METHODS

Overview of the INSPIRE study

INSPIRE is a current population-based birth cohort of previously healthy, term infants born between June and December of 2012 to 2013, designed so that the first RSV ARI during infancy could be studied. Eligible infants were enrolled mainly during a well-child visit at a participating general pediatric practice throughout the middle Tennessee region. The recruitment area encompasses urban, suburban, and rural areas. At enrollment, 1 of the parents was administered an extensive questionnaire to obtain information on the infant's sociodemographic characteristics, birth and family history, and respiratory health.

In order to capture an infant's first RSV ARI, biweekly respiratory illness surveillance (by e-mail, phone, and/or in person) was performed during the winter viral season (November to March) of each infant's first year of life. An ARI was defined as parental report of (1) 1 of the following major symptoms or diagnoses: wheezing, difficulty in breathing, or presence of a positive RSV test, or (2) any 2 of the following minor symptoms or diagnoses: fever, runny nose/congestion/snotty nose, cough, ear infection, or hoarse cry. If an infant met these prespecified criteria, an in-person visit was conducted, which included a nasal wash for viral identification and characterization of the nasopharyngeal microbiome, as well as a physical examination for assessment of the ARI severity. The ARI severity was measured with the RSS, an ordinal scale based on respiratory rate, flaring or retractions, heart rate, and wheezing that was slightly modified from other scores derived for ARIs.[E1,E2] The RSS ranges from 0 to 12, with lower scores indicating a less severe disease. The nasal wash was collected by gently flushing 5 ml of sterile saline solution into 1 of the infant's nares. After sampling, the nasal washes were aliquoted and snap frozen at -80°C until further processing.

Annual follow-up to assess the development of childhood wheezing illnesses is ongoing. Data were collected and managed using the REDCap tool hosted at Vanderbilt University.[E3] One parent of each infant provided informed consent for participation. The Institutional Review Board of Vanderbilt University approved this study. The detailed methods for INSPIRE have been previously reported.[E4]

Characterization of the nasopharyngeal microbiome

Bacterial DNA from 100 to 200 μL of nasal wash solution was extracted using a phenol:chloroform:isopropanol method as previously described.[E5-E7] Amplicons targeting the V4 region of the bacterial 16S rRNA were generated by combining 7 μL of template, 12.5 μL MyTaq HS Mix (Bioline, London, United Kingdom), 0.75 μL dimethyl sulfoxide (Sigma, St. Louis, Mo), 1 μL PCR Certified Water (Teknova, Hollister, Calif), and 2 μL of each 10 μmol/L primer before each round of PCR. During the first round of PCR, the target region was amplified with primers 515F 5′-GTGCCAGCHGCYGCGGT-3′ and 806R 5′-GGACTACNNGGGTWTCTAAT-3′, with an initial denaturing step at 95°C for 3 minutes. This was followed by 10 cycles of 95°C for 30 seconds, 50°C for 30 seconds, and 72°C for 1 second, and a final extension at 72°C for 5 minutes. During the second round of PCR, 30 cycles with the same cycling condition as before were performed to add Illumina adaptors, standard Illumina sequence primer region, a 12-bp barcode, and random nucleotides to increase sequence diversity.

Each amplified sample was run on a 1.2% agarose gel to confirm reaction success. Amplicons were cleaned and normalized with the SequalPrep Normalization Kit (Thermo Fisher Scientific, Waltham, Mass). Normalized amplicons were pooled and cleaned with 1X AMPure XP beads (Beckman Coulter, Pasadena, Calif). The pool was run on a 1.5% agarose gel and the target size band was extracted and cleaned with the NucleoSpin Gel and PCR cleanup kit (Macherey-Nagel, Bethlehem, Pa). The pool was then sequenced on an Illumina MiSeq platform with 2 × 300 bp reads.

A water negative control and 2 samples with known taxonomic composition (provided by the National Institutes of Health/National Institute of Allergy and Infectious Diseases Biological and Emerging Infections [BEI] Program) were amplified and sequenced concurrently with the samples.[E8] The 2 BEI control reagents obtained through BEI Resources included (1) Genomic DNA from Microbial Mock Community B (Staggered, Low Concentration), v5.2L, for 16S rRNA Gene Sequencing, HM-783D, and (2) Genomic DNA from Microbial Mock Community B (Even, Low Concentration), v5.1L, for 16S rRNA Gene Sequencing, HM-782D. After sequencing, only a small fraction of 16S rRNA sequences were found in the negative control and the bacterial sequences recovered had little overlap with the infant samples. Both BEI controls returned a similar taxonomic profile to their expected taxon distributions.

Data processing and statistical analyses

A mothur-based automated annotation pipeline,[E9] YAP,[E10] was used to perform initial processing of the 16S rRNA gene sequencing datasets. Low-quality sequences, chimeras, and nonbacterial sequences are discarded as part of this pipeline. Samples with ≤1000 final reads (n = 1) were discarded prior to statistical analysis. Statistical analyses were performed with the open source MGSAT package in R.[E11,E12]

The MGSAT pipeline applies several types of statistical tests, normalizations, and plotting routines to the abundance count matrices that are typically the output of annotating (meta) omics datasets and it generates a structured HTML report that, in addition to results, shows method parameters and versions of the external packages.[E11] The user has fine-grained control over types of tests, parameters, and a description of a study design through a data structure that is provided as input to the top-level routine of the package.

To compare the overall taxonomic profile according to the outcomes of interest, we applied the permutation-based ANOVA test, as implemented in the Adonis function of the R vegan package.[E13,E14] For this test, we used the Bray-Curtis dissimilarity index computed on simple proportions (on which it is equivalent to the Manhattan index) and 4000 permutations.[E15]

For the comparisons of richness and diversity between infants with or without the outcomes of interest, we also used the R vegan package.[E14] Counts were randomly rarefied to the lowest library size, and then common incidence- and abundance-based richness estimates and α diversity indices were computed (eg, Chao1, observed taxa counts, Shannon index, and inverse Simpson index). This was repeated multiple times (n = 400) and the results were averaged. Linear models were fit to test for associations between richness and diversity estimates and the outcomes of interest.

For differential abundance analyses, we used unbiased metadata-independent filtering at each taxonomy level by eliminating all taxa that were detected with a mean proportional abundance of <0.0005. The absolute counts from the removed features were aggregated into a category "other," which was taken into account when computing simple proportions during data normalization, but were otherwise discarded. For the differential abundance analyses and plotting of individual taxa, normalization to simple proportions was used with the exception of the specific methods described herein. The simple proportions were computed by dividing the observed sequence count of a given taxa in a given microbiome sample by the total count of sequences in that sample.

Differential taxon abundance by the outcomes of interest was tested with the DESeq2 package.[E16] DESeq2 is a method for differential analysis of count data that uses shrinkage estimation for dispersions and fold changes to improve the stability and interpretability of estimates. DESeq2 models raw absolute counts of each taxon with a negative binomial distribution and uses the estimated depth of sequencing of each sample to scale the (unknown) relative abundance that is the parameter of the negative binomial distribution. Compared with using either simple proportion-based normalization or rarefaction for controlling for differential sequencing depth, the DESeq2 approach provides improved sensitivity and specificity.[E17] Reported Q values are the result of a Wald test with Benjamini and Hochberg correction for multiple comparisons.[E18] Default outlier detection and replacement was used as described in the original DESeq2 publication.[E16]

Stabsel is a stability feature selection approach as implemented in R stabs package.[E19,E20] The package function stabsel implements the stability selection procedure developed by Meinshausen et al[E21] with the improved error bounds described by Shah et al.[E22] Although the full description and justification of the method is provided by the original references cited, we briefly introduce here the intuition behind the technique and the description of the specific parameters used in our analysis. The stabsel stability selection approach aims

to build the relative ranking of the predictor variables (taxa in our case) according to their importance for predicting the outcome. It does so by building multiple "base" models on random subsamples of the data. Because each of these base models only looks at a fraction of observations, each individual base model is likely to be suboptimal when compared with the full dataset. The requirement to each base model is that it must be selecting only a relatively small subset of the full list of variables (ie, provide variable selection). All variables are then ranked according to the number of times each of them was selected by the individual base models (ie, probability of selection). For example, if 100 models are built and *Lactobacillus* abundance is included as 1 of the predictors in 97 models, its probability of selection would be 0.97. We do not report the parameters of the individual base models, because the intended use of the stabsel method is a stable ranking of predictors rather than fitting of the best model. We have used the elastic net model from the R package glmnet as the base feature selection method to be wrapped by the stability protocol.[E23] In the elastic net, the correlational structure of the predictor matrix is considered when building each model. This contrasts with other variable ranking methods such as GeneSelector, which apply a univariate test to each predictor separately. As the original article of Meinshausen et al[E21] explains, the L1 penalty employed by glmnet to enforce the variable selection (model sparsity) makes the selection unstable with regard to small perturbations in the training data. As a consequence, the variable rankings obtained from a single sparse glmnet model built on a full set of observations can generalize poorly to other independently collected datasets, such as those from follow-up validation studies. The wrapping algorithm of Meinshausen et al[E21] provided the stability property to the final variable ranking that is based on the selection probability, hence leading to the name "stability selection." For our study, base models were built with a binomial family using the outcome of interest as a response and the matrix of the taxon abundance values as predictors. The mixing parameter α of the glmnet was selected based on a 15-fold cross-validation that computed deviance on the full dataset for a range of α values, repeated the procedure 400 times on randomly generated folds, averaged the deviance profile, and then selected an α that corresponded to the minimum averaged deviance. This resulted in an α of 1.0, indicating a pure lasso penalty in the model. The predictors were normalized to simple proportions within each multivariate observation followed by a variance-stabilizing transformation with inverse hyperbolic sign $\log(x+\sqrt{(x^2+1)})$. We also explored alternative normalization approaches such as centered log ratio transform and additive log ratio transform to account for a compositional nature of the dataset.[E24] We found that stabsel analysis after inverse hyperbolic sign transform generated ranking of taxa that was more concordant with the ranking from both DESeq2 and GeneSelector, and the diagnostic ordination plots exhibited smaller artificial trending effects characteristic for compositional data. Following the normalization, predictors were standardized to 0 means and unit variances before being used to build the models. With its multivariate base feature selection method, the stabsel protocol can potentially detect those correlated groups of biologically relevant features that will be missed by the univariate methods such as DESeq2 or GeneSelector. The ranking of taxa and their probability of being selected into the model were reported, as well as the probability cutoff corresponding to the per-family error rate (PFER) that is controlled by this method. Our PFER cutoff was set to 0.05, and the target number of features selected by the base classifier was set to 10, computed as $\sqrt{(0.8\times p)}$, where p is the total number of features.[E21] This resulted in a corresponding probability cutoff of 0.99, as computed by the parameter selection procedure within stabsel. In our experience with omics datasets, the PFER control in this method is indeed very conservative,[E20] and we typically look at the ranking of all features as opposed to only concentrating on features that pass the PFER cutoff. The number of subsampling replicates (B) was kept as a default stabsel value of 50, as recommended by Shah et al,[E22] to avoid violating the r-concavity assumptions at higher values of B. We have tested different values of B on different microbiome datasets and found the value of 50 to be sufficient for providing stable rankings.

The R GeneSelector package was used as a stability feature ranking method that is based on a nonparametric univariate test.[E25] In brief, the same ranking method (package function RankingWilcoxon) was applied to multiple random subsamples of the full set of observations (400 replicates, sampling 50% of observations without replacement). RankingWilcoxon ranks features in each replicate according to the test statistic from Wilcoxon rank-sum test with regard to the outcome group (eg, subsequent wheeze vs no subsequent wheeze). Consensus ranking between replicates was then found with a Monte Carlo procedure (package function AggregateMC) and the features were reported in the order of that consensus. The consensus ranking is expected to be more stable with regard to sampling error as compared to ranking obtained just once for the entire dataset. To account for different sequencing depth, the absolute abundance counts were normalized to simple proportions within each observation (with the Wilcoxon rank-sum test, the ranking would have been invariant to any additional monotonous transformation of the proportions). For each feature, we also obtained several types of the effect size, such as common language effect size and rank biserial correlation.[E26]

RESULTS

Additional analyses of the association of nasopharyngeal *Lactobacillus* and *Staphylococcus* during RSV ARI in infancy with subsequent wheeze

In addition to those shown in the main text, we performed several additional analyses to closely examine the reliability of the association for genera of interest with subsequent wheeze. Because *Lactobacillus* and *Staphylococcus* were the only genera that were statistically significant in the DESeq2 models while controlling for other important covariates (Table E2), we are describing here their sample distributions in more detail, although we performed the same review for the 8 genera ranked at the top in the initial DESeq2 test.

As described in the main text, in a GeneSelector stability ranking procedure that wraps a nonparametric Wilcoxon rank-sum test, *Lactobacillus* was ranked first among all genera (rank-biserial correlation effect size of subsequent wheeze relative to the group without subsequent wheeze = −0.23), while *Staphylococcus* was ranked 32nd (rank-biserial correlation effect size of subsequent wheeze relative to the group without subsequent wheeze = 0.01). The effect size for *Staphylococcus* in the Wilcoxon rank-sum test is opposite to the effect size in the $\log_2$-fold change from the initial DESeq2 test, which was -1.47. Examining the relative abundance of *Staphylococcus* expressed as simple proportions, we found that while the sample mean is actually lower in the group with subsequent wheeze when compared with that without subsequent wheeze (0.007 vs 0.04), the corresponding sample median is higher (0.0006 vs 0.0004), with non-0 counts in 98% versus 95% of samples in each group, respectively. On the contrary, the statistics and rankings for *Lactobacillus* are consistent across all tests. For the simple proportions abundance of *Lactobacillus* across those with and without subsequent wheeze, the sample means were 0.00002 versus 0.002, and the non-0 counts were 21% versus 41%, respectively.

Fig E2 reveals that there was a subset of 9 patients with a much higher relative abundance of *Lactobacillus* than the rest of the cohort. This subset is called "LactoHigh" in the following discussion. Each relative abundance value on this plot is computed as a simple proportion of a given taxa within a given microbiome sample. The lowest *Lactobacillus* proportion observed in the LactoHigh group is ~3.4 times higher than the highest proportion observed in the rest of the dataset, reflecting a visible separation of this group from the rest of the microbiome samples as shown in the figure. A similar relation held for the absolute count. Both relative abundance value and the absolute count are shown as labels attached to each patient's sample on the scatter plot. The key observation is that none of the patients from the LactoHigh subset developed subsequent wheeze (P = .0002 in a

G test of independence between LactoHigh cluster membership and subsequent wheeze status).

Fig E2 also shows that the 4 patients with the highest levels of *Staphylococcus* did not develop subsequent wheeze (1 of those patients also belonged to the LactoHigh group). The overall small number of patients with high *Staphylococcus* abundance and a lack of visible separation from the lower-abundance observations make it difficult to interpret. In one such patient, the relative *Staphylococcus* abundance was 99.5% of all sequences in the microbiome sample, likely indicating a *Staphylococcus* bacterial superinfection.

Interestingly, the majority of observations in the LactoHigh group (6 of 9) came from infants delivered vaginally as opposed to via a cesarean section (marked by the shape of the dots on Fig E2). However, this ratio was not statistically significantly different from the ratio observed over the entire cohort in the *G* test of independence ($P = .8$). Observations for those patients who were exposed to antibiotics after birth are marked with rings on Fig E2. There was no significant association between exposure to antibiotics after birth with the LactoHigh group membership in the *G* test of independence ($P = .6$).

We found very similar results to those described for *Lactobacillus* and *Staphylococcus* after rarefication to a lower library size (Fig E3). We rarefied all samples to the common sequencing depth ("sampling effort"). The target common depth of 6077 sequences was selected as a minimum total read count observed among those samples that had the original non-0 count of *Lactobacillus*. The justification for the described selection of the rarefication depth is that the 0 initial *Lactobacillus* counts do not change after the rarefication regardless of the rarefication depth (ie, they remain 0). Four such samples had sequence counts below 6077 and no identified *Lactobacillus*.

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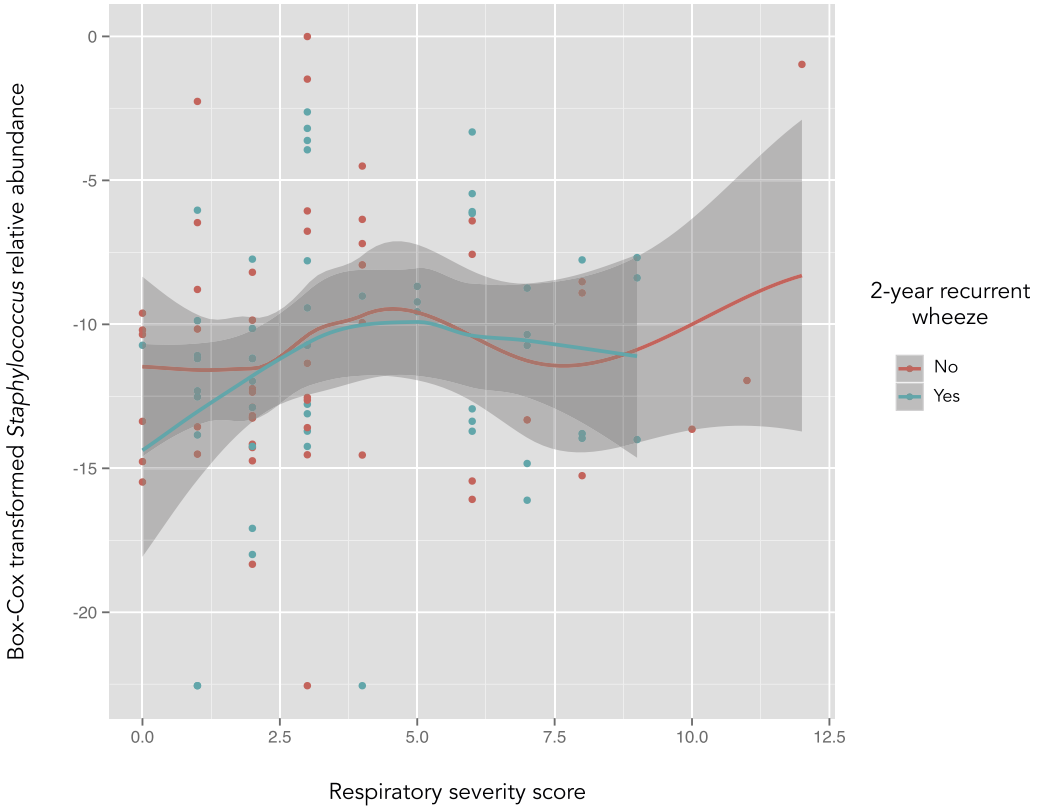

FIG E1. Box-Cox transformed nasopharyngeal relative abundance of *Staphylococcus* in infants with RSV ARI with *(blue line)* and without *(red line)* 2-year subsequent wheeze, plotted by RSS. Lines are local regression (LOESS) smoothed curves and gray areas are the 95% CIs. For the y-axis, values closer to 0 indicate a higher abundance. Not all individual data points are shown; a single data point is displayed for infants who had the same RSS and *Staphylococcus* abundance.

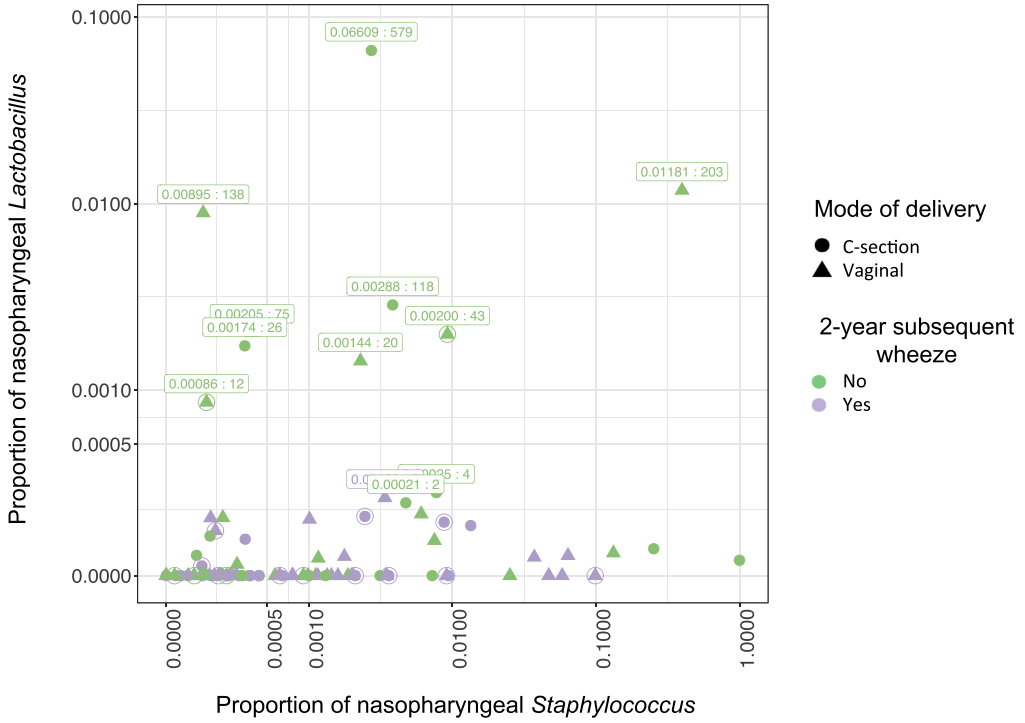

FIG E2. Scatter plot of the relative abundance of *Lactobacillus* (y-axis) and *Staphylococcus* (x-axis) using an unrarefied dataset. Relative abundance is computed as a simple proportion of a given genus in a given microbiome sample. The biexponential plot axes are used, with values from 0 to 0.0005 shown on an approximately linear scale and above that on a logarithmic scale. The point color and point shape denote the 2-year subsequent wheeze outcome and mode of delivery, respectively. Open rings around the points indicate exposure to antibiotics after birth. Labels above each point show simple proportion and absolute sequence count of *Lactobacillus*, separated by a colon. To avoid overplotting, the point labels in the range below 0.0005 of the *Lactobacillus* relative abundance are shown only for the top 3 highest points.

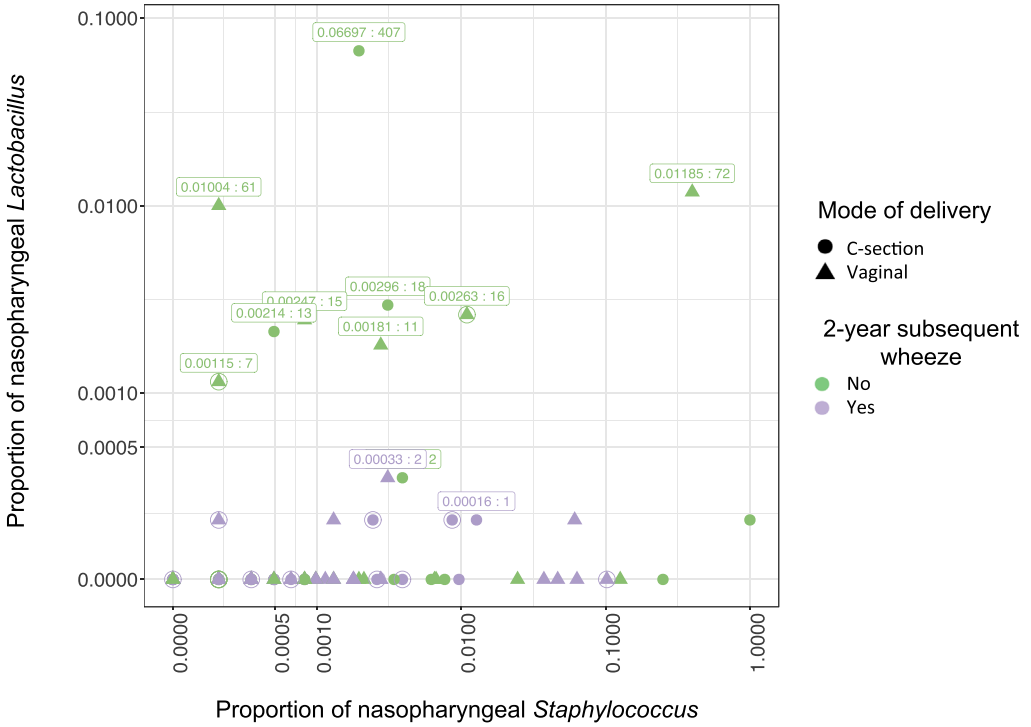

FIG E3. Scatter plot of the relative abundance of *Lactobacillus* (y-axis) and *Staphylococcus* (x-axis) using a rarefied dataset. Relative abundance is computed as a simple proportion of a given genus in a given microbiome sample. The biexponential plot axes are used, with values from 0 to 0.0005 shown on an approximately linear scale and above that on a logarithmic scale. The point color and point shape denote the 2-year subsequent wheeze outcome and mode of delivery, respectively. Open rings around the points indicate exposure to antibiotics after birth. Labels above each point show simple proportion and absolute sequence count of *Lactobacillus*, separated by a colon. To avoid overplotting, the point labels in the range below 0.0005 of the *Lactobacillus* relative abundance are shown only for the top 3 highest points.

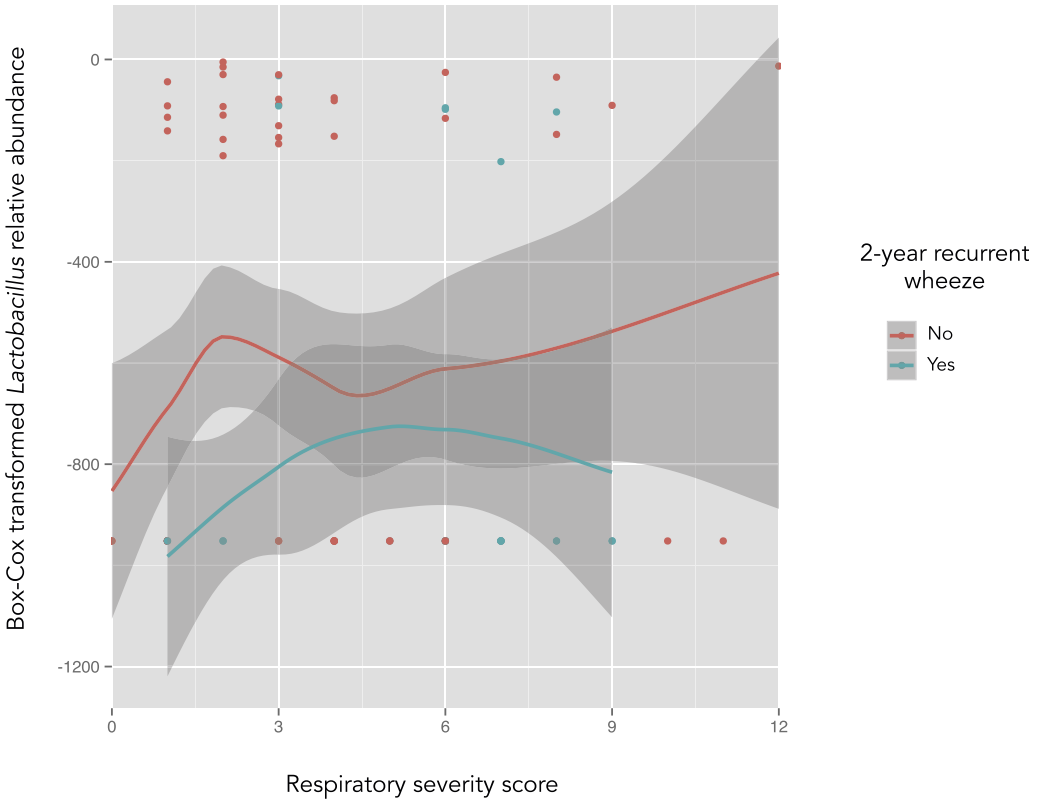

FIG E4. Box-Cox transformed nasopharyngeal relative abundance of *Lactobacillus* in infants with RSV ARI with *(blue line)* and without *(red line)* 2-year recurrent wheeze, plotted by RSS. Lines are local regression (LOESS) smoothed curves and gray areas are the 95% CIs. For the y-axis, values closer to 0 indicate a higher abundance. Not all individual data points are shown; a single data point is displayed for infants who had the same RSS and *Lactobacillus* abundance.

TABLE E1. Baseline characteristics of infants with RSV ARI included in this study (n = 118) by childhood wheezing illnesses

	Subsequent wheeze*		Recurrent wheeze*	
	No (n = 67)	Yes (n = 46)	No (n = 76)	Yes (n = 36)
Age (wk)	21.4 (13.3-27.6)	22.9 (12.1-27.1)	21.2 (13.0-27.4)	22.9 (12.4-27.4)
Female sex	33 (49.3)	17 (37.0)	38 (50.0)	12 (33.3)
Race or ethnicity				
Black non-Hispanic	11 (16.4)	7 (15.2)	11 (14.5)	6 (16.7)
White non-Hispanic	43 (64.2)	30 (65.2)	50 (65.8)	23 (63.9)
Hispanic	5 (7.5)	5 (10.9)	6 (7.9)	4 (11.1)
Other†	8 (11.9)	4 (8.7)	9 (11.8)	3 (8.3)
Gestational age (wk)	39 (38-40)	39 (39-40)	39 (38-40)	39 (39-40)
Birth weight (g)	3377 (2894-3859)	3377 (3260-3859)	3377 (2894-3859)	3420 (2894-3859)
Birth by cesarean section	24 (35.8)	17 (37.0)	26 (34.2)	15 (41.7)
Exposure to antibiotics *in utero* or after birth	31 (46.3)	30 (65.2)	36 (47.4)	24 (66.7)
Any breastfeeding	49 (73.1)	34 (73.9)	55 (72.4)	27 (75.0)
Maternal smoking at enrollment	16 (23.9)	8 (17.4)	18 (23.7)	6 (16.7)
Maternal asthma	10 (14.9)	9 (19.6)	10 (13.2)	8 (22.2)
Respiratory severity score	3 (2-4)	3 (2-6)	3 (2-4)	4 (2-7)‡
Insurance type				
Medicaid	31 (46.3)	23 (50.0)	35 (46.1)	18 (50.0)
Private	34 (50.8)	22 (47.8)	39 (51.3)	17 (47.2)
Other	2 (3.0)	1 (2.2)	2 (2.6)	1 (2.8)

Data are presented as median (interquartile range) for continuous variables or n (%) for binary variables.
Percentage calculated for children with complete data.
*See main text for the definitions of the outcomes.
†Other includes mixed race and unknown.
‡$P < .05$ for comparison between groups using Mann-Whitney *U*-test or Fisher exact test, as appropriate.

TABLE E2. Mean relative abundance and SD of nasopharyngeal bacterial genera in infants with RSV ARI according to 2-year subsequent wheeze

Genus	2-year subsequent wheeze	Mean relative abundance	SD
Lactobacillus	No	0.001942758	0.009399022
Lactobacillus	Yes	2.47E-05	5.51E-05
Staphylococcus	No	0.036712113	0.152407114
Staphylococcus	Yes	0.006885463	0.018418984
Enterococcus	No	0.00012783	0.000408534
Enterococcus	Yes	0.001540195	0.007631301
Porphyromonas	No	0.001708625	0.006103841
Porphyromonas	Yes	0.00355293	0.010752724
Pseudomonas	No	0.000540211	0.00154762
Pseudomonas	Yes	0.009317244	0.050410306
Unclassified *Neisseriaceae*	No	0.003615014	0.010331853
Unclassified *Neisseriaceae*	Yes	0.011664653	0.057269642
Rothia	No	0.002832255	0.007617005
Rothia	Yes	0.001668454	0.0027666
Neisseria	No	0.012270897	0.056884128
Neisseria	Yes	0.004710482	0.021636135
Rhodanobacter	No	0.009296451	0.020571983
Rhodanobacter	Yes	0.017646174	0.046968479
Methylobacterium	No	0.000202755	0.000910573
Methylobacterium	Yes	0.00117315	0.008429472
Dolosigranulum	No	0.039000725	0.087160534
Dolosigranulum	Yes	0.050445553	0.080538676
Acinetobacter	No	0.001620769	0.006587765
Acinetobacter	Yes	0.005935488	0.025037033
Streptococcus	No	0.203472708	0.251240675
Streptococcus	Yes	0.202740851	0.203957383
Corynebacterium	No	0.085921783	0.172343488
Corynebacterium	Yes	0.109429383	0.139440755
Moraxella	No	0.372198928	0.371905573
Moraxella	Yes	0.324286227	0.297850868
Unclassified *Prevotellaceae*	No	0.00488979	0.011894647
Unclassified *Prevotellaceae*	Yes	0.005652231	0.012985728
Prevotella	No	0.004060933	0.011409723
Prevotella	Yes	0.008375445	0.02979668
Haemophilus	No	0.146703009	0.251652889
Haemophilus	Yes	0.158412998	0.261873229
Granulicatella	No	0.001463183	0.003981424
Granulicatella	Yes	0.001162611	0.003180168
Unclassified *Actinomycetales*	No	0.000524594	0.001072039
Unclassified *Actinomycetales*	Yes	0.000487652	0.000625912

See main text for the definitions of the outcome.
The top 20 ranked genera in the stabsel stability selection model are shown; all other genera are not shown in this table.
Genera are ordered from highest to lowest ranked.