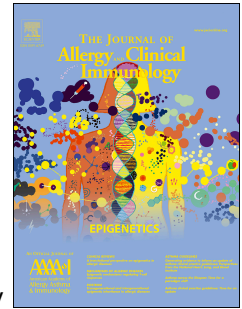


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Increased *Moraxella* and *Streptococcus* following severe bronchiolitis is associated with recurrent wheezing

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Increased *Moraxella* and *Streptococcus* following severe bronchiolitis is associated with recurrent wheezing

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Conflict of Interest

Dr. Petrosino owns shares at Diversigen Inc., a microbiome research company. Dr. Piedra provided bronchiolitis-related consultation for Gilead, Novavax, Ablynx and Regeneron and received grant support from Novavax, Gilead, Regeneron, Janssen and Ablynx. The other authors have no financial relationships relevant to this article to disclose.

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ABSTRACT

Background: The role of the airway microbiome in the development of recurrent wheezing and asthma remains uncertain, particularly in the high-risk group of infants hospitalized for bronchiolitis.

Objective: Examine the relation of nasal microbiota at bronchiolitis hospitalization and 3 later points to the risk of recurrent wheezing by age 3 years.

Methods: In 17 US centers, researchers collected clinical data and nasal swabs from infants hospitalized for bronchiolitis. Trained parents collected nasal swabs 3 weeks post-hospitalization and, when healthy, during summer and 1 year post-hospitalization. We applied 16S rRNA gene sequencing to all nasal swabs. We used joint modeling to examine the relation of longitudinal nasal microbiota abundances to risk of recurrent wheezing.

Measurements and Main Results: Among 842 infants hospitalized for bronchiolitis, there was 88% follow-up at 3 years and 31% developed recurrent wheezing. The median age at enrollment was 3.2 months (IQR, 1.7-5.8 months). In joint modeling analyses adjusting for 16 covariates, including viral etiology, a 10% increase in relative abundance of *Moraxella* or *Streptococcus* 3 weeks after day 1 of hospitalization was associated with an increased risk of recurrent wheezing (HR 1.38; 95% highest density interval [HDI] 1.11-1.85; and HR 1.76; 95%HDI 1.13-3.19, respectively). Increased *Streptococcus* the summer after hospitalization was also associated with a higher risk of recurrent wheezing (HR 1.76; 95%HDI 1.15-3.27).

Conclusions: Enrichment of *Moraxella* or *Streptococcus* after bronchiolitis hospitalization was associated with recurrent wheezing by age 3 years, possibly providing new avenues to ameliorate the long-term respiratory outcomes of infants with severe bronchiolitis.

Abstract word count: 250

Clinical Implications: In a prospective multicenter cohort of infants hospitalized with bronchiolitis, we found the post-hospitalization reassembly of the nasal microbial ecosystem was associated with recurrent wheezing by age 3 years.

Capsule Summary: Enrichment of *Moraxella* or *Streptococcus* after bronchiolitis hospitalization was associated with recurrent wheezing by age 3 years. This finding identifies a potential window of opportunity for clinicians to ameliorate these infants' long-term respiratory outcomes.

Key words: Bronchiolitis, recurrent wheezing, *Moraxella*, *Streptococcus*, *Haemophilus*, respiratory syncytial virus, rhinovirus, longitudinal studies, microbiome

Abbreviations: RSV: respiratory syncytial virus; RV: rhinovirus; ARI: acute respiratory infections; ED: emergency department; NPA: nasopharyngeal aspirate; HDI: high density interval; CAS: Childhood Asthma Study

Manuscript word count: 3,466 (original submission count)

INTRODUCTION

Bronchiolitis is an acute respiratory infection and the leading cause of hospitalization for U.S. infants, accounting for ~130,000 hospitalizations annually.¹ In addition to this acute severity of illness, these hospitalized infants are 3 to 4 times more likely than healthy controls to develop recurrent wheezing and childhood asthma.² Unfortunately, the infants who will develop this long-term respiratory morbidity after a bronchiolitis hospitalization remain incompletely defined.

To date, most longitudinal bronchiolitis cohort studies have utilized 1 of the 2 most common viral etiologies of bronchiolitis, respiratory syncytial virus (RSV) and rhinovirus (RV), as the primary exposure.² However, respiratory viruses infect infants in airways colonized with highly functional bacteria.^{3,4} And emerging evidence suggests that the bacterial composition of the airway is associated not only with acute severity outcomes,⁵ but also long-term respiratory outcomes.⁶

More than a decade ago, Bisgaard and colleagues using conventional culture reported that hypopharyngeal colonization by *Moraxella*, *Haemophilus*, or *Streptococcus* in asymptomatic 1 month old infants was associated with recurrent wheezing and childhood asthma.⁷ More recently, cross sectional, culture-independent studies have shown that when compared with healthy controls, children and adults with asthma have airway microbiota compositions enriched with Proteobacteria (e.g., *Moraxella* and *Haemophilus*).^{4, 8-11} Furthermore, longitudinal microbiota studies in the first 2 years of life, have found that *Moraxella*, *Haemophilus*, and *Streptococcus* dominate nasopharyngeal communities during acute respiratory infections (ARI)^{6, 12} and in sensitized children, are associated with chronic wheeze at age 5 years.⁶

To date, however, no study has examined in a multicenter cohort of infants hospitalized for bronchiolitis, the association between the longitudinal composition of the nasal microbiota and risk of recurrent wheezing of childhood. Indeed, this is the first longitudinal microbiota analysis of the present cohort.^{5, 13} We hypothesized that a post-hospitalization increase in *Moraxella* relative abundance is associated with a higher risk of recurrent wheezing by age 3 years.

METHODS

Study design, setting, and participants

As previously described,⁵ site teams at 17 hospitals in 14 U.S. states enrolled infants (age less than 1 year) hospitalized for bronchiolitis into the 35th Multicenter Airway Research Collaboration (MARC-35; http://www.emnet-usa.org/Marc_35/M35.htm). Attending physicians diagnosed an infant's bronchiolitis based on the American Academy of Pediatrics definition¹⁴. Enrollment occurred over 3 consecutive winter seasons (i.e., November to April) from 2011-2014. The institutional review board at the 17 participating hospitals approved this study and all families signed an informed consent prior to participation.

Data collection

Research teams at each of the sites not only extracted clinical data from the emergency department (ED) and inpatient charts, but also conducted structured interviews with parents / legal guardians during the hospitalization for demographic, historical and environmental information.

Nasopharyngeal aspirate collection

All site researchers collected nasopharyngeal aspirates (NPAs) within 24 hours of hospitalization using the same equipment (e.g., sample traps and suction catheters from Medline Industries [Mundelein, IL]) and a standardized protocol as previously described.¹⁵

Nasal swab collection

Within 24 hours of hospitalization, site teams collected an index nasal swab from both anterior nares using a single nylon, pediatric FLOQSwab (Copan, Brescia, Italy).¹⁵ This swab was collected either before the NPA collection or >2 hours after the NPA collection. During the

hospitalization, site teams taught parents how to collect the nasal swab sample. When possible during the index sample collection, the site team swabbed 1 nare and had the parent practice their technique by swabbing the other nare. Three weeks after the date of hospitalization, using the same sample collection methods, trained parents collected the “clearance swab”. The summer (June, July, or August) following the hospitalization, when the children were healthy, the parents collected the “summer swab”. The year following the hospitalization, children were randomly assigned to a specific season for their healthy swab collection. Therefore, 1/4 of parents were assigned to collect the “seasonal swab” over summer (June, July, August), fall (September, October, November), winter (December, January, February), or spring (March, April, May). All collected swabs were processed as previously described.¹⁵

16S rRNA gene sequencing

As previously described,¹⁵ we sequenced the 16S rRNA gene V4 region of the nasal swab bacteria on the Illumina MiSeq platform. We utilized microbiota data with sufficient sequence depth (i.e., $\geq 1,000$ reads per sample) for all analyses (see Analysis).

Quantitative reverse-transcriptase polymerase chain reaction assays

Using the NPA samples from hospitalization, quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) assays were conducted at Baylor College of Medicine as previously described for 17 viruses: RSV (types A and B), rhinovirus, influenza (types A and B, and 2009 novel H1N1), human metapneumovirus, human bocavirus, parainfluenza viruses (types 1, 2 and 3), adenovirus, enterovirus, coronaviruses (NL-63, OC-43, HKU1, and 229E).¹⁶

Outcome measures

Based on biannual parent phone interviews, the primary outcome for the present analysis was

recurrent wheezing by age 3 years, as defined by the 2007 National Institutes of Health (NIH)'s Expert Panel Report 3 (EPR-3) – i.e., having at least 2 corticosteroid-requiring exacerbations in 6 months or having at least 4 wheezing episodes in 1 year that last at least 1 day and affect sleep.¹⁷ There is no required or specified time frame between episodes of wheezing. Since wheezing in early childhood may be transient,¹⁸ we extended this outcome to children who had recurrent wheezing by age 3 years and also had asthma at age 4 years. We defined asthma at age 4 years using an epidemiologic definition: between ages 3 and 4 years having both a physician-diagnosis of asthma and either asthma medication use (e.g., albuterol inhaler, inhaled corticosteroids) or asthma-related symptoms (e.g., wheezing, nocturnal cough).¹⁹

Statistical analyses

Analyses only included subjects with an index microbiota sample of sufficient quality. In order to maintain consistency between cross-sectional and the longitudinal time-to-event analyses, microbiota samples collected after the onset of recurrent wheezing were excluded. Covariates for patient characteristics and clinical presentation collected at index were compared between children with and without recurrent wheezing using chi-squared tests or Wilcoxon rank sum tests, as appropriate.

The unrarefied 16S rRNA gene sequence read counts were combined at the genus level. We defined the top 10 genera as those with the highest mean relative abundance across all samples. Kruskal-Wallis rank sum tests assessed the significance of the differences in age, alpha-diversity (Shannon index), and relative abundances of the top genera across all 4 time points, with P-values for the top genera comparisons using the Bonferroni correction for multiple testing. To examine the relation of genus abundance to recurrent wheezing, we first performed cross-sectional analyses on the data. We calculated the overall mean microbial abundances for each

genus of interest using the relative abundance across all time points. Based on microbial abundances at each of the 4 time points, we categorized the nasal samples into 2 groups: those above the mean and those below. Chi square tests determined significance of the association between these groups and recurrent wheezing. Multiple comparison correction was not used in these analyses as they served as exploratory analyses for the joint modeling .

Next, we performed longitudinal analyses by constructing joint models for the longitudinal microbial data to quantify its relationship with the onset of recurrent wheezing.²⁰ Specifically, we created a longitudinal mixed-effects generalized linear model to predict relative genus abundances over time using the 8 most abundant taxa and clinical covariates as the fixed effects, time as the random slope, and subject as the random intercept. These relative abundances and their interaction by time point were both scaled such that a 10% change in abundance was equivalent to a unit change and incorporated as time-dependent covariates into a Cox proportional hazards model with the outcome recurrent wheezing by age 3 years. We used Bayesian methods via a modified version of the *rstanarm* R package assuming scaled default prior distributions. This Bayesian approach produces conservative model estimates without the multiple comparison problems found in frequentist approaches.²¹ Significance was determined using the 95% high density interval (HDI) of the posterior distribution of the log hazard ratio within a region of practical equivalence (ROPE) around zero.

To confirm the joint model results, we generated Kaplan-Meier curves stratified by groups of participants identified by the joint model as being at higher risk for recurrent wheezing and used log-rank tests to determine statistical significance. We also conducted a joint model sensitivity analysis restricting the outcome to children who had recurrent wheezing by age 3 years and also had asthma at age 4 years.

For each of the high-risk groups identified by the joint model, we generated locally weighted scatterplot smoothing (i.e., loess) curves with 95% confidence intervals (CI) for the relative abundances for the time following day 1 of hospitalization. The process of creating smoothed curves for each of the risk groups was repeated to include 8 of the top 10 genera; we removed 2 genera which showed imperceptible changes over time. To further improve clarity, we used a rug plot of sample times (instead of 95% CIs) to indicate the distribution of the observations.

The overlap between participant memberships in the risk groups identified by the joint model was assessed using a Venn diagram. After removing group overlap, patient characteristics and clinical presentation were compared between the risk groups using Fisher exact tests and Kruskal-Wallis tests, as appropriate.

The analysis used R version 3.3.3. All P-values were two-tailed, with $P < 0.05$ considered statistically significant.

RESULTS

Patient characteristics

Of 921 infants in the longitudinal cohort, 842 had sufficient sequence depth (i.e., ≥ 1000 reads per sample) and were included in the present analysis. The analytic and non-analytic cohorts differed in antibiotic use prior to hospitalization ($P=0.02$), with a higher percentage of infants in the non-analytic cohort having used antibiotics (see **Table E1** in online repository). The analytic cohort had a median age at hospitalization of 3.2 months (IQR, 1.7-5.8 months), 60% were male, and 80% had no history of wheezing. We characterized the bacterial microbiota using 2,086 nasal swabs (842 index, 599 clearance, 379 summer, and 266 seasonal) collected during and after hospitalization for bronchiolitis. We had 88% follow-up at age 3 years. Overall, 265 (31%) infants developed recurrent wheezing by age 3 years. **Table 1** summarizes the patient characteristics among children who did and did not develop recurrent wheezing. In general, infants who developed recurrent wheezing were older upon study entry, had a mother with asthma, a personal history of eczema, and RSV-negative bronchiolitis.

Composition of the nasal microbiota during and after bronchiolitis hospitalization

The median age at the time of nasal swab collection, Shannon index, and abundances of top 10 nasal microbiota genera by swab collection time point are shown in **Table E2** (in online repository). Nasal swab collections continued until a median age of 16 months. The alpha diversity was lowest at the clearance time point and highest at the seasonal time point. Among the genera of interest, *Moraxella*, *Haemophilus*, and *Streptococcus* were all in the top 10 genera by relative abundance.

Relative abundance of *Moraxella*, *Haemophilus*, and *Streptococcus* by time point and risk of developing recurrent wheezing by age 3 years

In cross sectional analyses, we found that upon hospitalization the relative abundance of the risk bacteria (i.e., *Moraxella*, *Haemophilus*, and *Streptococcus*) were not associated with recurrent wheezing by age 3 years. By contrast, at the clearance time point (i.e., 3 weeks after day 1 of hospitalization), infants with a relative abundance of *Moraxella* greater than the mean had significantly higher risk of recurrent wheezing by age 3 years than infants with relative abundances below the mean ($P=0.04$; **Figure 1A**). In parallel analyses, *Haemophilus*' association with recurrent wheezing did not reach significance at any of the time points (see **Figure E1** in online repository). However, infants with *Streptococcus* abundances greater than the mean at either the clearance or summer time point had significantly higher risk of recurrent wheezing by age 3 years when compared with infants who had below mean *Streptococcus* abundances ($P=0.01$ and $P=0.03$ respectively; **Figure 1B**).

Longitudinal *Moraxella*, *Haemophilus*, and *Streptococcus* abundance after hospitalization and hazard of developing recurrent wheezing by age 3 years

The aforementioned cross sectional results were also shown in our longitudinal analysis using joint modeling. In the joint model, increased *Moraxella* relative abundance at the clearance time point was associated with development of recurrent wheezing by age 3 years after adjusting for 16 confounders such as maternal asthma, birth mode, history of eczema, sensitization, viral etiology, and intensive care use (HR 1.38, 95% HDI 1.11-1.85; **Figure 2**). Thus, for every 10% increase in the abundance of *Moraxella* at clearance, there was a 38% increase in the risk of developing recurrent wheezing by age 3 years. Similar to our cross sectional analyses, *Haemophilus* abundance did not reach significance in the joint model analyses (see **Figure E1** in online repository). By contrast, we found that increased *Streptococcus* abundance at clearance and the summer following hospitalization were both associated with recurrent wheezing by age 3 years (HR 1.76, 95%HDI 1.13-3.19; and HR 1.76; 95%HDI 1.15-3.27, respectively; **Figure 2**).

In sensitivity analyses, we created Kaplan-Meier curves stratified by the 3 risk groups (i.e., increased *Moraxella* at clearance, increased *Streptococcus* at clearance, and increased *Streptococcus* at summer) identified in our cross sectional and joint modelling analyses (**Figure 3**). In these survival analyses, we again demonstrated the increased hazard of recurrent wheezing by age 3 years for infants with high *Moraxella* or *Streptococcus* at clearance (both $P=0.01$) or high *Streptococcus* at summer ($P<0.01$).

Longitudinal *Moraxella* and *Streptococcus* abundance after hospitalization among children with recurrent wheezing by age 3 years who also developed asthma at age 4 years

To address the potential heterogeneity of recurrent wheezing, we examined children who not only developed recurrent wheezing by age 3 years, but also had asthma at age 4 years.

Supportive of the recurrent wheezing results, we found in joint models that children with higher *Moraxella* and *Streptococcus* abundance at clearance had an increased risk of recurrent wheezing accompanied by age 4 year asthma (HR 1.75, 95%HDI 1.13-3.18; and HR 2.84, 95%HDI 1.07-9.03, respectively; **Figure 4**). Children with increased *Streptococcus* abundance at summer had a borderline significant increased risk of recurrent wheeze with age 4 year asthma (HR 2.51; 95%HDI 0.96-8.43; **Figure 4**).

Stability of nasal microbiota risk group composition after hospitalization

The 3 nasal microbiota risk groups – A) increased *Moraxella* at clearance; B) increased *Streptococcus* at clearance; and C) increased *Streptococcus* at summer – have minimal overlap (see **Figure E2** in online repository). Comparing the characteristics and clinical presentation of infants within each of the 3 risk groups - after excluding infants in more than one risk group - we found that children with elevated *Moraxella* or *Streptococcus* at clearance were wheezing upon hospitalization more than children with increased *Streptococcus* at summer. Otherwise, the 3 risk

groups had similar demographics and clinical presentations (see **Table E3** in online repository). We also found that the elevated abundances found in each of the 3 risk groups were not present at the subsequent sample time point (**Figure 5 and Figure E3** in online repository). Examining whether the transient nature of the increased abundances could be due to antibiotic administration, we found that most patients did not receive antibiotics either before or during the hospitalization, in accordance with the current bronchiolitis practice guidelines.¹⁴ Moreover, there were no significant differences in antibiotic administration between patients with above or below mean taxa abundances (see **Table E4** in online repository).

DISCUSSION

In this prospective, multicenter cohort of 842 infants hospitalized for bronchiolitis, we identified in cross-sectional, survival, and joint modeling analyses that having increased relative abundance of nasal *Moraxella* or *Streptococcus* 3 weeks after day 1 of hospitalization – or increased nasal *Streptococcus* the summer after hospitalization – was associated with recurrent wheezing by age 3 years. Our data corroborate the relation of infant *Moraxella* and *Streptococcus* abundance to childhood wheezing outcomes,^{6, 12} and extend this prior research by focusing not only on a high-risk cohort of infants hospitalized for bronchiolitis, but also by analyzing hospitalization and post-hospitalization samples using robust longitudinal analytic techniques (i.e., joint modeling). Although replication is needed, and causality has not been established, we may have identified, for infants hospitalized with bronchiolitis, a short window of opportunity (i.e., the weeks after hospitalization) to influence the secondary succession of the nasal microbial ecosystem, and potentially, the risk of developing of recurrent wheezing by age 3 years^{22, 23} and recurrent wheezing accompanied by asthma at age 4 years.

Over the past 60 years, multiple cohort studies have demonstrated that up to 60% of infants hospitalized with bronchiolitis will develop recurrent wheezing within 3 years of hospitalization.² Furthermore, compared with healthy controls, children hospitalized with bronchiolitis are also at much higher risk of developing childhood asthma² and even, longer term respiratory morbidity.²⁴ Many of these bronchiolitis cohorts focused on the viral infection (e.g., RSV). However, viruses infect infants within a complex microbial ecosystem (i.e., microbiome).²⁵ And research results over the last decade have demonstrated that airway bacteria, including nasal microbiota, are also associated with wheezing outcomes in children.^{6, 7, 12, 26}

Several studies of airway microbiota have implicated *Moraxella*, *Haemophilus*, and/or *Streptococcus* as risk bacteria for wheezing and asthma using both cross sectional^{4, 8-11, 26} and prospective^{6, 7, 12} study designs. Teo and colleagues found in the Australian Childhood Asthma Study (CAS; n=234) that asymptomatic nasopharyngeal colonization with a high abundance of *Streptococcus* around age 2 months was associated with wheeze at age 5 years.¹² In the same cohort, higher frequency of detecting nasopharyngeal *Moraxella*, *Haemophilus*, or *Streptococcus* at times of health between age 6 months and 2 years was associated in sensitized children (n=73) with higher odds of chronic wheeze at age 5 years and in non-sensitized children (n=64) with any wheeze in the first 3 years of life (i.e., transient wheeze).⁶

For the first time, we have examined the longitudinal nasal abundances of these 3 risk bacteria (i.e., *Moraxella*, *Haemophilus*, and *Streptococcus*) in a large, diverse multicenter cohort of U.S. infants hospitalized for bronchiolitis. The relative abundances of these risk bacteria upon hospitalization were not associated with recurrent wheezing by age 3 years. However, in the weeks after severe viral infection, enrichment of an infant's nasal ecosystem with either *Moraxella* or *Streptococcus* increased the risk of developing recurrent wheezing by age 3 years and recurrent wheezing accompanied by asthma at age 4 years. Similar to CAS,¹² we also found a separate group of children who at a median age of 9 months (i.e., around 6 months after hospitalization) while healthy, had increased *Streptococcus* abundance and a higher risk of recurrent wheezing by age 3 years. It remains unclear, however, why these infants have a later increase in *Streptococcus* abundance and as seen in Figure 3, a later onset of recurrent wheezing. The lack of a significant association with *Haemophilus* may have been due to inadequate power and/or a weaker association. Importantly, we confirmed all of these findings using multiple analytic techniques. Moreover, our joint models demonstrated that the present microbiota

findings are independent of multiple other potential confounding factors associated with childhood asthma, including viral etiology and IgE sensitization in infancy.

These findings suggest that interventions that alter the nasal microbiome after hospitalization for bronchiolitis may be beneficial. Indeed, Beigelman and colleagues found in a small (n=39) randomized, placebo-controlled trial that azithromycin for 14 days in infants hospitalized with RSV bronchiolitis²³ reduced nasal lavage *Moraxella* abundance at 2 weeks.²² Furthermore, in concordance with our findings, decreased *Moraxella* abundance at 14 days, no matter the group assignment, was associated with reduced odds of recurrent wheezing (i.e., 3 or more wheezing episodes) over the subsequent 12 months.²² This potential approach to bronchiolitis treatment needs further study and thoughtful consideration about possible unintended consequences of widespread antibiotic use. Indeed, bronchiolitis and its long-term respiratory outcomes may be best understood as a complex adaptive system.²⁷

Taken together, the potential health effects of the airway microbiome are tantalizing. We note, however, that early *Moraxella* colonization in healthy infants was associated with lower rates of respiratory infections.²⁸ Thus, understanding the potential protective role of *Moraxella* in healthy populations, compared with its consistent identification as a risk bacterium (not only before and during acute respiratory infections^{6, 11} but also in asthma⁸), will be critical to advancing our understanding of the role of this genus (and ultimately, the strain of *Moraxella*) in respiratory conditions. It is also possible that the post-hospitalization increases in *Moraxella* or *Streptococcus* may simply be a marker of children who are already predisposed to develop recurrent wheezing. Alternatively, the association between post-hospitalization increases in *Moraxella* or *Streptococcus* and recurrent wheezing may be causal – i.e., microbiota may influence host immune response to viral infection.^{23, 29, 30} If true, influencing microbiome

reassembly after hospitalization for bronchiolitis either with antibiotics^{22, 23} or preferably, with intranasal administration of protective species³¹ may be an approach to help prevent asthma in this high-risk group.

This study has potential limitations. First, although bronchiolitis is the leading cause of hospitalization in infants, we suggest caution when generalizing our results beyond the ~130,000 U.S. infants hospitalized with bronchiolitis each year.³² Second, bronchiolitis is a condition that affects the entire airway from nose to lungs.³² While our samples from the nose may not represent the pathobiology in the lung, nasal cells and bronchial cells have similar responses to stimulation and have significant mediator release correlations.³³ Third, there was no healthy control group in MARC-35, but our study objective was not to examine the association between the microbiome and the development of bronchiolitis within the general population, but rather to determine the relation of the nasal microbiota to recurrent wheezing within the high-risk population of infants hospitalized with bronchiolitis. Fourth, although we have 88% follow-up at age 3 years, the number of nasal swabs included in the analyses decreased over time due to parents forgetting to collect swabs and the exclusion of samples collected after the onset of recurrent wheezing as required by the joint model. Fifth, the present results are unable to untangle how host environmental conditions, including bacterial-bacterial interactions at the time of infection, may enhance the survival of certain species.³⁴ However, our group is currently examining the role of airway microbe-host relations in the development of childhood asthma. Sixth, these analyses will need to be repeated when the children in the cohort turn age 6 years and are formally evaluated for asthma. Finally, the present analysis neither accounted for potential genetic differences within our sample³² nor different strains of *Moraxella* and *Streptococcus*, nor the function of the microbiome. We are currently pursuing these important research questions.

In summary, we have identified in a large, prospective, multicenter cohort of diverse U.S. infants hospitalized with bronchiolitis that increased abundance of nasal *Moraxella* or *Streptococcus* in the weeks and months following hospitalization was related to recurrent wheezing by age 3 years. These results not only confirm that *Moraxella* and *Streptococcus* are risk bacteria, but also identify a potential window of opportunity for clinicians to ameliorate the chronic respiratory outcomes for this high-risk group of children. Indeed, these findings may serve as a starting point to begin investigations that would change management for a common pediatric condition that has no proven effective intervention beyond supportive care.

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Table 1. Characteristics and clinical presentations for 842 infants hospitalized for bronchiolitis, by recurrent wheezing outcome

	Recurrent wheezing by 36 months		
	No	Yes	
Variables	n = 577 (69%)	n = 265 (31%)	P-value
Characteristics			
Age at index (months), median (IQR)	3.0 (1.5, 5.8)	3.6 (2.1, 5.9)	0.01
Sex – Female	232 (40)	106 (40)	1.00
Race/ethnicity			0.11
Non-Hispanic white	245 (43)	127 (48)	
Non-Hispanic black	127 (22)	65 (25)	
Hispanic	180 (31)	67 (25)	
Other	25 (4)	6 (2)	
Maternal asthma	100 (17)	77 (29)	<0.001
Maternal smoking during pregnancy	69 (12)	45 (17)	0.06
C-section delivery	188 (33)	90 (34)	0.76
Low birth weight (<2.3 kg)	29 (5)	22 (8)	0.09
Mostly breastfed during first 3 months	254 (44)	121 (46)	0.71
Postnatal smoke exposure	90 (16)	38 (14)	0.71
Child history of eczema	72 (13)	49 (19)	0.03
Daycare attendance	129 (22)	67 (25)	0.40
Presentation at hospitalization for bronchiolitis			
History of antibiotics use prior to index	172 (30)	88 (33)	0.36
History of hospitalization prior to index	87 (15)	44 (17)	0.64
RSV*	494 (86)	196 (74)	<0.001
Rhinovirus*	111 (19)	62 (23)	0.20
IgE sensitization†	118 (21)	46 (17)	0.34
Intensive care use‡	82 (14)	45 (17)	0.35
All data are no. (%) unless otherwise indicated. P-values are computed using chi-squared tests (categorical variables) and Wilcoxon rank sum tests (continuous variables).			
Abbreviations: IQR, interquartile range; RSV, respiratory syncytial virus; IgE, immunoglobulin E.			
* Detected via nasopharyngeal aspirate samples collected at index.			
† Defined as detection of any positive values for serum allergen-specific IgE at index.			
‡ Defined as admission to intensive care unit and/or use of mechanical ventilation (continuous positive airway pressure and/or intubation during inpatient stay, regardless of location) at any time during the index hospitalization.			

Figure 1. Cross-sectional comparison of *Moraxella* and *Streptococcus* relative abundance and the percentage of recurrent wheezing outcomes

The microbiota samples were classified into above mean abundance (orange) and below mean abundance (purple) groups by comparing the relative abundance of a genus at one of 4 time points (i.e., index, clearance, summer, and seasonal) with the mean of this genus across all time points. The percentage of recurrent wheezing by age 3 years was computed at every time point for each group. The association between the abundance group (i.e., above mean or below mean) and recurrent wheezing outcome at each time point was analyzed using chi-squared tests. Abundance group membership was not consistent across time points; a taxon may be protective at one time point and a risk factor for recurrent wheezing at a different time point. Samples collected after the onset of recurrent wheeze were excluded. (1) Increased abundance of *Moraxella* (mean = 0.08) at the clearance time point is significantly associated with a higher percentage of recurrent wheezing ($P=0.04$). (2) Increased abundance of *Streptococcus* (mean = 0.04) at the clearance ($P=0.01$) and summer ($P=0.03$) time points are significantly associated with a higher percentage of recurrent wheezing.

Figure 2. Associations between longitudinal *Moraxella* and *Streptococcus* relative abundances and hazard of recurrent wheezing by age 3 years

The hazard ratios for the relative abundance and abundance by time point interaction for *Moraxella* (left) and *Streptococcus* (right) were calculated using a Bayesian implementation of the joint model for longitudinal and time-to-event data. Bars and points indicate the 95% high density intervals (HDI) and medians, respectively, for the posterior distributions of the log hazard ratios. The shaded area represents the region of practical equivalence (ROPE) around zero, defined as $[-0.05, 0.05]$. Variables with HDIs that do not overlap the ROPE were considered statistically significant. The time-to-event models adjusted for clinical variables recorded at the index time point, with maternal asthma and RSV infection being significant in both models.

Abbreviations: HDI, high density intervals; ROPE, region of practical equivalence; HR, hazard ratio; RSV, respiratory syncytial virus; RV, rhinovirus; IgE, immunoglobulin E

Figure 3. Kaplan-Meier curves stratified by *Moraxella* and *Streptococcus* risk groups for the onset of recurrent wheezing by age 3 years

Using the mean cutoffs for *Moraxella* (mean=0.08) and *Streptococcus* (mean=0.04) at the relevant time points, infants were classified into the 3 risk groups identified by the joint model: (A) increased *Moraxella* at clearance ($n=102$), (B) increased *Streptococcus* at clearance ($n=48$), and (C) increased *Streptococcus* at summer ($n=58$). Kaplan-Meier curves were generated for each of the 3 risk groups for the outcome, recurrent wheezing by age 3 years. The curves were stratified by membership in the risk group. The table shows the number of subjects still at risk for recurrent wheeze based on event occurrences and study dropout. Infants may be included in more than 1 risk group. Log-rank test results indicated that the differences in the hazard functions of the stratified curves were significant for all risk groups.

Figure 4. Associations between longitudinal *Moraxella* and *Streptococcus* relative abundances and hazard of recurrent wheezing by age 3 years accompanied by asthma at age 4 years

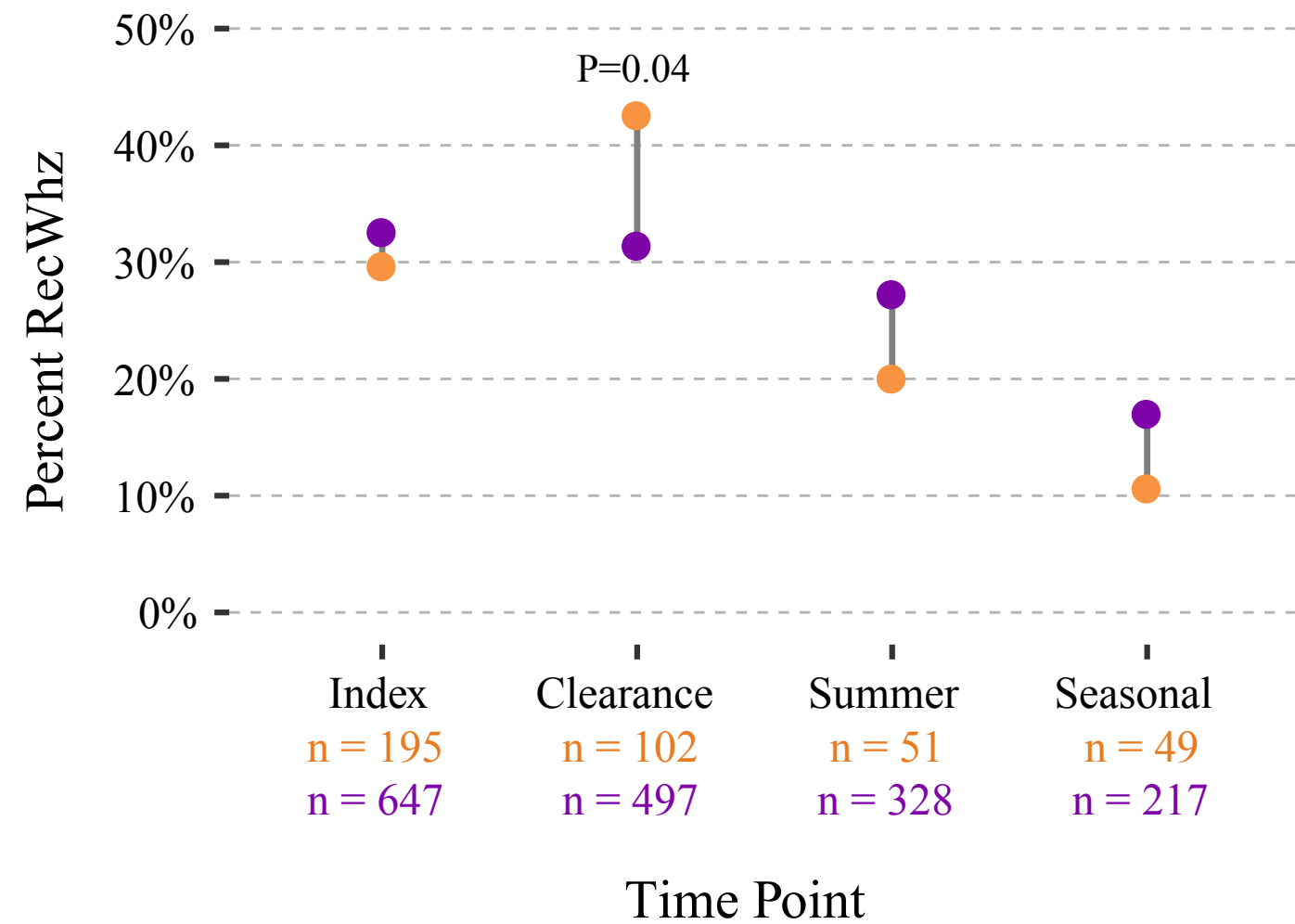
The hazard ratios for the relative abundance and abundance by time point interaction for *Moraxella* (left) and *Streptococcus* (right) were calculated using a Bayesian implementation of the joint model for longitudinal and time-to-event data. Bars and points indicate the 95% high density intervals (HDI) and medians, respectively, for the posterior distributions of the log hazard ratios. The shaded area represents the region of practical equivalence (ROPE) around zero, defined as $[-0.05, 0.05]$. Variables with HDIs that do not overlap the ROPE were considered statistically significant. The time-to-event models adjusted for clinical variables recorded at the index time point, with maternal asthma, RSV infection, and history of eczema being significant in both models.

Abbreviations: HDI, high density intervals; ROPE, region of practical equivalence; HR, hazard ratio; RSV, respiratory syncytial virus; RV, rhinovirus; IgE, immunoglobulin E

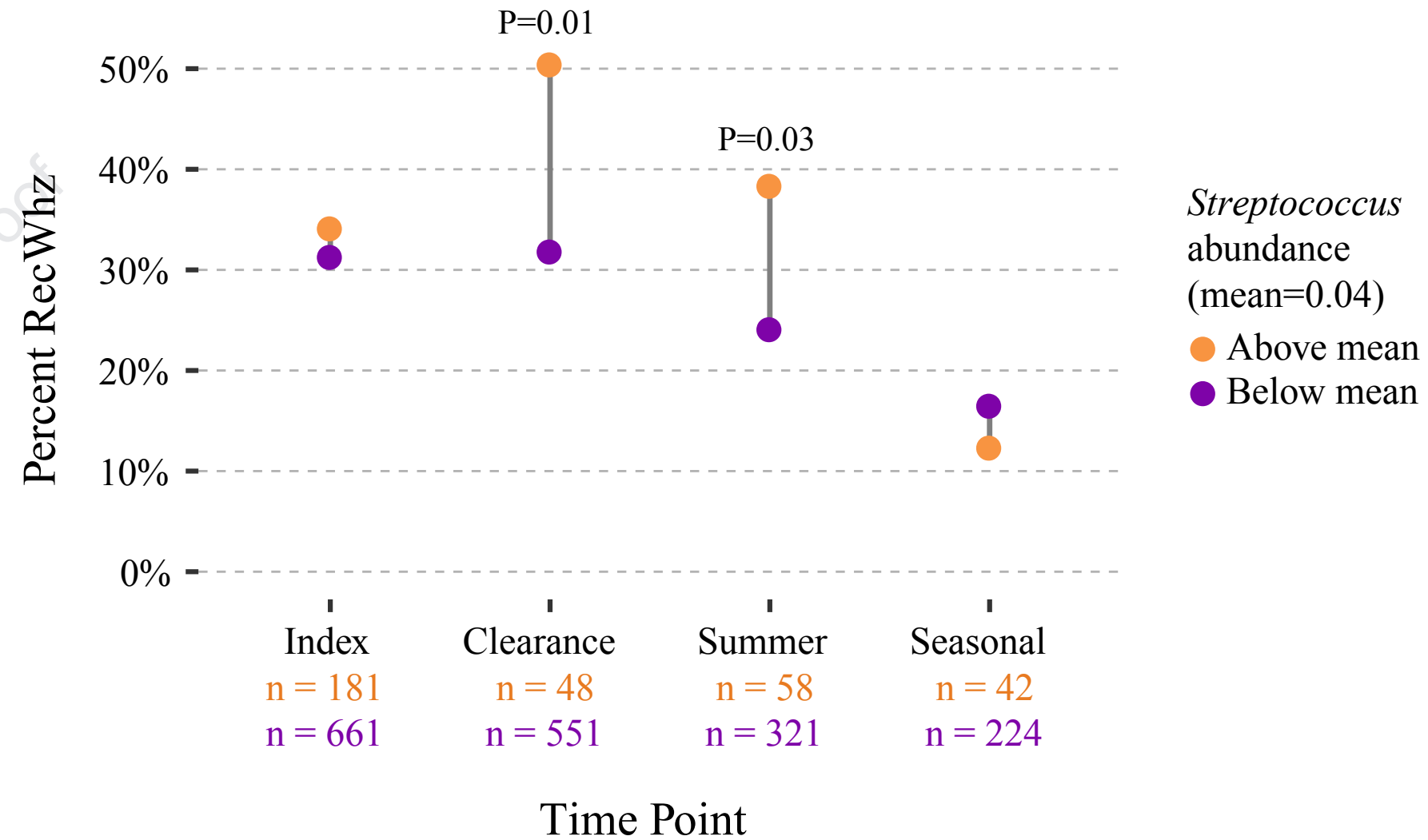
Figure 5. Longitudinal variation of relative abundances for *Moraxella* and *Streptococcus* risk groups in the time after hospitalization

Loess curves were fit for the relative abundance of *Moraxella* (blue) and *Streptococcus* (red) using the time since day 1 of hospitalization for infants in the 3 risk groups identified by the joint model: (A) increased *Moraxella* at clearance ($n=102$), (B) increased *Streptococcus* at clearance ($n=48$), and (C) increased *Streptococcus* at summer ($n=58$). Infants were classified into risk groups using the mean cutoffs for *Moraxella* (mean=0.08) and *Streptococcus* (mean=0.04) at the relevant time points and may have been included in more than 1 risk group. Shaded regions indicate 95% confidence intervals. The curves show the transience of the increased abundance of the risk genera.

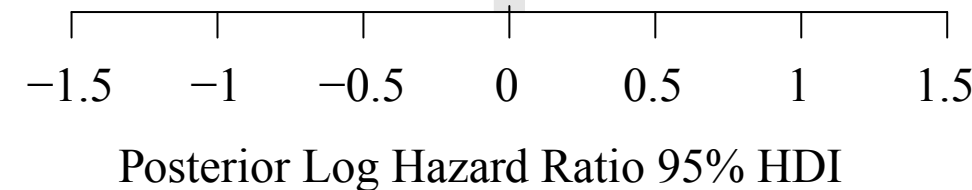
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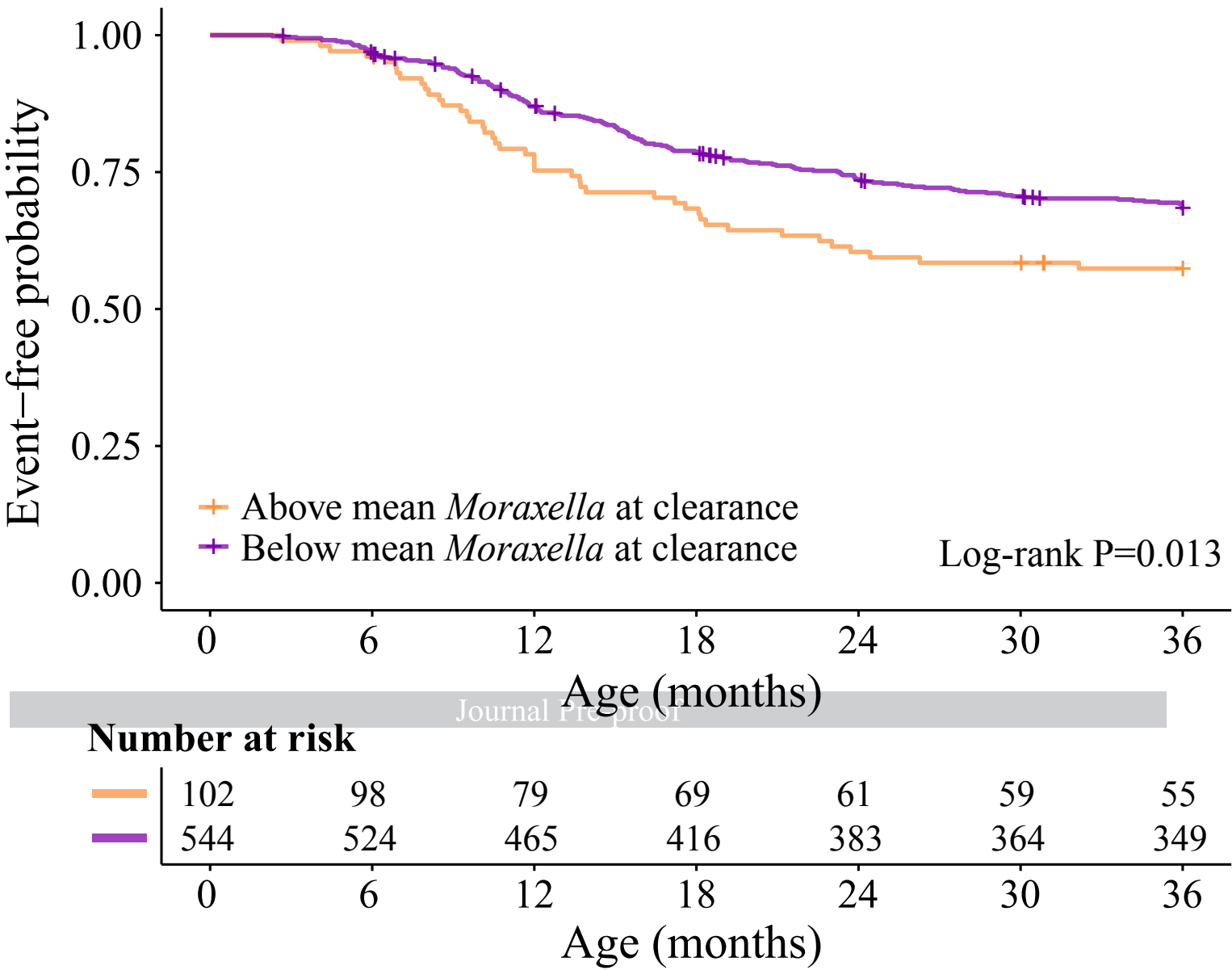
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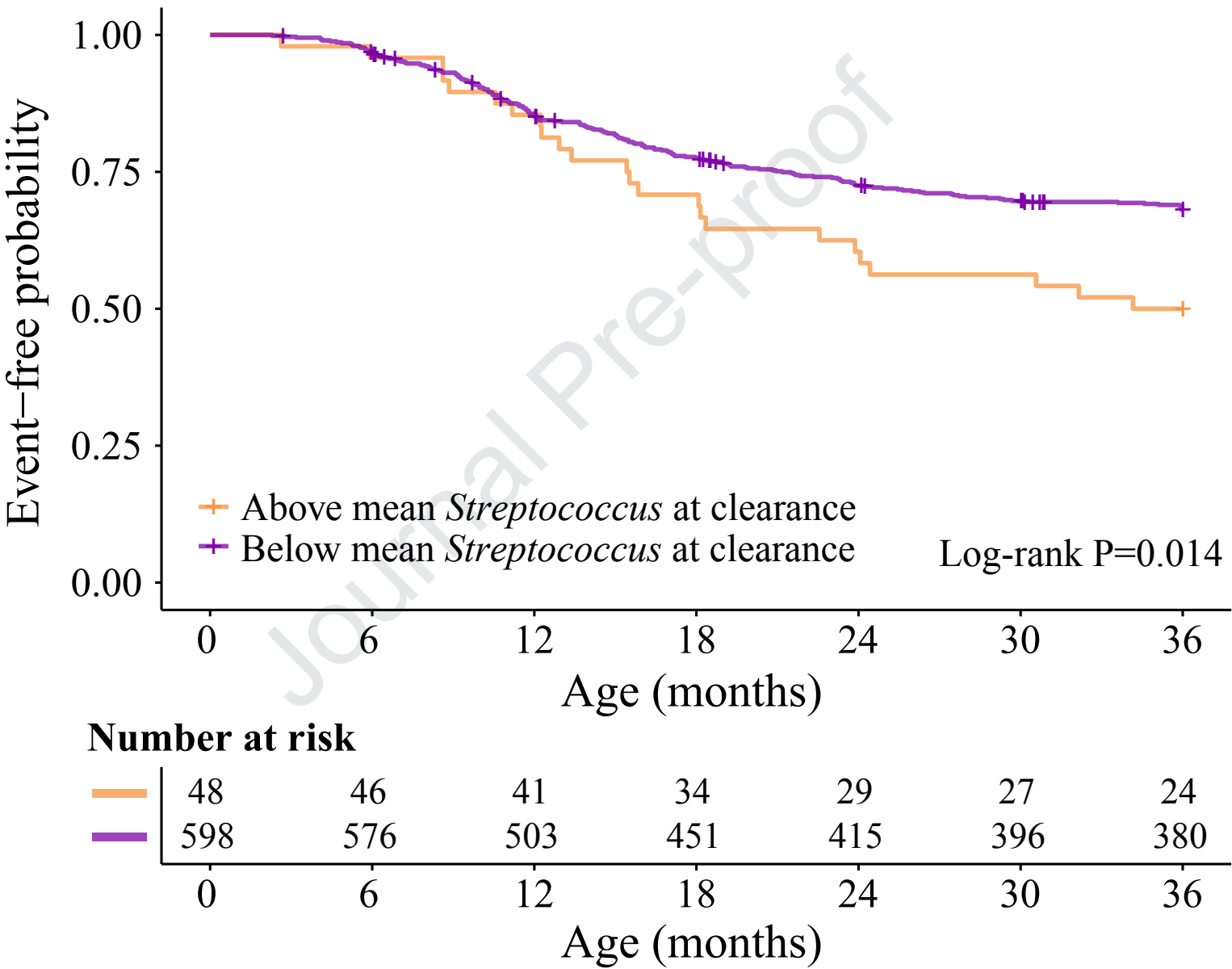
Posterior Log Hazard Ratio 95% HDI



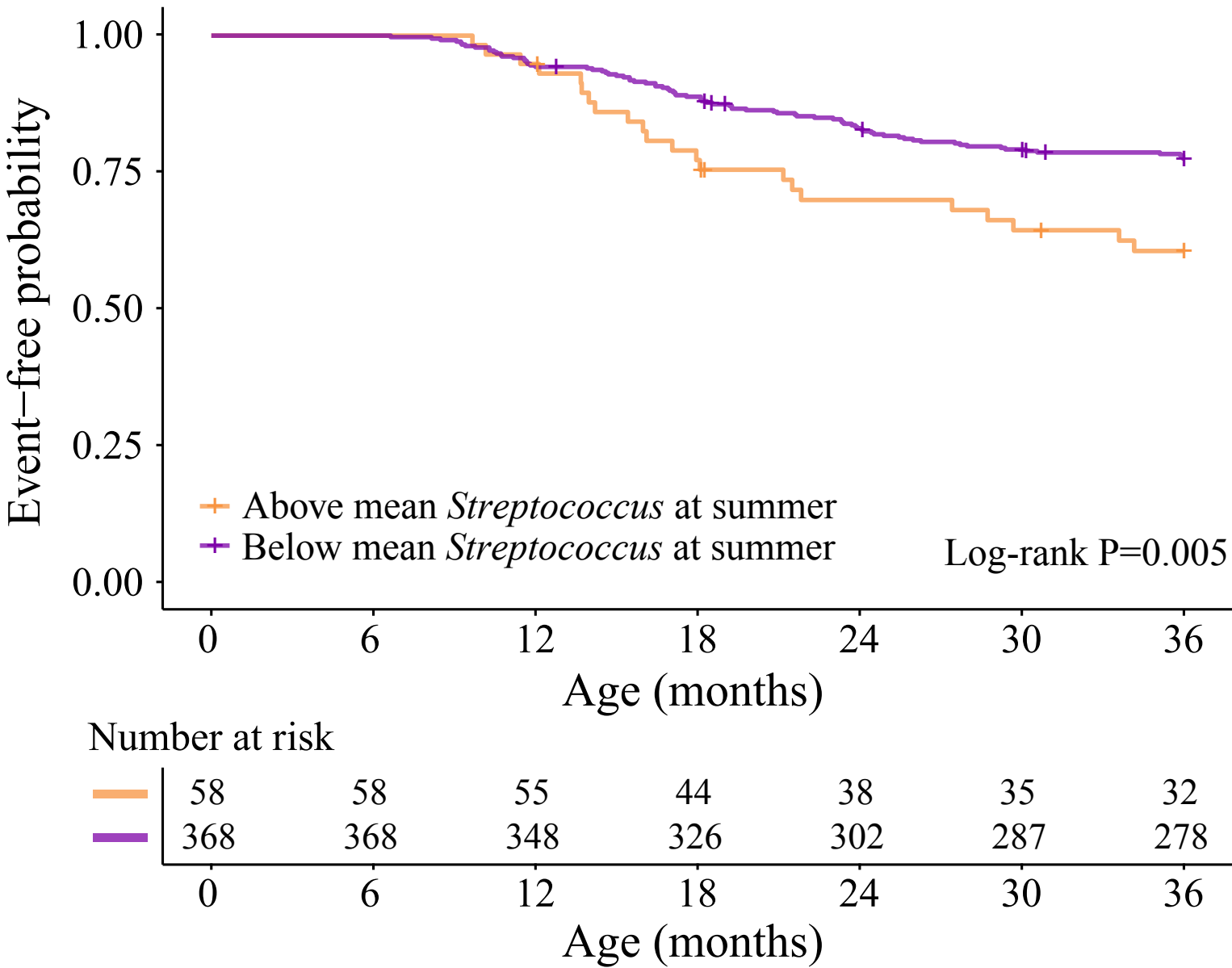
A - Increased *Moraxella* at clearance



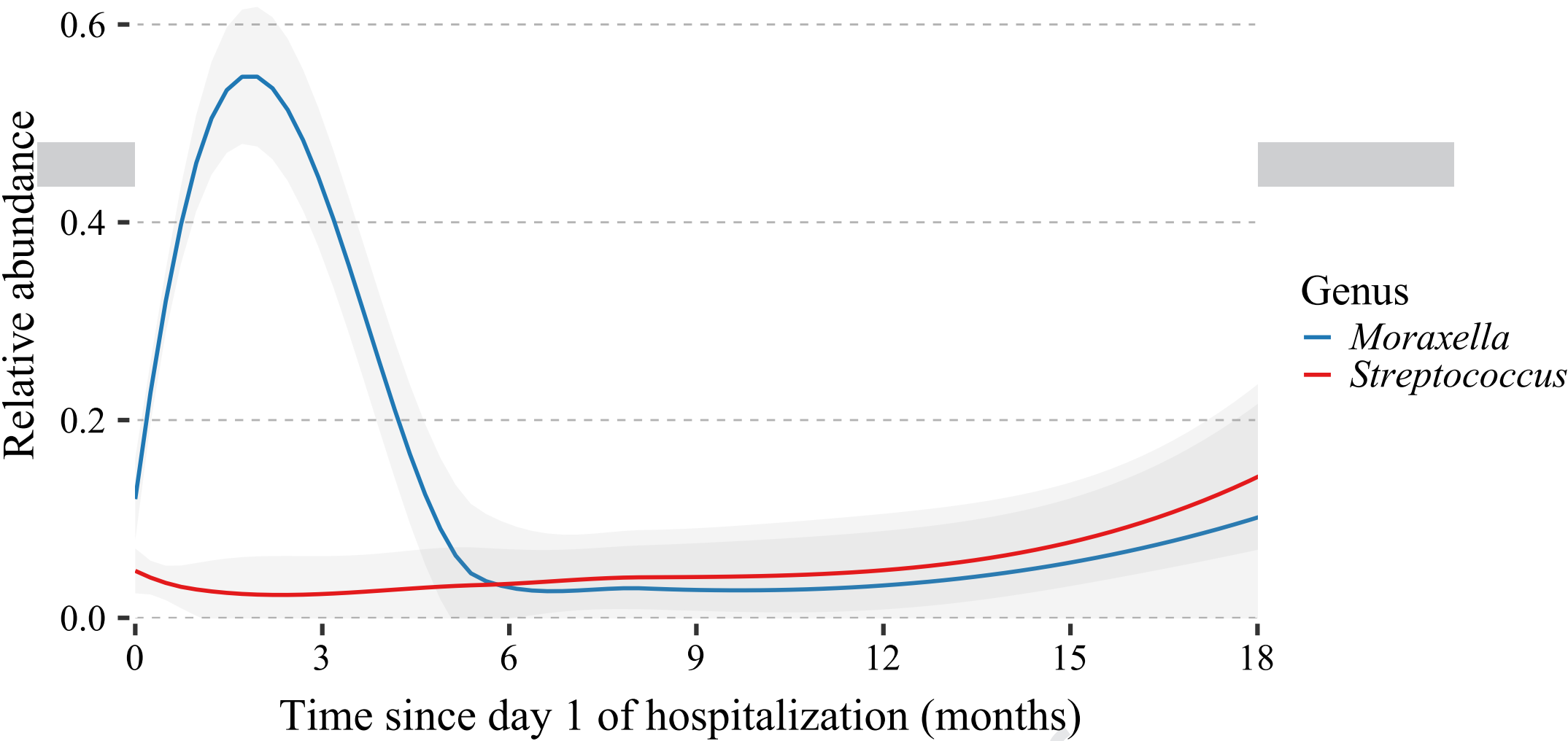
B - Increased *Streptococcus* at clearance



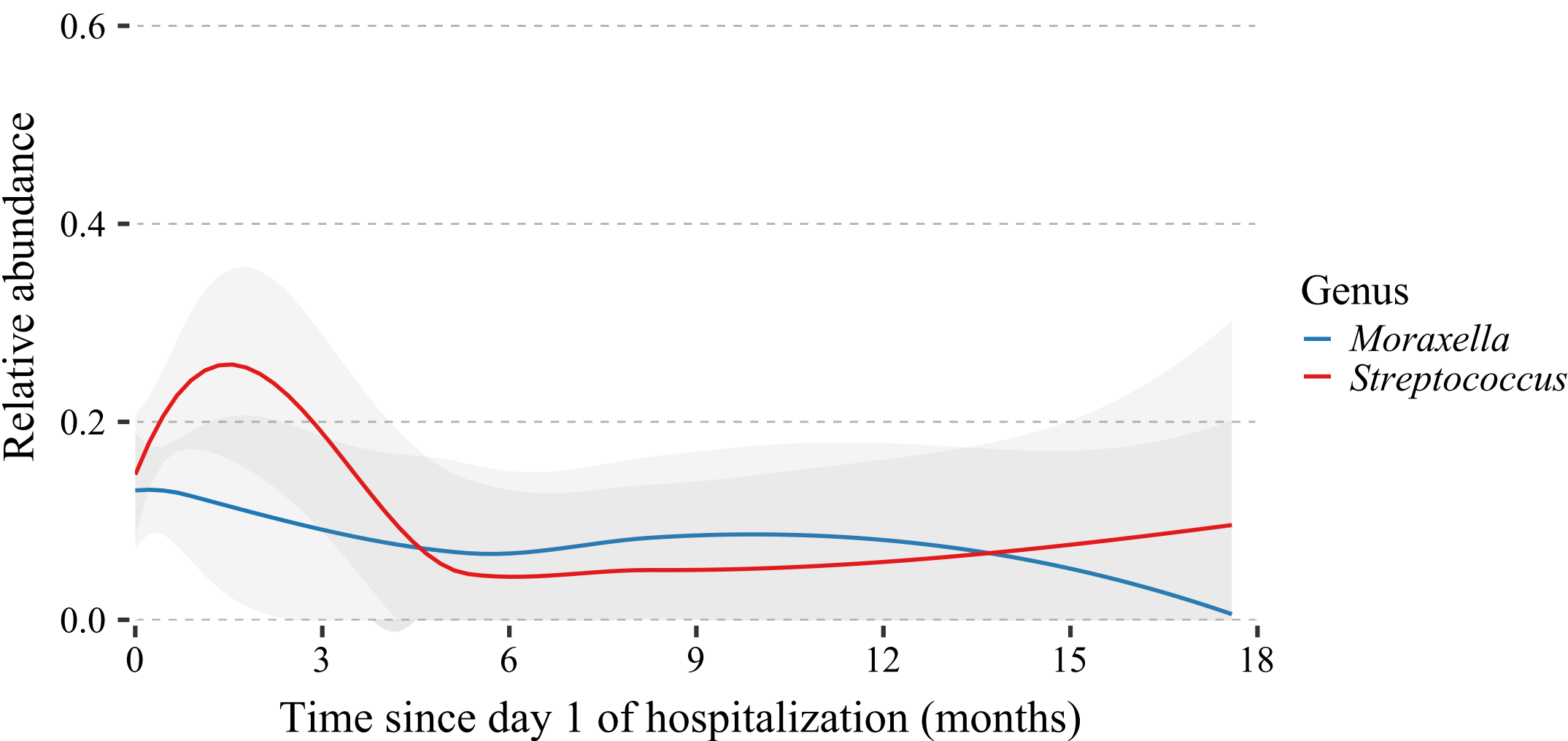
C - Increased *Streptococcus* at summer



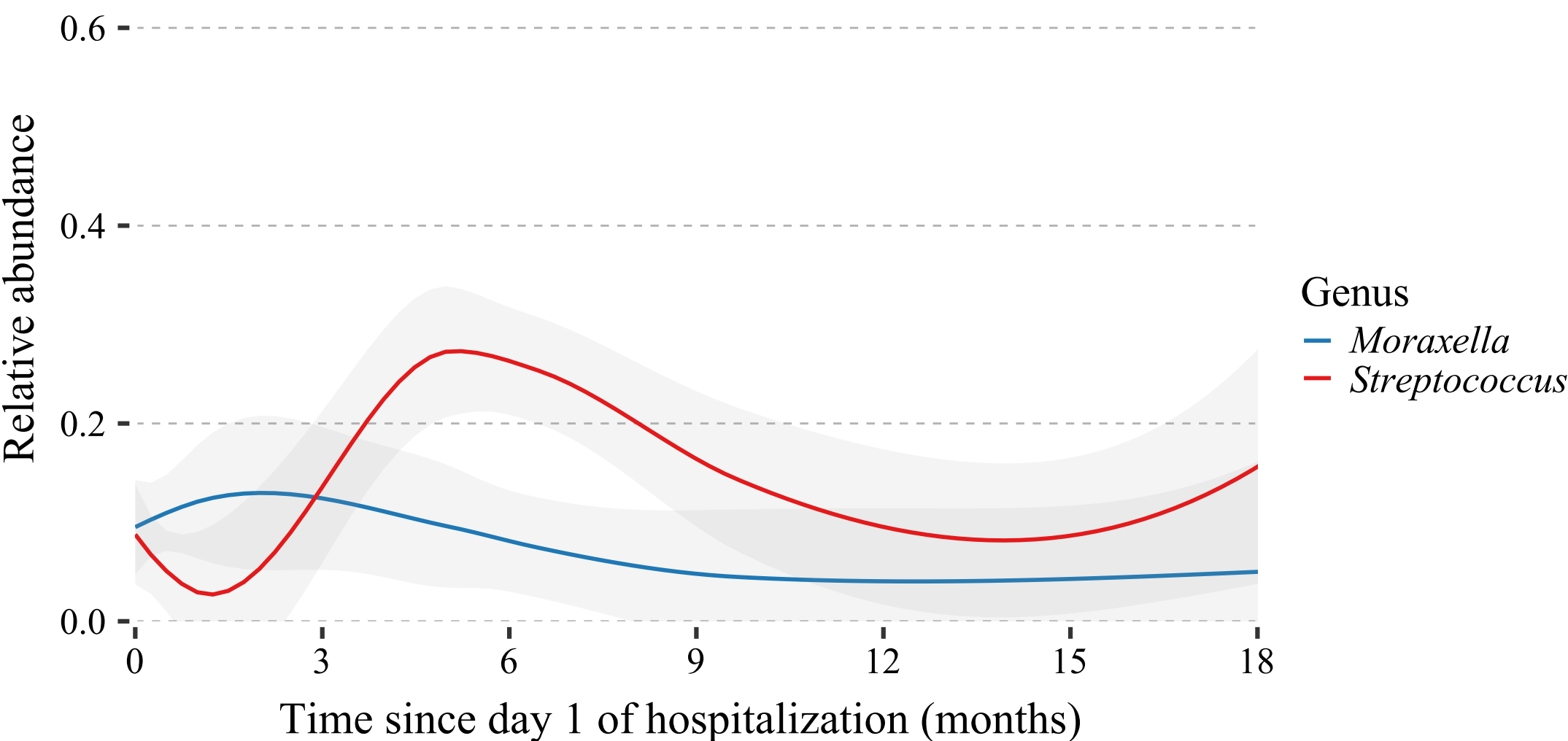
A - Increased *Moraxella* at clearance

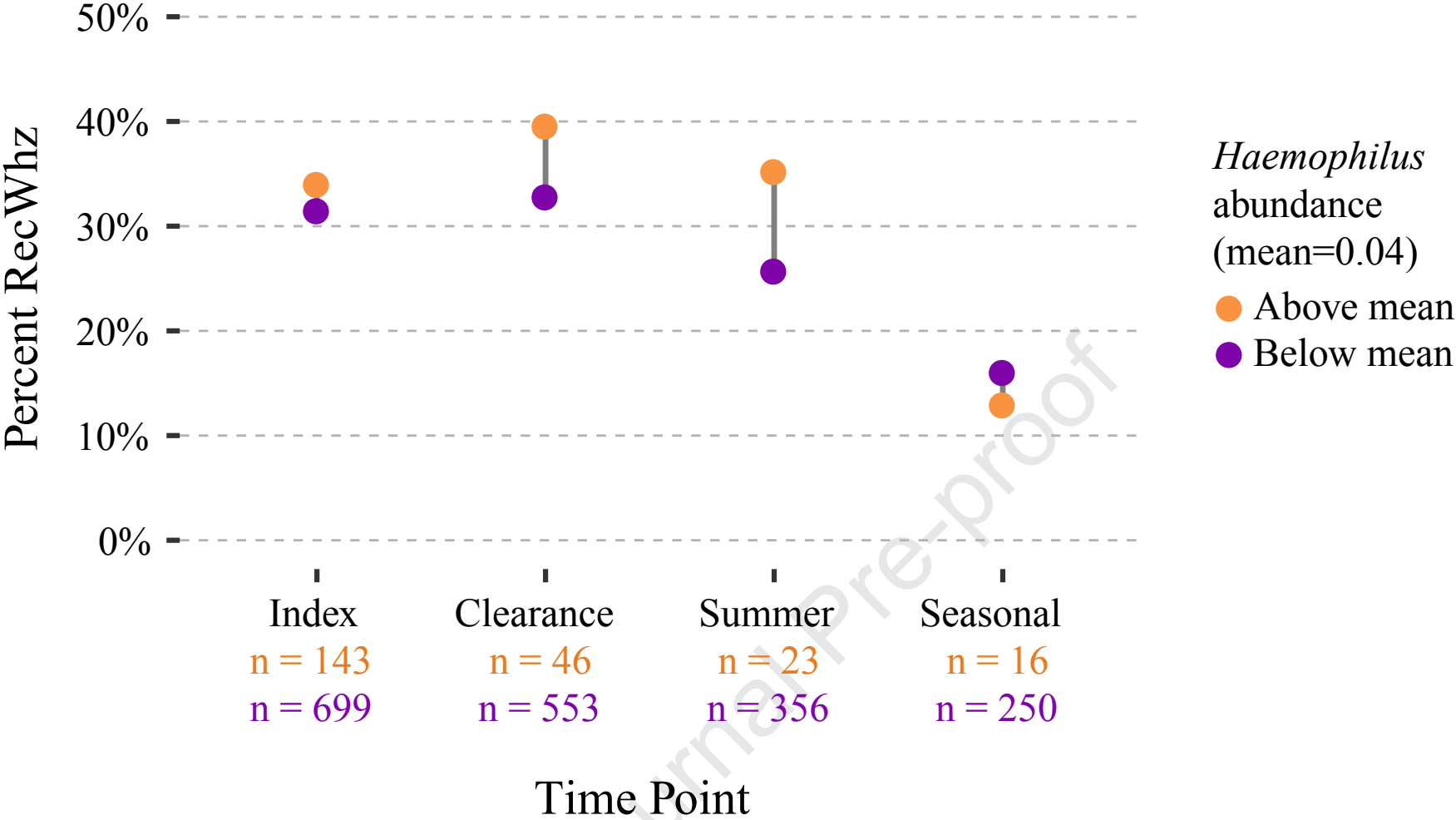


B - Increased *Streptococcus* at clearance

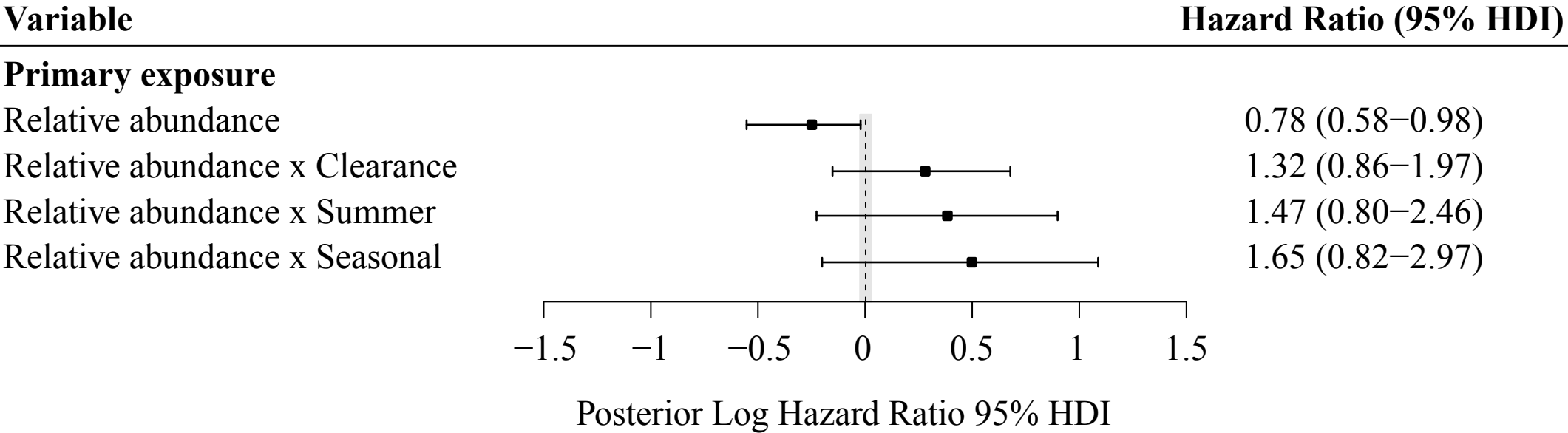


C - Increased *Streptococcus* at summer

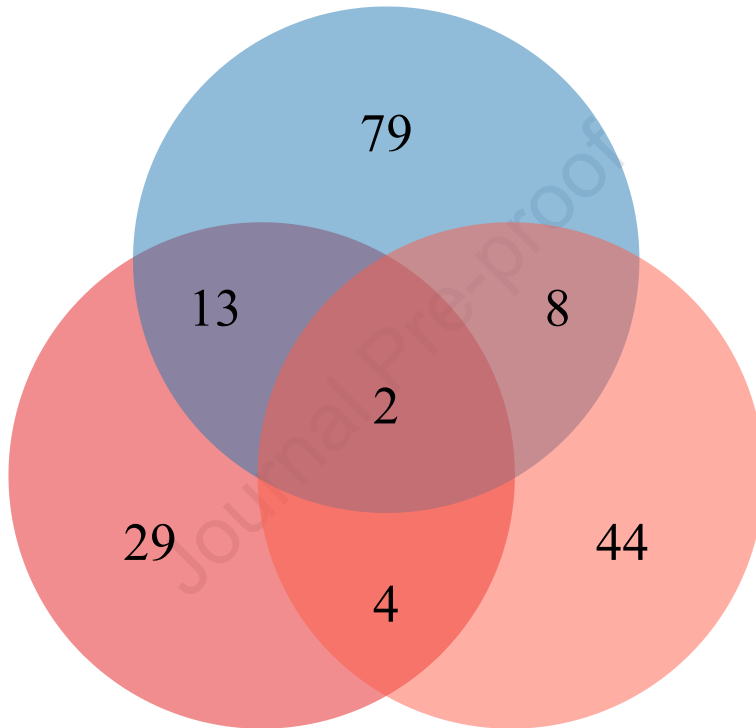




2



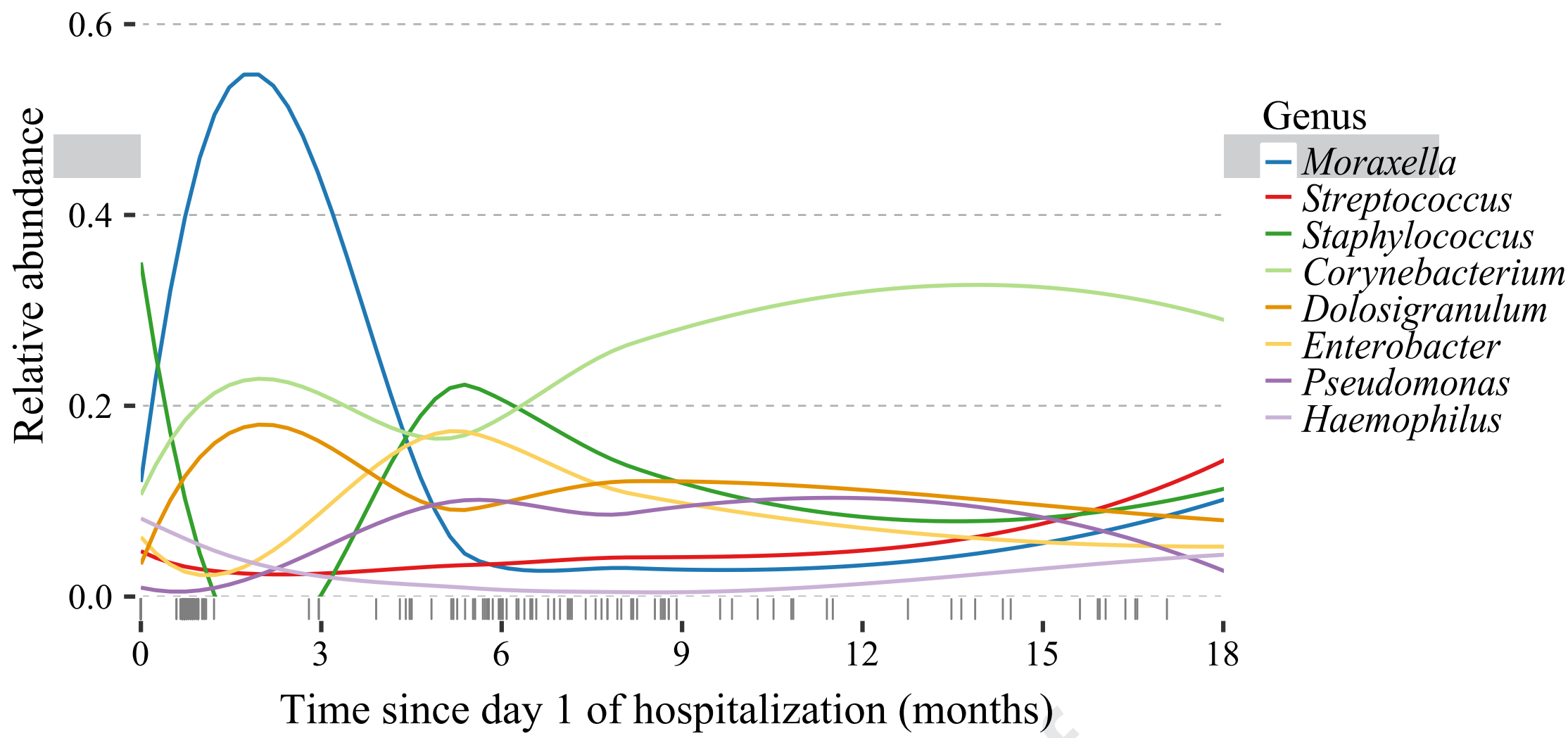
A - Increased *Moraxella*
at clearance
(n=102)



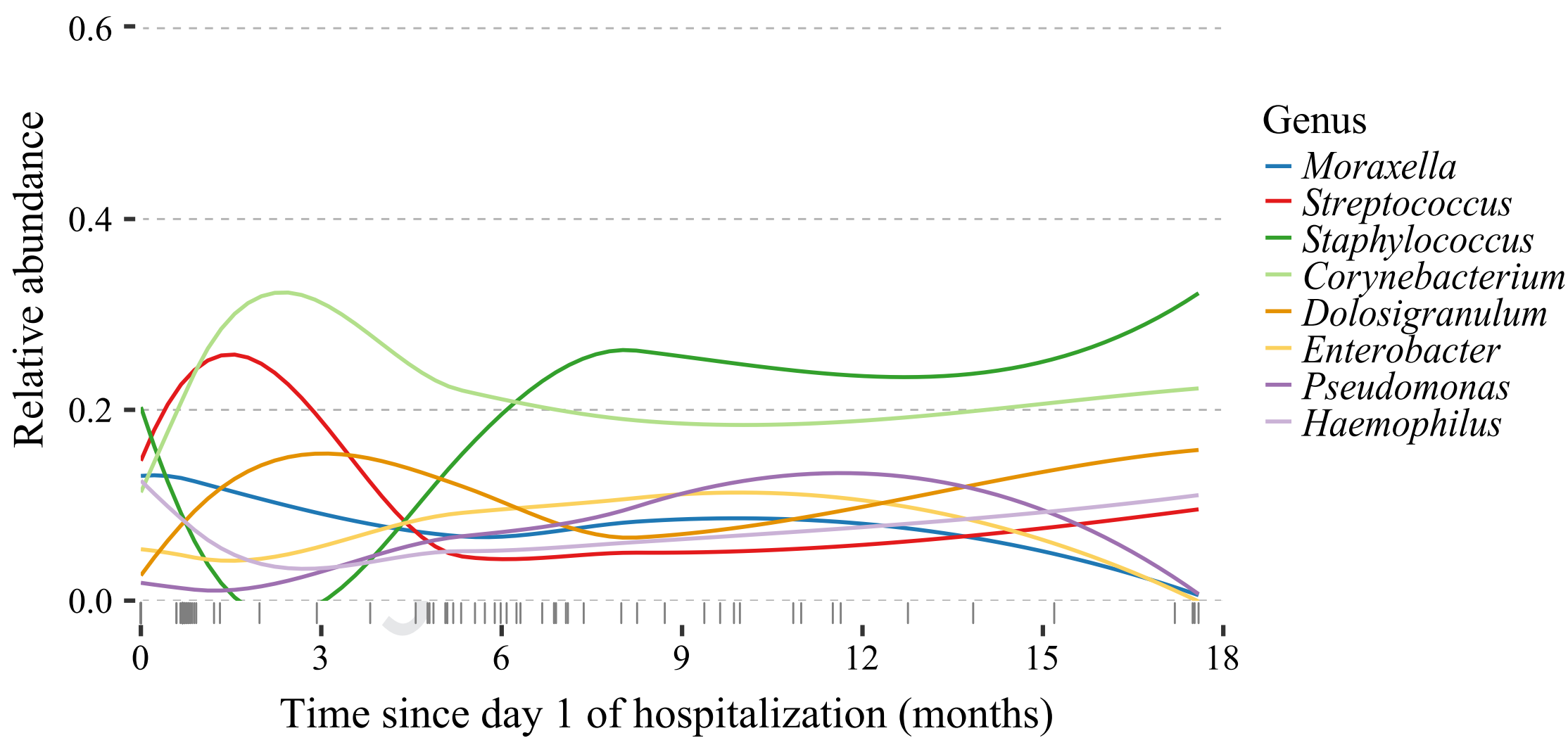
B - Increased
Streptococcus
at clearance
(n=48)

C - Increased
Streptococcus
at summer
(n=58)

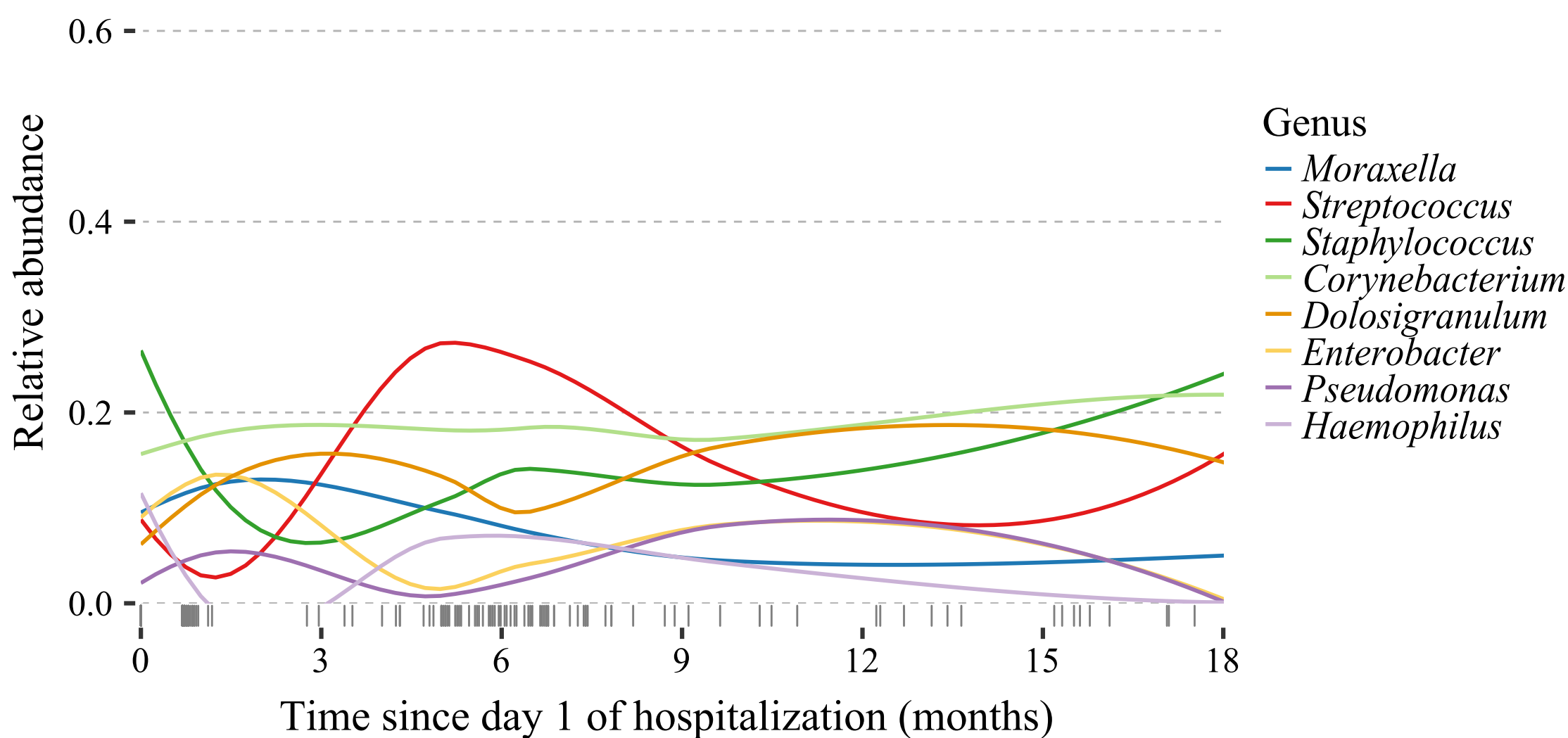
A - Increased *Moraxella* at clearance



B - Increased *Streptococcus* at clearance



C - Increased *Streptococcus* at summer



Supplementary materials – Figures

Increased *Moraxella* and *Streptococcus* following severe bronchiolitis is associated with recurrent wheezing

Jonathan M. Mansbach, MD, MPH; Pamela N. Luna, PhD; Chad A. Shaw, PhD; Kohei Hasegawa, MD, MPH; Joseph F. Petrosino, PhD; Pedro A. Piedra, MD; Ashley F. Sullivan, MS, MPH; Janice A. Espinola, MPH; Christopher J. Stewart, PhD; and Carlos A. Camargo, Jr., MD, DrPH

Figure E1. Relation of *Haemophilus* relative abundance to recurrent wheezing by age 3 years

(1) Based on the mean abundance of *Haemophilus* across all time points (mean = 0.04), microbiota samples were classified into above mean (orange) and below mean (purple) groups. The percentage of recurrent wheezing was computed at every time point for each abundance group. Abundance group membership was not consistent across time points, and samples collected after the onset of recurrent wheeze were excluded. (2) The hazard ratios for the relative abundance and abundance by time point interaction for *Haemophilus* were calculated using a Bayesian implementation of the joint model for longitudinal and time-to-event data. Bars and points indicate the 95% high density intervals (HDI) and medians, respectively, for the posterior distributions of the log hazard ratios. The shaded area represents the region of practical equivalence (ROPE) around zero, defined as [-0.05, 0.05]. Variables with HDIs that did not overlap the ROPE were considered statistically significant. The time-to-event model adjusted for clinical variables recorded at the index time point, with maternal asthma and RSV infection being significant in both models (data not shown).

Figure E2. Overlap of participants in the *Moraxella* and *Streptococcus* risk groups

Using the mean cutoffs for *Moraxella* (mean=0.08) and *Streptococcus* (mean=0.04) at the relevant time points, infants were classified into the risk groups identified by the joint model: (A - blue) increased *Moraxella* at clearance (n=102); (B - red) increased *Streptococcus* at clearance (n=48); and (C - light red) increased *Streptococcus* at summer (n=58). The overlap between these 3 risk groups is depicted using a Venn diagram. There is minimal overlap between the risk groups, with only 2 subjects belonging to all 3 risk groups and 6 subjects having increased *Streptococcus* at both clearance and summer.

Figure E3. Longitudinal variation of relative abundances for highly abundant genera in the time after hospitalization, categorized by the *Moraxella* and *Streptococcus* risk groups

Loess curves were fit for the relative abundances of 8 of the top ten genera using the time since day 1 of hospitalization for infants in each of the risk groups identified by the joint model: (A) increased *Moraxella* at clearance (n=102), (B) increased *Streptococcus* at clearance (n=48), and (C) increased *Streptococcus* at summer (n=58). Infants were classified into risk groups using the mean cutoffs for *Moraxella* (mean=0.08) and *Streptococcus* (mean=0.04) at the relevant time points and may have been included in more than 1 risk group. The rug plot along the x-axis denotes the distribution of observation times for subjects in the specified risk group. *Acinetobacter* and *Bacillus* did not have noticeable variation over time and were excluded to improve readability.

Supplementary materials – Tables

Increased *Moraxella* and *Streptococcus* abundance following severe bronchiolitis is associated with recurrent wheezing

Jonathan M. Mansbach, MD, MPH; Pamela N. Luna, PhD; Chad A. Shaw, PhD; Kohei Hasegawa, MD, MPH; Joseph F. Petrosino, PhD; Pedro A. Piedra, MD; Ashley F. Sullivan, MS, MPH; Janice A. Espinola, MPH; Christopher J. Stewart, PhD; and Carlos A. Camargo, Jr., MD, DrPH

Table E1. Comparison of characteristics and clinical presentations for analytic and non-analytic cohorts

Variables	Analytic cohort n = 842*	Non-analytic n = 78	P-value
Genus abundances, mean (SD)			
<i>Moraxella</i> at clearance	0.07 (0.19)	0.10 (0.24)	0.56
<i>Streptococcus</i> at clearance	0.02 (0.09)	0.06 (0.18)	0.46
<i>Streptococcus</i> at summer	0.04 (0.14)	0.04 (0.13)	0.97
Outcomes			
Recurrent wheezing by age 3 years	265 (31)	31 (40)	0.17
Recurrent wheezing by age 3 years accompanied by asthma at 4 years	119 (14)	19 (24)	0.03
Characteristics			
Age at index (months), median (IQR)	3.19 [1.68, 5.78]	3.73 [1.61, 7.32]	0.17
Sex – Female	338 (40)	29 (37)	0.70
Race/ethnicity			0.47
Non-Hispanic white	372 (44)	28 (36)	
Non-Hispanic black	192 (23)	18 (23)	
Hispanic	247 (29)	28 (36)	
Other	31 (4)	4 (5)	
Maternal asthma	177 (21)	18 (23)	0.82
Maternal smoking during pregnancy	114 (14)	12 (15)	0.78
C-section delivery	278 (33)	33 (42)	0.12
Low birth weight (<2.3 kg)	51 (6)	8 (10)	0.23
Mostly breastfed during first 3 months	375 (45)	36 (46)	1.00
Postnatal smoke exposure	128 (15)	10 (13)	0.69
Child history of eczema	121 (14)	16 (21)	0.20
Daycare attendance	196 (23)	15 (19)	0.50
Presentation at hospitalization for bronchiolitis			
History of antibiotics use prior to index	260 (31)	35 (45)	0.02
History of hospitalization prior to index	131 (16)	16 (21)	0.33
RSV†	690 (82)	61 (78)	0.51
Rhinovirus†	173 (21)	13 (17)	0.50
IgE sensitization‡	164 (19)	18 (23)	0.54
Intensive care use §	127 (15)	14 (18)	0.61

All data are no. (%) unless otherwise indicated. P-values are computed using chi-squared tests (categorical variables), Wilcoxon rank sum tests (continuous variables), and Kruskal-Wallis rank sum tests (relative abundances).

Abbreviations: IQR, interquartile range; RSV, respiratory syncytial virus; IgE, immunoglobulin E; SD, standard deviation

* One index swab was lost during shipment.

† Detected via nasopharyngeal aspirate samples collected at index.

‡ Defined as detection of any positive values for serum allergen-specific IgE at index for both food and aeroallergens.

§ Defined as admission to intensive care unit and/or use of mechanical ventilation (continuous positive airway pressure and/or intubation during inpatient stay, regardless of location) at any time during the index hospitalization.

Table E2. Nasal swab microbiota summaries, by collection time point

	Collection Time Point				P-value*
	Index	Clearance	Summer	Seasonal	
Variable	n = 842	n = 599	n = 379	n = 266	
Age (months), median (IQR)	3.2 (1.7, 5.8)	3.8 (2.3, 6.5)	8.9 (7.1, 10.9)	16.4 (12.5, 19.8)	<0.001
Alpha-diversity, median (IQR)					
Shannon index	0.61 (0.09, 1.12)	0.57 (0.19, 0.94)	0.68 (0.32, 1.05)	0.77 (0.42, 1.12)	<0.001
Relative abundances of top 10 genera, mean (SD)					
<i>Staphylococcus</i>	0.40 (0.42)	0.32 (0.40)	0.26 (0.37)	0.19 (0.33)	<0.001
<i>Corynebacterium</i>	0.11 (0.22)	0.13 (0.24)	0.19 (0.28)	0.16 (0.26)	<0.001
<i>Dolosigranulum</i>	0.05 (0.14)	0.08 (0.18)	0.08 (0.17)	0.12 (0.22)	<0.001
<i>Moraxella</i>	0.09 (0.20)	0.07 (0.19)	0.05 (0.14)	0.07 (0.16)	<0.001
<i>Streptococcus</i>	0.05 (0.13)	0.02 (0.09)	0.04 (0.14)	0.04 (0.13)	<0.001
<i>Enterobacter</i>	0.06 (0.21)	0.13 (0.29)	0.13 (0.30)	0.13 (0.29)	0.004
<i>Acinetobacter</i>	0.02 (0.12)	0.06 (0.21)	0.03 (0.14)	0.05 (0.16)	0.41
<i>Bacillus</i>	0.02 (0.14)	0.03 (0.15)	0.04 (0.15)	0.05 (0.17)	<0.001
<i>Pseudomonas</i>	0.01 (0.08)	0.05 (0.18)	0.04 (0.17)	0.07 (0.20)	<0.001
<i>Haemophilus</i>	0.07 (0.20)	0.02 (0.11)	0.02 (0.07)	0.02 (0.08)	<0.001

Comparisons across time points use Kruskal-Wallis rank sum tests. Relative abundances calculated using unrarefied sequence read counts.

Abbreviations: IQR, interquartile range; SD, standard deviation

*P-values for relative abundances adjusted for multiple testing using the Bonferroni correction.

Table E3. Characteristics and clinical presentations for infants hospitalized for bronchiolitis, by identified risk groups

Variable	Risk group			P-
	<i>Moraxella</i> n = 79	<i>Streptococcus</i> n = 29	<i>Streptococcus</i> n = 44	
Characteristics				
Age at time of collection (months), median (IQR)	4.3 (2.8, 7.1)	6.1 (3.4, 10.1)	9.6 (7.7, 11.1)	<0.001
Sex - Female	37 (47)	10 (35)	20 (46)	0.52
Race/ethnicity				0.21
Non-Hispanic white	38 (48)	14 (48)	27 (61)	
Non-Hispanic black	13 (17)	7 (24)	8 (18)	
Hispanic	26 (33)	6 (21)	6 (14)	
Other	2 (3)	2 (7)	3 (7)	
Maternal asthma	17 (22)	4 (14)	8 (18)	0.70
Maternal smoking during	10 (13)	2 (7)	7 (16)	0.52
Birth season				0.15
Spring	8 (10)	5 (17)	4 (9)	
Summer	11 (14)	8 (28)	10 (23)	
Fall	43 (54)	7 (24)	19 (43)	
Winter	17 (22)	9 (31)	11 (25)	
C-section delivery	32 (41)	12 (41)	13 (30)	0.40
Low birth weight (<2·3 kg)	4 (5)	0 (0)	3 (7)	0.45
Mostly breastfed during first	41 (52)	16 (55)	24 (55)	0.93
Postnatal smoke exposure	13 (17)	2 (7)	7 (16)	0.48
Child history of eczema	14 (18)	7 (24)	6 (14)	0.50
Daycare attendance	21 (27)	9 (31)	11 (25)	0.83
Presentation at hospitalization for bronchiolitis				
Age at index (months),	3.6 (2.0, 6.3)	5.5 (2.2, 8.7)	3.9 (2.1, 5.2)	0.25
History of antibiotics use	28 (35)	10 (35)	19 (43)	0.66
History of hospitalization	11 (14)	6 (21)	5 (11)	0.56
RSV+ at index*	62 (79)	22 (76)	36 (82)	0.80
RV+ at index*	15 (19)	6 (21)	12 (27)	0.59
IgE sensitization at index†	16 (20)	8 (28)	9 (21)	0.70
Wheezing at index				0.01
No	31 (39)	2 (7)	17 (39)	
Yes	45 (57)	24 (83)	25 (57)	
Unknown	3 (4)	3 (10)	2 (5)	
Antibiotics during pre-	21 (27)	13 (45)	18 (41)	0.11
Intensive care use‡	6 (8)	4 (14)	7 (16)	0.31

All data are no. (%) unless otherwise indicated. Subjects in more than 1 risk group are excluded. P-values are computed using Fisher exact tests (categorical variables) and Wilcoxon rank sum tests (continuous variables).

Abbreviations: IQR, interquartile range; RSV, respiratory syncytial virus; IgE, immunoglobulin E

* Detected via nasopharyngeal aspirate samples collected at index

† Defined as detection of any positive values for serum allergen-specific IgE at index for both food and aeroallergens.

‡ Defined as admission to intensive care unit and/or use of mechanical ventilation (continuous positive airway pressure and/or intubation during inpatient stay, regardless of location) at any time during the index hospitalization.

Table E4. Comparison of above and below mean taxa abundances by antibiotics administration

Genus abundance	History of antibiotics use prior to index		P-value
	No n = 562 (67%)	Yes n = 280 (33%)	
<i>Moraxella</i> at clearance			0.10
Below mean	486 (87)	254 (91)	
Above mean	76 (14)	26 (9)	
<i>Streptococcus</i> at clearance			0.26
Below mean	534 (95)	260 (93)	
Above mean	28 (5)	20 (7)	
<i>Streptococcus</i> at summer			0.95
Below mean	524 (93)	260 (93)	
Above mean	38 (7)	20 (7)	
All data are no. (%). P-values are computed using chi-squared tests.			

Table E5. Principal investigators at the 17 participating sites in MARC-35

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