between *S. epidermidis* or *S. aureus* (Table S7). Cultures were cryopreserved at -80°C in Brain Heart Infusion (BHI; BD Biosciences) broth containing 15% (v/v) sterile glycerol.

Bacterial biofilm culture and supernatant preparation

Five strains exhibiting highest homology (Table S7) to the V4 16S rRNA sequence of the dominant *Moraxella*, *Staphylococcus* and *Corynebacterium* OTUs identified in our study of children with asthma, were selected for *in vitro* experiments. A modified method adapted from Schwarzer(22) and Lynch(23) was used to culture biofilms and expose airway epithelial monolayers. In brief, strains were grown aerobically in 5 mL of BHI broth for 24 hours at 37°C. Each culture was diluted in fresh BHI broth to OD₆₀₀=0.1 and 100uL of this suspension was used to inoculate pre-cut, sterilized nylon membrane (GE Healthcare Amersham Hybond-N) squares (3 cm²) placed on BHI agar plates. Plates were incubated aerobically for 72 hours at 37°C to permit biofilm formation and harvested by aseptically scraping the biofilm into 800ul of sterile phosphate buffered saline (PBS) per nylon square. Cellular material was removed by centrifugation (13,000 rpm for 1 minute) and supernatants were passed through a 0.22um syringe filter (Millipore). Sterile biofilm supernatants were also obtained from *Lactobacillus sakei* ATCC15521 control strain using the same procedure except that it was cultivated in Tryptic Soy media at 30°C.

Epithelial cell assay

Human alveolar epithelial A549 cells were seeded at a confluent density in 96-well plates in fresh RPMI-1640 media (Thermo Fisher Scientific) supplemented with 10% heat-inactivated fetal bovine serum (FBS; USA Scientific), 100 U/ml penicillin-streptomycin (Life Technologies) 24 hours before exposure to biofilm extracts. Cells were treated with sterile biofilm supernatants (SBS; diluted in fresh media to 20% v:v) for 24 hours. Controls included PBS buffer (for gene expression) and 10 ug/mL LPS (Sigma; for lactate dehydrogenase assays). Total RNA was isolated with RNAqueous-Micro Kit (Thermo Fisher Scientific) and contaminant DNA digested

with DNase I (Sigma) for 20 minutes at 37°C. First-strand cDNA was synthesized using 300 ng RNA with High-Capacity RNA-to-cDNA Kit (Thermo Fisher Scientific) according to the manufacturer's instructions.

Real-time quantitative PCR was performed in triplicate with SYBR Green master mix (Life Technologies) in the QuantStudio™ 6 Flex Real-Time PCR System (Applied Biosystems) using primers listed in Table S8. The PCR reaction conditions were as follows: 50°C for 2 min, 95°C for 10 min (1 cycle); 95°C for 15 s and 60°C for 1 min (40 cycles); and a final melting curve cycle of 95°C for 15 s, 60°C for 1 min and 95°C for 15 s. Gene expression was normalized to β -actin expression and relative fold change in expression determined by the $2^{-\Delta\Delta CT}$ (where C_T is threshold cycle) method using PBS exposed cells as control.

Cytotoxicity assay

Epithelial cell damage was assessed using the Pierce LDH Cytotoxicity Assay Kit (Thermo Scientific) following manufacture's protocol. In brief, 50 μ L of A549 cell supernatants were added to 50 μ L of lactate dehydrogenase (LDH) reaction mix and incubated for 30 minutes at room temperature in the dark. This was followed by addition of 50 μ L of stop solution, after which LDH activity was determined by subtracting absorbance measured at 680 nm from that measured at 490 nm. LDH activity was reported relative to that released following LPS stimulation of A549 cells.

Statistical Analyses

All statistical analyses were completed using R(24) and QIIME(8). Faith's phylogenetic diversity was calculated in QIIME(8) to evaluate phylogenetic diversity within samples. A Weighted

UniFrac(25) distance matrix was constructed using QIIME. Relationships between beta-diversity and a range of clinical and viral infection variables, considered univariately, were assessed using PERMANOVA (*adonis*) in the *vegan*(26) package in R, using the initial sample received from each patient (baseline). To confirm PERMANOVA observations made using baseline samples, “Bootstrapped” PERMANOVA analysis was performed by random resampling of a single sample per participant with replacement, repeating this process 500 times. This was performed to permit inclusion of repeated samples obtained from participants, as *adonis* does not currently support random effects in unbalanced study designs. This bootstrapping method generated R^2 values and p-values for each iteration, the means of which were calculated and presented as bootstrapped values. In addition, the minimum and maximum value of the R^2 values and p-values are presented for reference.

We performed elastic nets on the baseline samples to determine taxa that may be predictive of future exacerbation risk. The OTU table was scaled using the *scale* function in R and default parameters. Alpha and lambda parameters were tuned on the full dataset using the *train* function from the *glmnet* package in R and 10-fold cross validation. The tuned parameters were subsequently used in a *glmnet* model to obtain the predictions. A 95% accuracy was obtained, and the p-value associated with an accuracy greater than the No Information Rate [the probability of choosing the majority class (here: Non-Exacerbation at 86%)] was less than 0.001.

Significant differences in taxon relative abundance were assessed as previously described(27), using a three-model approach (Poisson, Negative Binomial, and Zero-Inflated Negative Binomial). Mixed effects models were utilized to adjust for unbalanced repeated

sampling across individuals. To reduce spurious findings, we further-filtered rare OTUs detected in less than 15% of the samples, using a data-driven method to determine this threshold. The *glmmTMB* (generalized linear mixed models using Template Model Builder) package in R was used to generate all summary statistics for each of the models to maintain consistency in the model-building method across model types. The Akaike Information Criterion (AIC) was employed to identify the model that best fit the data. Results from models presenting convergence warnings were excluded from consideration in model selection for each OTU. Observations were corrected for false discovery using the Benjamini-Hochberg method, and those with a q-value of less than 0.1 were considered significant. The coefficient from the regression model determined enrichment or depletion. Weighted mean differences, presented in Table S4, were calculated by generating weighted counts for each individual (multiplying counts by the inverse of the total number of samples from that individual). These weighted counts were then averaged together, and the difference in the means between groups was calculated to generate the weighted mean difference.

Samples were stratified based on dominant taxon i.e. taxon that exhibited the largest proportion of sequence reads in each sample, and the genus-level identity of the dominant taxon was used to classify each sample. For OTUs unclassified at the genus level, we used the next-available classification. Risk ratios (RRs) were computed using generalized estimating equations (GEE) in the *geepack* package in R. An exchangeable correlation structure within each child was used to generate the estimates. The outcome of the regression equation was a binary variable comparing each microbiota against all other groups (the reference group). Q-values were derived

from a Benjamini-Hochberg False Discovery Rate across p-values for each microbiota within variables of interest; q-values less than 0.15 were considered significant.

For analyses related to infection events, all samples associated with an RV infection were selected. Clinical event information (cold symptoms, exacerbation or neither) was mapped to RV infections. Generalized Estimating Equations using an exchangeable correlation structure within each child was used to generate Relative Risks and P-values between clinical events as well as microbiota assemblages. When groups had no observations (i.e., exacerbation did not lead to *Corynebacterium* microbiota in this sub-analysis), samples were considered independent and a test for differences in two proportions was used to determine if significant differences were apparent.

Pair-wise assessment of microbiota state transitions in repeated measures samples was performed for each participant; the frequency with which the same or distinct microbiota (based on dominant genus) occurred in the subsequent sample obtained from an individual was determined(28). A heat map with transition counts was generated for the overall population as well as for the first three samples provided from each participant, and from samples taken at least a week apart. The latter two approaches were taken to normalize the number of samples assessed per participant and to account for potential biases in sample collection timing.

To determine factors that differentiated participants who were stably-colonized by a *Moraxella* or *Staphylococcus* community, individuals with stably-maintained microbiota were defined as those with >50% of longitudinal samples being dominated by a either *Moraxella* or

Staphylococcus. Generalized Linear Models with a binomial distribution and a Benjamini-Hochberg false-discovery correction was applied to test multiple participant characteristics.

Co-occurrence networks of OTUs were constructed using the *SparCC* and *WGCNA*(29) packages in R, using all samples from all participants. The rarified OTU table was filtered to include only those taxa present in at least 25% of the samples, resulting in 150 OTUs being used for network analysis. *SparCC*(30) generated the correlation matrix, which was transformed to an adjacency matrix using soft thresholding, and a dissimilarity matrix was generated. The dissimilarity matrix was then hierarchically clustered and the resulting dendrogram was cut using *dynamicTreeCut* in the *stats* package in R to generate modules. Closely related modules were combined using transformations of the module eigengenes, which generated a dissimilarity matrix for further hierarchical clustering, and the final network was visualized using *WGCNA* and *phyloseq*(31) in R. A connection is defined as being in the 75th percentile in the topological overlap matrix. Hub OTUs are defined as those with a greater number of connections within their module than between modules, while connector OTUs are those with a greater number of connections between modules than within.

Statistical analyses were performed in close collaboration with the Statistical Data Coordination Center, Rho, Inc. (Chapel Hill, NC).

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Figure Legends

Figure E1. Taxa exhibiting relative enrichment or depletion at baseline in children who go

on to experience exacerbation in the outcome period. **(A)** Heat map indicating specific bacterial taxa that exhibit significantly higher (red) or lower (blue) relative abundance in baseline samples from children that go on to experience an asthma exacerbation. Three count-based mixed-effects models (Poisson, Negative Binomial, or Zero-Inflated Negative Binomial) were used to identify taxa that differed significantly in relative abundance between groups, with results from the best-fitting model being selected. Bacterial taxa are sorted by phylogeny. Significance was defined as a false discovery q-value < 0.1 ; the beta coefficient was used to determine the strength of the association. **(B)** Multiple taxa were identified as weakly predictive (beta-coefficients typically less than 1) of exacerbation (red) or non-exacerbation (blue) in Elastic Nets analysis. Beta coefficients and color indicate the directionality of the association.

Figure E2. Multiple taxa belonging to specific bacterial genera associate with exacerbation,

RV detection, and respiratory illness. Heat map indicating specific bacterial taxa that exhibit significantly higher (red) or lower (blue) relative abundance in **A.** children with asthma who experience at least one exacerbation (n=54 subjects) during the period of observation versus those who do not (all samples collected from exacerbation or non-exacerbation subjects were used in this analysis). **B.** samples in which RV was detected (RV detection samples; irrespective of associated respiratory illnesses) versus those in which RV was not detected (non-RV infected samples); **C.** samples collected during respiratory illnesses (as indicated by symptom questionnaires) versus samples not associated with respiratory illnesses. Three count-based mixed-effects models (Poisson, Negative Binomial or Zero-Inflated Negative Binomial) were used to identify taxa that differed significantly in relative abundance between groups, with

results from the best-fitting model being selected. Bacterial taxa are sorted on phylogeny.

Significance was defined as a false discovery q-value < 0.1 ; sign of the coefficient was used to determine directionality of the relationship (enriched or depleted).

Figure E3. Multiple taxa belonging to a relatively small number of genera co-associate with detection of specific Rhinovirus strains. Heat map indicating specific bacterial taxa that exhibit significantly higher (red) or lower (blue) relative abundance in samples with RV-A detection, RV-B detection, or RV-C detection, respectively. Three count-based mixed-effects models (Poisson, Negative Binomial, or Zero-Inflated Negative Binomial) were used to identify taxa that differed significantly in relative abundance between groups, with results from the best-fitting model being selected. Bacterial taxa are sorted by phylogeny. Significance was defined as a false discovery q-value < 0.1 ; sign of the coefficient was used to determine directionality of the relationship (enriched or depleted). Abbreviations: RV: Rhinovirus

Figure E4. Age and ECP concentration at randomization relates to the dominant microbiota of the first post-randomization sample collected from each subject. Numbers below boxplots indicate the total number of samples included for each microbiota. Significant differences across microbiota ($q < 0.05$) are indicated with an asterisk (*).

Figure E5. Association of Microbiota Assemblages with RV infection-associated outcomes. Distribution of nasal airway microbiota states in RV infection samples with no associated clinical events, exacerbation or respiratory illness symptoms. Relative risks (RR) and P-values (P) compare samples with clinical events (“Exacerbation” or “Respiratory Illness”) to samples without clinical events (“No Clinical Events”) across each microbiota group using Generalized Estimating Equations (GEE) with an exchangeable correlation structure within each child.

Figure E6. *Moraxella*- and *Staphylococcus*-dominated microbiota exhibit the greatest temporal stability irrespective of analysis method employed. Frequency of inter- or intra-microbiota transitions across samples collected **A.** between 7 and 13 days apart (n=1536 subsequent samples from N=390 participants), and **B.** the first three samples (n=826 subsequent samples from N=413 participants) collected from each child with asthma. The color intensity reflects the frequency with which microbiota groups remain stable or transition to a different microbiota state.

Figure E7A. *Moraxella*- and *Staphylococcus*-dominated microbiota frequently persist despite asthma exacerbation or RV infection. A total of 54 participants from whom pre-, peri- and post-exacerbation samples were available were examined. Individual PCoA plots illustrate temporal (x-axis) bacterial composition changes with RV (letters represent species, M = mixed detection of more than one species) and exacerbation (filled circles). Y-axis (PC1) denotes bacterial microbiota compositional variability; colors denote microbiota group. Size of sphere indicates the relative abundance of the dominant genus within the microbiota. **B.** A relatively small number of taxa belonging to the six genera of interest exhibit temporal fluctuations in relative abundance upon RV infection and exacerbation. Individual plots of taxon-specific relative abundance (y-axis) for all taxa per subject, indicate that only a small number of taxa exhibit large-scale shifts in relative abundance during exacerbation and RV infection events. RV infection strain denoted by letters (M indicates detection of more than one species), exacerbation by closed circles. Line colors denote the genus-level classification of each taxon.

Figure E8. Biofilm-derived products of bacterial isolates dominating nasal airway microbiota differentially influence epithelial responses *in vitro*. Statistical significance was determined using

426 Kruskal-Wallis rank test. Results were obtained from two or more independent experiments
427 using three biological replicates. KW: Kruskal-Wallis; P: PROSE; U:URECA