Bacterial microbiota of the upper respiratory tract and childhood asthma

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Background: Patients with asthma and healthy controls differ in bacterial colonization of the respiratory tract. The upper airways have been shown to reflect colonization of the lower airways, the actual site of inflammation in asthma, which is hardly accessible in population studies.
Objective: We sought to characterize the bacterial communities at 2 sites of the upper respiratory tract obtained from children from a rural area and to relate these to asthma.
Methods: The microbiota of 327 throat and 68 nasal samples from school-age farm and nonfarm children were analyzed by 454-pyrosequencing of the bacterial 16S ribosomal RNA gene.
Results: Alterations in nasal microbiota but not of throat microbiota were associated with asthma. Children with asthma had lower α- and β-diversity of the nasal microbiota as compared with healthy control children. Furthermore, asthma presence was positively associated with a specific operational taxonomic unit from the genus *Moraxella* in children not exposed to farming, whereas in farm children *Moraxella* colonization was unrelated to asthma. In nonfarm children, *Moraxella* colonization explained the association between bacterial diversity and asthma to a large extent.
Conclusions: Asthma was mainly associated with an altered nasal microbiota characterized by lower diversity and *Moraxella* abundance. Children living on farms might not be susceptible to the disadvantageous effect of *Moraxella*. Prospective studies may clarify whether *Moraxella* outgrowth is a cause or a consequence of loss in diversity. (J Allergy Clin Immunol 2017;139:826-34.)

Key words: *Asthma, childhood, upper respiratory tract, throat, nose, microbiota, 16S rRNA gene*

Human microbes are key players in host immune responses and metabolism, as well as in the development of chronic disease.[1] Perturbation in the structure of the complex microbial communities, also known as dysbiosis, impacts on health and may impair immune system development.[2] There are 3 variants of dysbiosis: (1) loss of beneficial microbial organisms, (2) expansion of pathobionts or potentially harmful microorganisms, and (3) loss of overall microbial diversity.[2] The bacterial microbiota of the lower airways is a prominent example that has been implicated in the pathogenesis of asthma and airway inflammation.[3-6] It has also been related to asthma severity and therapy response.[7,8]

However, assessment of microbial communities is difficult in the lower respiratory tract, and accurate sampling methods such as bronchial brushings or lavage cannot be applied in population studies for practical and ethical reasons. Alternatively, easily accessible sampling sites such as the oropharynx are likely to reflect the lower bacterial airway microbiota sufficiently.[4,9]

In the context of the united airways hypothesis, it is conceivable that upper and lower airways share physiologic and pathologic response patterns.[10] Symptoms at either site might be manifestations of the same underlying inflammatory process.

The development of childhood wheeze has been predicted in different studies from early life colonization of the upper respiratory tract with potential pathogens such as *Haemophilus influenzae*, *Moraxella catarrhalis*, and *Streptococcus pneumoniae*[11] as well as the genus *Streptococcus*.[12] The contribution of the above-mentioned forms of dysbiosis to initiation and further persistence of childhood asthma is largely unclear.

The aim of the present study was to assess diversity and composition of the bacterial communities residing on 2 sites of the upper respiratory tract (throat or nose) in a rural population of

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Abbreviations used
GABRIELA: Multidisciplinary Study to Identify the Genetic and Environmental Causes of Asthma in the European Community–Advanced Studies
OTU: Operational taxonomic unit
OR: Odds ratio
qPCR: Quantitative PCR
16S rRNA: 16S ribosomal RNA

school-age farm or nonfarm children and relate those to childhood asthma.

METHODS

Study design and subjects

The current study population is part of the cross-sectional Multidisciplinary Study to Identify the Genetic and Environmental Causes of Asthma in the European Community–Advanced Studies (GABRIELA), which involved 6- to 12-year-old children from 5 rural areas of Europe.[13] The GABRIELA study has been approved by the ethics committees of the participating universities and the regional data protection authorities.

For the analysis of bacterial communities, a stratified random sample of 333 throat samples was drawn from the Bavarian arm of the GABRIELA study population including farm and nonfarm children. *Farm children* were defined as children living on a farm run by the family. *Nonfarm children* were defined as children not living on a farm and not exposed to farm environments or cow's milk directly bought from a farm.[14] Furthermore, 75 nasal samples were selected from the population available for the analysis of throat swabs (see Fig E1 in this article's Online Repository at www.jacionline.org). *Asthma* was defined as either parent-reported wheeze during the last 12 months, a positive answer to the question "Did your child ever use an asthma spray?" or a reported doctor's diagnosis of asthma at least once or of wheezy bronchitis more than once."[13,14] From the fieldwork, we used the following information: the month at taking the swabs, the age of children at taking swabs, the report of a cold or a runny nose (nasal swabs), and the use of antibiotics in the last 4 weeks (available only for throat swabs).

Sample collection and processing

Nasal and throat samples were collected by trained field workers using sterile dry cotton-headed swabs (MASTASWAB MD 559, MAST Diagnostica GmbH, Reinfeld, Germany). Material from the nose was taken by 5 circular rubbings about 1 cm from the nare. Secretion and other material were collected from the soft and moving part of the nose. Throat samples were obtained by gently sweeping tonsils and velum without contacting the buccal mucosa or the gingiva (Fig 1, *A*). The nasal samples were collected between May and July 2007 and the throat samples during the same months in 2008 (Fig 1, *B*).

After sampling, the swab was immediately placed back in the collection tube and stored within 24 hours at −80°C (nasal swab) or at −20°C (throat swab).

DNA was extracted from the swabs with the QIAmp DNA Mini Kit (Qiagen Ltd, Manchester, United Kingdom) using an adapted protocol.[15] In the case of nasal samples, 0.001 g acid-washed glass beads were added to the extraction tube and vortexed for 20 seconds at the beginning of the extraction. DNA was stored at −20°C until further processing. Negative extraction controls were performed for both extraction protocols and evaluated by gel analysis after PCR amplification.

Determination of bacterial load by quantitative PCR (qPCR) was performed using the Applied Biosystems ViiA 7 Real Time PCR System (Thermo Fisher Scientific, Warrington, United Kingdom). The V4 region of the bacterial 16S ribosomal RNA (16S rRNA) gene was amplified with the forward primer 520F and the reverse primer 802R (see Table E1 in this article's Online Repository at www.jacionline.org).[16]

For pyrosequencing of the bacterial 16S rRNA gene, the V3 to V5 region of the gene was amplified with the forward primer 357F and the reverse primer 926R (tagged with barcodes[17]) (see Table E1).[18] For the throat swab samples, quadruplicate 25 μL PCRs were set up and processed as previously described.[15] For amplification of the nasal DNA, Platinum SuperMix High Fidelity reagents (Life Technologies, Paisley, United Kingdom) was used (see this article's Online Repository at www.jacionline.org). After purification with Agencourt AMPure XP beads (Beckman Coulter Ltd, High Wycombe, United Kingdom), concentrations of the 16S rRNA gene fragments were determined using Quant-iT PicoGreen (Life Technologies).

Pyrosequencing and data processing

Unidirectional sequencing of the 16S rRNA gene fragments starting from the reverse primer 926R was performed using the 454-GS FLX Titanium (Roche Diagnostics, Burgess Hill, United Kingdom) protocol. Raw data were processed by the 454 data processing pipeline v2.8 for shotgun reads. Denoising and removal of chimeric reads was achieved by Ampliconnoise,[19] resulting in about 400-bp long fragments. Subsequently, reads from all throat and nasal samples were concatenated and clustered in operational taxonomic units (OTUs) with at least 97% sequence similarity by UCLUST[20] using the *de novo* picking method in QIIME[21] version 1.7. For taxonomy assignment, OTUs were aligned against SILVA database version111 NR. OTUs classified as *Chloroplasts* were excluded from further analysis. Furthermore, potential contaminants were eliminated as determined by OTU distribution relative to biomass.[22] In detail, OTUs with relative abundances inversely and significantly correlated (Spearman correlation ≤ -0.4) to bacterial load were removed.

Finally, singletons were removed and samples with less than 1000 reads in either throat ($n = 6$) or nasal samples ($n = 7$) were excluded from further analysis, resulting in 327 throat and 68 nasal samples (see Fig E1).

Data analysis

For the analysis of bacterial load, the values of gene copy numbers were $\log_2$-transformed because of the skewed distribution. The α-diversity indices such as species richness and Shannon index were calculated with the R package vegan.[23] Richness was $\log_{10}$-transformed for regression analysis. To adjust for varying read counts between individuals, all samples were rarefied to the lowest individual sequence count ($n = 1008$) for the analysis of α-diversity; this process was performed in 1000 replicates per sample and subsequently averaged. Unweighted UniFrac was used as distance measurement for β-diversity using R package GUnifrac.[24,25] Wilcoxon tests were used to test for group differences in bacterial load or diversity.

Main taxa were defined as taxa being represented by at least 1% of all reads per sample. Those taxa were compared between groups for relative abundance, whereas rare taxa were analyzed only as an individual variable representing all remaining taxa. Results are shown for all OTUs as well as classified by phyla or genera; unclassified phyla or genera were included in sensitivity analyses. Logistic regression models were used to test for differences in richness and relative abundances between children with asthma and healthy controls adjusted for farming, the main environmental exposure. A product term was used to test for interaction. A P value of .05 was considered statistically significant. Sensitivity analyses focusing on children with adequate coverage of OTUs, that is, more than the median of reads, were done to analyze associations of diversity or relative abundances with disease status.

Correction for multiple comparisons was achieved by the Bonferroni method considering the numbers of main taxa at the respective taxonomic level. Significant P values after adjustment were indicated.

Survey-weighted variants of the statistical procedures (R package survey; https://cran.r-project.org/web/packages/survey/index.html) were applied to account for the stratified sampling design of the GABRIELA study.[13] All statistical analyses were performed with R 3.02[26] and the phyloseq package[27] if not further specified.

RESULTS

Study population

The analyzed subpopulations of 327 children with throat samples and 68 children with nasal samples did not differ from

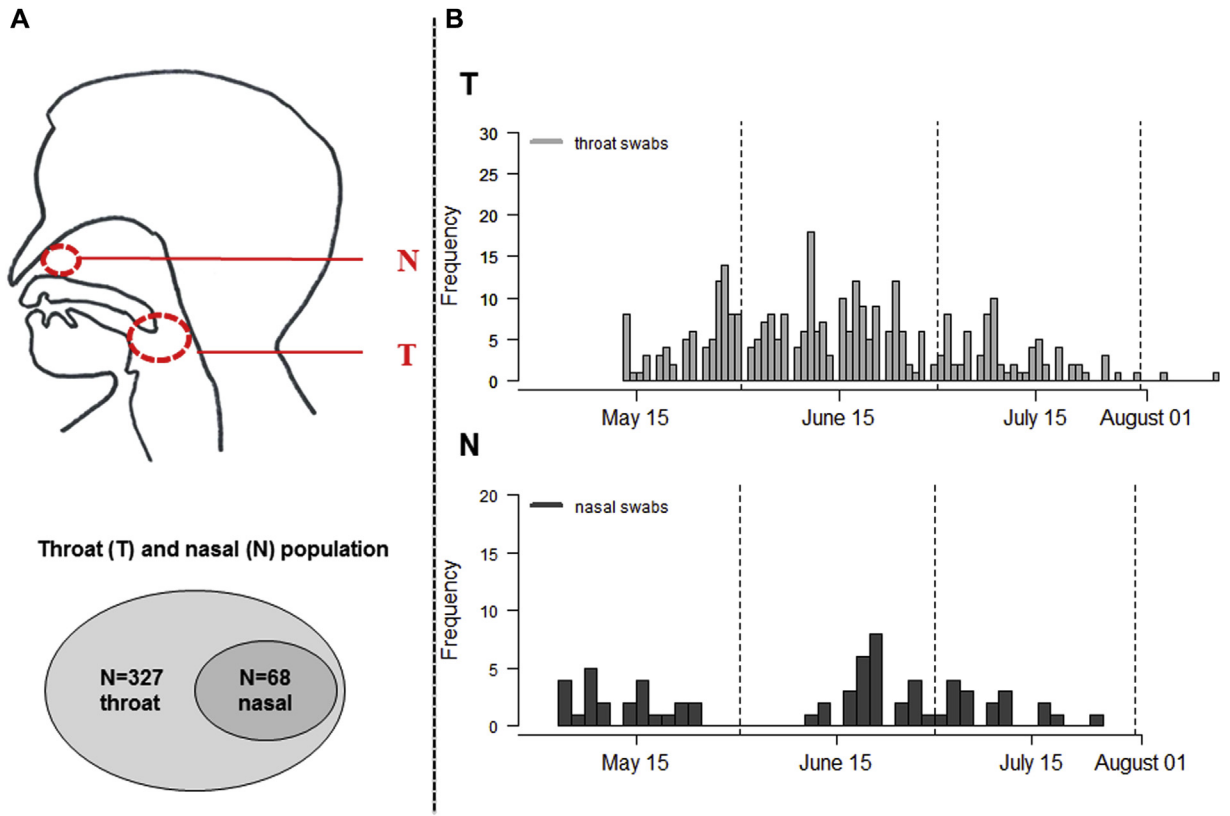

FIG 1. Sampling sites and timing. **A,** Anatomic location of the throat (T) and nasal (N) sampling site. **B,** Distribution of sampling time points of the nasal and throat samples. Methodical differences in sample processing preclude direct comparison of throat and nasal samples.

TABLE I. Description of the different study populations

Characteristic	GABRIELA population (Bavaria only) (N = 1759)	Throat population (N = 327)	Nasal population (N = 68)
Farming (%)	21.2	21.4	23.9
Any asthma (%)	18.1	16.2	17.4
Doctor diagnosis of hay fever (%)	7.9	6.5	3.3
Atopic sensitization ≥0.70 IU/mL (%)	37.8	39.3	38.2
Number of positive IgE tests (mean)	1.37	1.37	1.26
Family history of atopy (%)	51.9	50.0	50.3
At least 2 siblings (%)	39.5	33.7	36.5
Sex: female (%)	46.2	41.9	41.1
Smoking in pregnancy (%)	9.2	9.2	2.9
High education of parents (%)	21.6	23.7	39.3
Effect of farming on asthma (OR)	0.68	0.70	0.66

All percentages and means are given for the representative sample after weighting back to the recruitment population (Fig E1).

the Bavarian GABRIELA study population with respect to asthma frequency and general demographic variables except for the 2 interrelated variables parental education and smoking in pregnancy (Table I). Within the throat and nasal population, there was no difference between cases and controls for age, sampling season, and health information as shown by Table E2 in this article's Online Repository at www.jacionline.org.

Pyrosequencing performance and bacterial community composition

Because of methodological differences between sample preparation of throat and nasal samples, direct comparison of the respective results is not possible. After processing, 1,660,035 reads were obtained from all 327 throat samples and 578,551 reads from all 68 nasal samples (see Fig E2 in this article's Online Repository at www.jacionline.org). Multiple rarefaction curves confirmed adequate sequence depth at 1008 reads for throat samples (Fig 2). Conversely, the rarefaction pattern of richness among nasal samples suggested an incomplete coverage in individuals with read counts below the median of 8120 reads (Fig 2, *A*), which was explained by the previous removal of contaminant sequences (data not shown). However, the rarefaction curves of the Shannon index leveled off. As this index stresses OTUs with intermediate frequencies, the plateauing demonstrates acceptable coverage of OTUs for both throat and nasal samples (Fig 2, *B*).

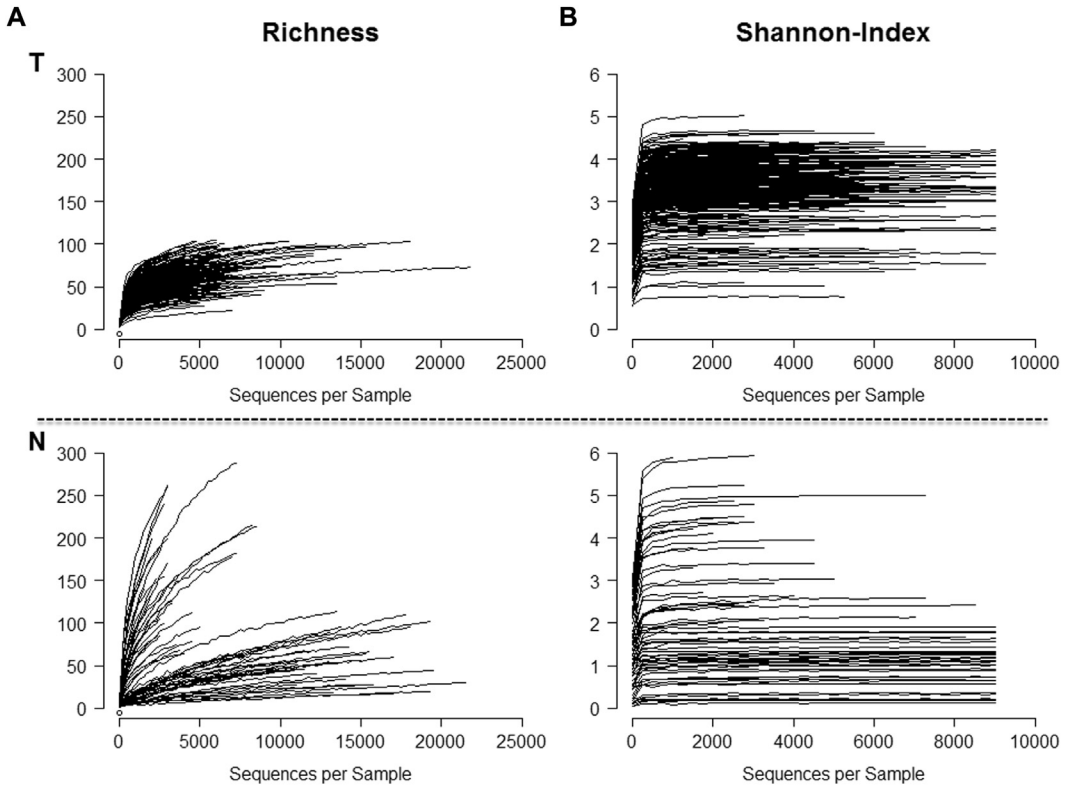

FIG 2. Multiple rarefaction curves for richness **(A)** and Shannon index **(B)** for all 327 throat (T) and 68 nasal (N) samples, respectively. Shannon index is shown up to a rarefaction of only 9100 sequences per sample.

On the phylum level, 0.011% of all reads were unclassified in throat and 0.008% in nasal samples, respectively. The throat microbiota was dominated by 42.4% *Firmicutes*, 29.3% *Bacteroidetes*, 17.4% *Proteobacteria*, 6.7% *Fusobacteria*, and 4.0% *Actinobacteria* (Fig 3, *A*). A similar composition of throat microbiota was found in the subset of children with nasal samples (see Fig E3 in this article's Online Repository at www.jacionline.org.). In contrast, the composition of the nasal microbiota was dominated by 42.6% *Actinobacteria*, 29.9% *Proteobacteria*, and 26.4% *Firmicutes* (Fig 3, *A*). *Bacteroidetes* were present at only 0.8% and *Fusobacteria* at 0.2%.

At the genus level, 0.14% were unclassified reads in the throat samples and 0.48% in the nasal samples, respectively. Throat microbiota mainly contained *Prevotella* (26.3%), *Streptococcus* (23.2%), and *Veillonella* (13.8%), whereas nasal samples were characterized by *Corynebacterium* (41.4%), *Moraxella* (23.5%), *Staphylococcus* (11.9%), and *Streptococcus* (10.1%) (Fig 3, *B*). Among the main genera (≥1% relative abundance), only *Streptococcus* and *Haemophilus* were common to both sampling sites. Relative abundance of *Streptococcus* and *Haemophilus* was increased in children with a cold or a runny nose (see Fig E4 in this article's Online Repository at www.jacionline.org).

Associations of the bacterial microbiota from the upper respiratory tract with asthma

Bacterial load and diversity. There was no association of bacterial load with asthma status or farming exposure, neither in throat nor in nasal samples (see Fig E5 in this article's Online Repository at www.jacionline.org). Unweighted UniFrac distance matrices (β-diversity parameter) were used to test whether bacterial communities from children with asthma and control children differed with respect to their phylogenetic trees. For the nasal microbiota, a greater phylogenetic similarity was found within the cases as compared with controls ($P = .014$; Fig 4, *A*, lower panel). For the throat microbiota, there was no difference between cases and controls.

Also, the α-diversity of the nasal microbiota varied with asthma status; children with asthma had a lower bacterial richness ($P = .052$; Fig 4, *B*). This was equally seen in farm and nonfarm children (see Table E3 in this article's Online Repository at www.jacionline.org). Sensitivity analyses focusing on children with adequate coverage of OTUs, that is, more than the median of reads, confirmed the association of richness of nasal microbiota with asthma (see Fig E6 in this article's Online Repository at www.jacionline.org). A similar trend was observed for the Shannon index (Fig 4, *C*). In contrast, α-diversity of the throat microbiota did not vary with asthma status (Fig 4, upper panel).

Relative abundance of taxa on different taxonomic levels. The relative abundance test was restricted to the main taxa (phyla, genera, and OTUs) represented by at least 1% of reads on average. In the throat microbiota, no association of the relative abundance of main phyla, genera, and OTUs with asthma presence was detected (see Tables E4 and E5 in this article's Online Repository at www.jacionline.org). In contrast, the nasal microbiota differed substantially, displaying a higher relative abundance of the phylum *Proteobacteria* in children with asthma (adjusted odds ratio [OR] and 95% CI for z-scaled relative abundance, 2.44 [1.07-5.59]; $P = .0384$). Among the main genera, a significant positive association of asthma with

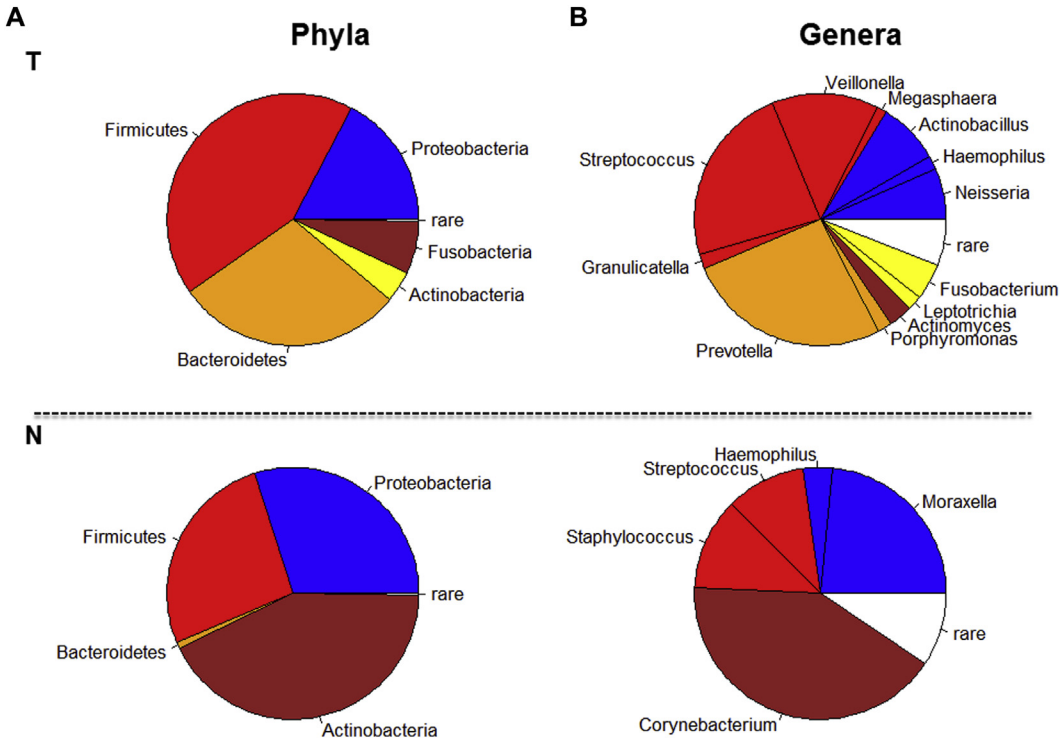

FIG 3. Taxonomic composition of the microbiota from throat and nose. Survey-weighted relative abundance of phyla **(A)** and genera **(B)** for throat (T) and nasal (N) samples. Genera belonging to the same phylum are represented by the color of the respective phylum. Phyla and genera representing less than 0.5% of all reads for phyla and less than 1% for genera in the combined throat and nasal sample, respectively, are grouped in "rare."

Moraxella (phylum *Proteobacteria*) was observed (adjusted OR and 95% CI, 3.78 [2.02-7.05]; $P = 9.76 \times 10^{-5}$; see Table E4). All the above associations were not affected by exclusion or inclusion of unclassified phyla and genera (data not shown) or selection of samples with at least 4635 reads (see Table E6 in this article's Online Repository at www.jacionline.org).

A highly significant association was observed for OTU 1462 (OR, 3.73; 95% CI, 2.02-6.91; $P = 9.01 \times 10^{-5}$; see Table E5). The association remained significant after adjustment for multiple testing. The reference sequence for this OTU mapped to the 16S rRNA gene fragment of both *Moraxella catarrhalis* and *M nonliquefaciens* with 100% identity. In the nasal samples, the genus *Moraxella* was predominantly formed by OTU 1462 and the far less frequent OTU 867; other *Moraxella* OTUs were rather uncommon.

The above-described association of *Proteobacteria* with asthma was almost completely explained by the genus *Moraxella*, and again by OTU 1462. A sensitivity analysis stratified for farming revealed that the association between this OTU and asthma was restricted to nonfarm children ($OR_{nonfarm}$, 7.88; 95% CI, 2.75-22.53; $P = .0006$; OR_{farm}, 0.94; 95% CI, 0.34-2.63; $P = .904$; P for interaction = .006; Fig 5). Conversely, the association of asthma and this *Moraxella* OTU did not vary with age or month of sample collection (data not shown).

OTU 1462 was also explored in the throat samples. This OTU was present in 22% of the children with a mean relative abundance of 0.12%, and showed a trend for a positive association with asthma (adjusted OR, 1.47; 95% CI, 0.98-2.21; $P = .0621$).

Moraxella OTU 1462 and richness of the nasal microbiota in relation to asthma. Richness was inversely correlated to relative abundance of OTU 1462 (farm children: $r = -0.37$, $P = .03$; nonfarm children: $r = -0.35$, $P = .05$; Fig 6). The mutual relations of the effects of richness and *Moraxella* OTU 1462 on asthma were compared in nonfarm children, where asthma was associated with this OTU (Table II). When including both variables in the same model, the magnitude of the association between OTU 1462 and asthma hardly changed; rather, it explained 60% of the effect of richness on asthma (Table II).

All reported associations were not confounded by age, sampling season, a cold or a runny nose, or atopy status (atopic sensitization or hay fever).

DISCUSSION

The key finding of this study was an association of asthma with reduced α- and β-diversity of the nasal microbiota and the relative abundance of an OTU belonging to the genus *Moraxella*. The association of asthma and *Moraxella*, however, was restricted to children not living on farms. In contrast to the nasal samples, the throat microbiota in terms of diversity and main taxa was not related to asthma.

Substantial differences between nasal and throat microbiota have previously been observed. Our findings are well in concordance with previous studies with respect to the bacterial composition at these anatomic regions.[4,15,28-35] However, a direct comparison between nasal and throat samples is not possible in

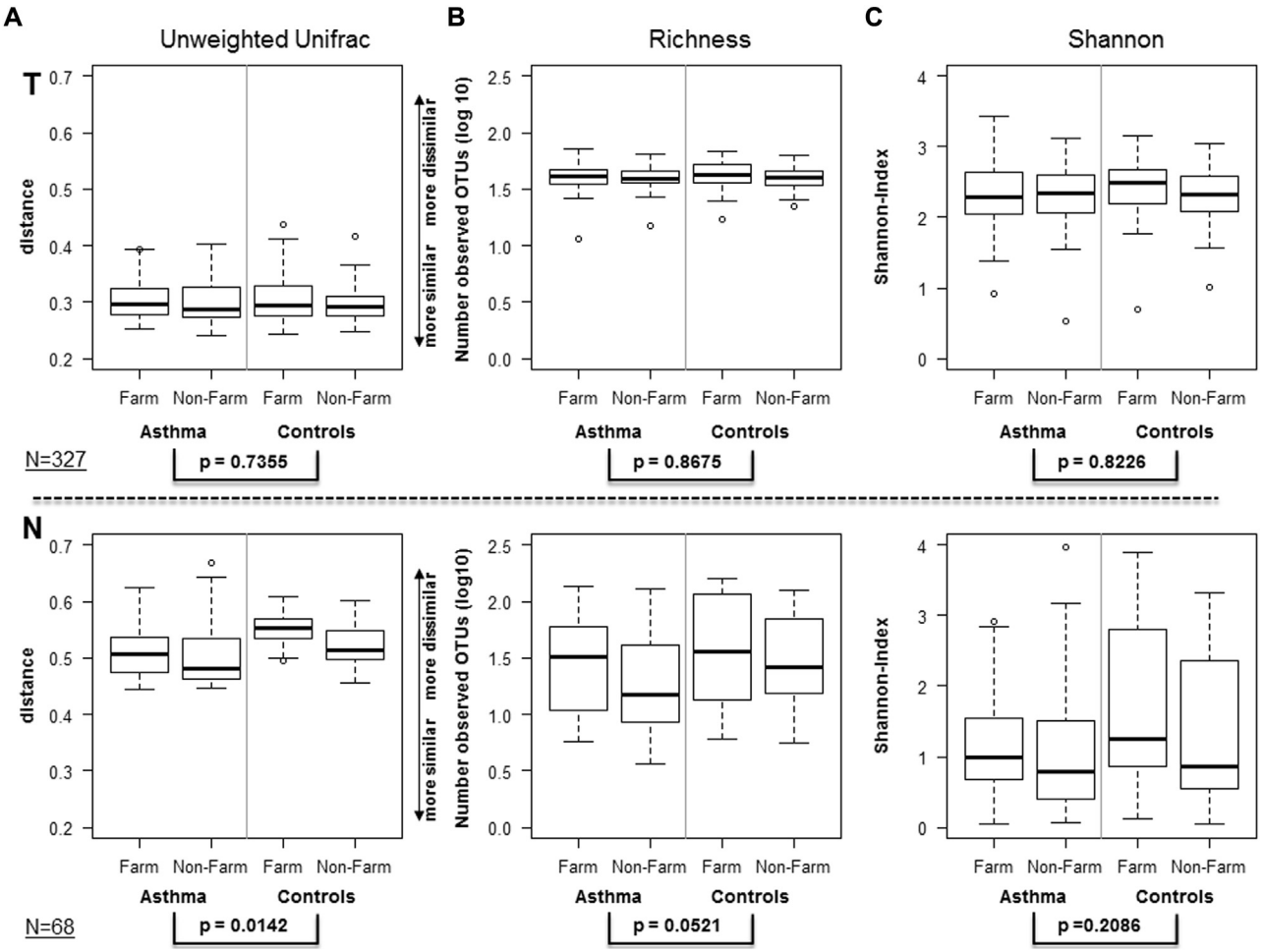

FIG 4. Bacterial diversity in throat (T) and nasal (N) samples from children with asthma and controls. Shown are β-diversity as unweighted UniFrac **(A)**, and α-diversity as richness **(B)** and Shannon index **(C)** for throat samples and nasal samples. The *P* values for Wilcoxon test comparing asthma versus control group are shown.

our study because the different types of specimens were processed from different laboratories and with several differences in extraction and PCR protocols. However, samples were taken from the same individuals, contamination during DNA processing was successfully eliminated from the data, and the most important discrepancies between nasal and throat samples are related to associations within the given sample type. Moreover, we detected associations for asthma with microbiota from exactly the sampling site for which fewer specimens were available and consequently the statistical power was lower (nose).

In contrast, the large sample size of 327 throat swabs excludes any associations of the throat microbiota with asthma with rather high probability and renders this negative result highly informative. Actually this finding contradicts other studies with smaller sample sizes[15,32] even when taking slightly different outcome definitions, other study designs, and sequencing protocols into consideration. Possibly, the throat microbiota is tightly regulated by the surrounding immune tissue, which functions as a strong barrier against environmental microorganisms.[36]

Conversely, the nasal microbiota may underlie less stringent regulatory processes, resulting in a more diverse microbiota susceptible to environmental influences. This notion is supported by the pronounced difference in the α-diversity between farm and nonfarm children and the strong interindividual variation in diversity measures in general (Fig 4). The relatively large interquartile ranges of these measures also reduce the power to detect differences between groups, which retrospectively stresses the detected association with asthma status.

In this study, the variation between individuals might have been underestimated because half of the nasal samples did not level off in the rarefaction curves of species richness. Despite this shortcoming, the association of asthma with richness in nasal samples was independent of sample selection (Fig 4 and Fig E6).

In a complementary approach, we assessed specific taxa and the association of their relative abundance with asthma. Here, a very strong positive association with a specific *Moraxella* OTU was observed. Based on the chosen 16S rRNA gene fragment, it was not possible to determine the OTU definitively. One candidate is *M catarrhalis*, a bacterium with pathogenic potential specifically for the human upper respiratory tract. In neonates, the colonization of the throat with *M catarrhalis* has been reported as a potential risk factor for the development of childhood asthma.[11] Also, an association of this species with acute wheezing in young children has been reported.[37] However, another study did not reveal an association of early colonization (within the first year of life) of the nasopharynx with *Moraxella*

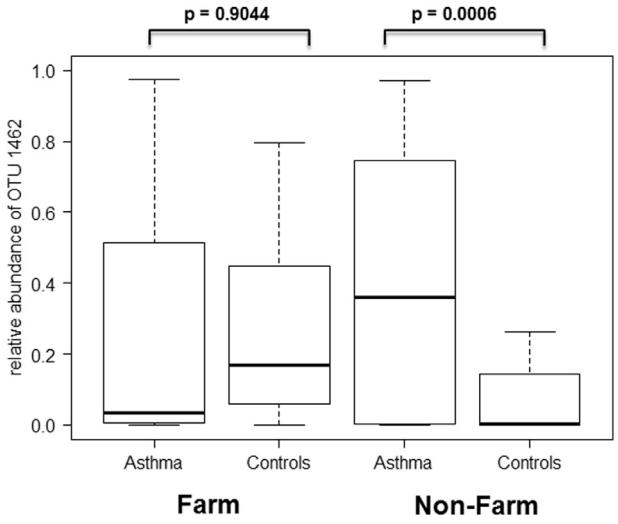

FIG 5. Relative abundance of *Moraxella* OTU 1462 in children with asthma and controls stratified for farming. The *P* value refers to the association with asthma in the respective stratum.

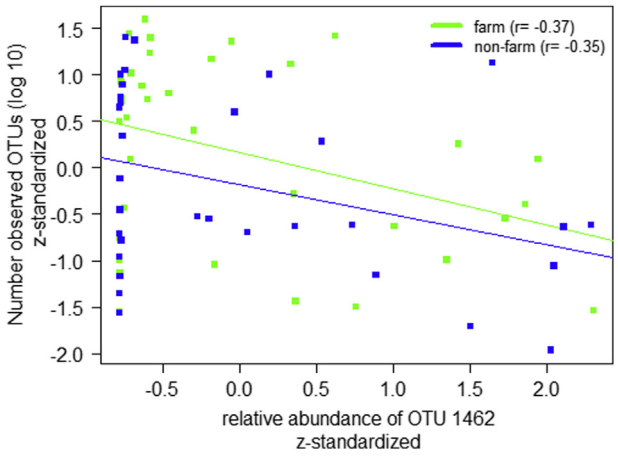

FIG 6. Inverse correlation of richness and relative abundance of *Moraxella* OTU 1462 in nasal samples stratified for farming.

with wheeze at year 5.[12] Although *M catarrhalis* seems to be in general more prevalent in young children,[12,38,39] it was also detected in sputum samples of adults with asthma, where it was associated with more severe airway obstruction and neutrophilic airway inflammation.[7] There is evidence that this species might play a dominant role in the active microbiota of the nasal cavities from children with asthma.[40] For 5 out of 8 asthma cases, the main proportion of the microbial RNA analyzed by shotgun sequencing belonged to *M catarrhalis*.[40] This bacterial species maintains several virulence factors that contribute to successful colonization and growth under adverse conditions in the respiratory tract, such as specific receptors to attach to mucosal surfaces, the ability to form biofilms, and the power to evade and survive host immune responses.[39]

In our study, the abundance of *Moraxella* spp. explained the association between species richness and asthma in the nonfarm children to a large extent. This may either imply that the effect of species richness is an epiphenomenon to the colonization with *Moraxella* spp. or that reduced bacterial diversity might create a favorable environment for these bacteria, thereby fostering the development of asthma. Longitudinal studies are needed to further elucidate the relation of *Moraxella* and diversity.

TABLE II. Comparison of *Moraxella* and richness effect on asthma in nasal samples of nonfarm children

Bacterial determinants	Unadjusted		Mutually adjusted	
	OR (95% CI)	*P* value	OR (95% CI)	*P* value
Richness	0.50 (0.21-1.21)	.1369	0.76 (0.25-2.31)	.6317
Moraxella (genus)	7.59 (2.66-21.64)	.0007	6.88 (2.27-20.86)	.0021
Richness	0.50 (0.21-1.21)	.1369	0.76 (0.25-2.34)	.6381
OTU 1462	7.88 (2.75-22.53)	.0006	7.18 (2.35-21.88)	.0018

All determinants are *z*-standardized.

Studies on the fecal microbiome suggest an association of reduced bacterial diversity during the first month of life with the development of childhood asthma.[41] A related example in terms of the pathologic effect of reduced richness might be found in another chronic lung disorder, cystic fibrosis. The bacterial microbiota of children with advanced cystic fibrosis is reduced in diversity, and certain species such as *Pseudomonas aeruginosa* prevail, whereas exacerbation of cystic fibrosis is delayed in individuals with a higher gut microbial diversity.[42]

Evidence comes from a murine model of sinusitis, which revealed an influence of the diversity of the sinus microbiome on the pathogenicity of *Corynebacterium tuberculostearicum*.[43] When reducing microbial diversity before inoculation with *C tuberculostearicum*, the pathogenic potential of the species increased. Ultimately, these analogies support the notion that reduced diversity promotes outgrowth of potentially harmful bacteria rather than vice versa.

In this context, the observation that the association of *Moraxella* with asthma was seen only in nonfarm children might be relevant because it suggests a particular diversity effect in farm children. Studies on environmental exposure to microorganisms have shown that children growing up on farms are exposed to a wider range of microbes,[44,45] which explains a substantial proportion of the protective effect of farming on asthma.[44] Possibly the farm exposure influences the nasal microbiota by inhalation of environmental microorganisms, their components, or metabolites. Eventually, the microbial diversity of the nasal mucosa might mirror the surrounding environment[46] and somehow interact with the mucosal immunity. Again, whether pathogens such as *Moraxella* are kept at bay by an established diverse microbiota or whether they primarily disturb the balance of the indigenous microbiota has to be elucidated in longitudinal studies.

We acknowledge that we did not study bronchial microbiota directly, and whether the tightly regulated throat microbiota truly reflects bronchial colonization remains doubtable. However, the association of disease status and nasal microbiota can be interpreted in 2 ways: Either the nasal microbiota reflects the colonization of a related villous epithelium, that is, the bronchial epithelium, or environmental microbes reflect asthma risk or protection without direct impact on the mucosal interface of the airways.

Taken together, we found an inverse association of asthma with bacterial diversity in the nasal cavity but not in the throat. In nonfarm children, this effect was partially explained by colonization with a possibly pathogenic *Moraxella* species. In

contrast, the risk effect of these bacteria was not seen in farm children, thereby suggesting that this environment neutralizes the detrimental effect of *Moraxella* colonization. Further research into environmental exposure and mucosal colonization might foster our understanding of the role of microorganisms in the development and persistence of asthma.

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Key messages

- **Asthma was associated with an altered diversity in the nasal, but not in the throat, microbiota and an increased *Moraxella* abundance in school-age children living in rural areas.**
- **The association of asthma with *Moraxella* colonization was restricted to nonfarm children.**

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METHODS

Sample collection

Material from the nose was taken by 5 circular rubbings about 1 cm from the nare. Throat samples were obtained by gently sweeping tonsils and velum without contacting the buccal mucosa or the gingiva. The nasal samples were collected between May and July 2007 and the throat samples during the same months in 2008.

Determination of total bacterial load by qPCR

Reactions were set up with 15-μL reactions in triplicate and amplified using 7.5 μL of SYBR Fast qPCR kit Master Mix (Kappa Biosystems, London, United Kingdom), 0.3 μL of primer 520F (10 μM) and primer 802R (10 μM), 1.9 μL PCR-grade water, and 5 μL of sample DNA. The qPCR cycling was performed with an initial incubation at 95°C for 3 minutes, followed by 40 cycles of 95°C for 20 seconds, 50°C for 30 seconds, and 72°C for 30 seconds. Gene copy numbers for each sample were determined using a standard curve generated from linearized plasmid DNA containing the full-length 16S rRNA gene from *Pseudomonas aeruginosa* PAO1. Tenfold serial dilutions of the plasmid DNA ranging from 10^4 to 10^8 provided data points for standard curve generation. PCR-grade water was used as negative control.

Preparation of nasal 16S rRNA gene fragments for pyrosequencing

For the amplification of the nasal DNA, Platinum SuperMix High Fidelity reagents (Life Technologies) with 1 μL (0.5 μM) of each primer and 1 μL sample DNA was used in triplicate. PCR conditions were set at 95°C for 90 seconds, followed by 34 cycles at 95°C for 30 seconds, 55°C for 60 seconds, 68°C for 60 seconds, with a final elongation at 68°C for 5 minutes. Concentrations of purified 16S rRNA gene fragments were determined using Quant-iT PicoGreen (Life Technologies).

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E2. Sim K, Cox MJ, Wopereis H, Martin R, Knol J, Li MS, et al. Improved detection of bifidobacteria with optimised 16S rRNA-gene based pyrosequencing. PLoS One 2012;7:e32543.

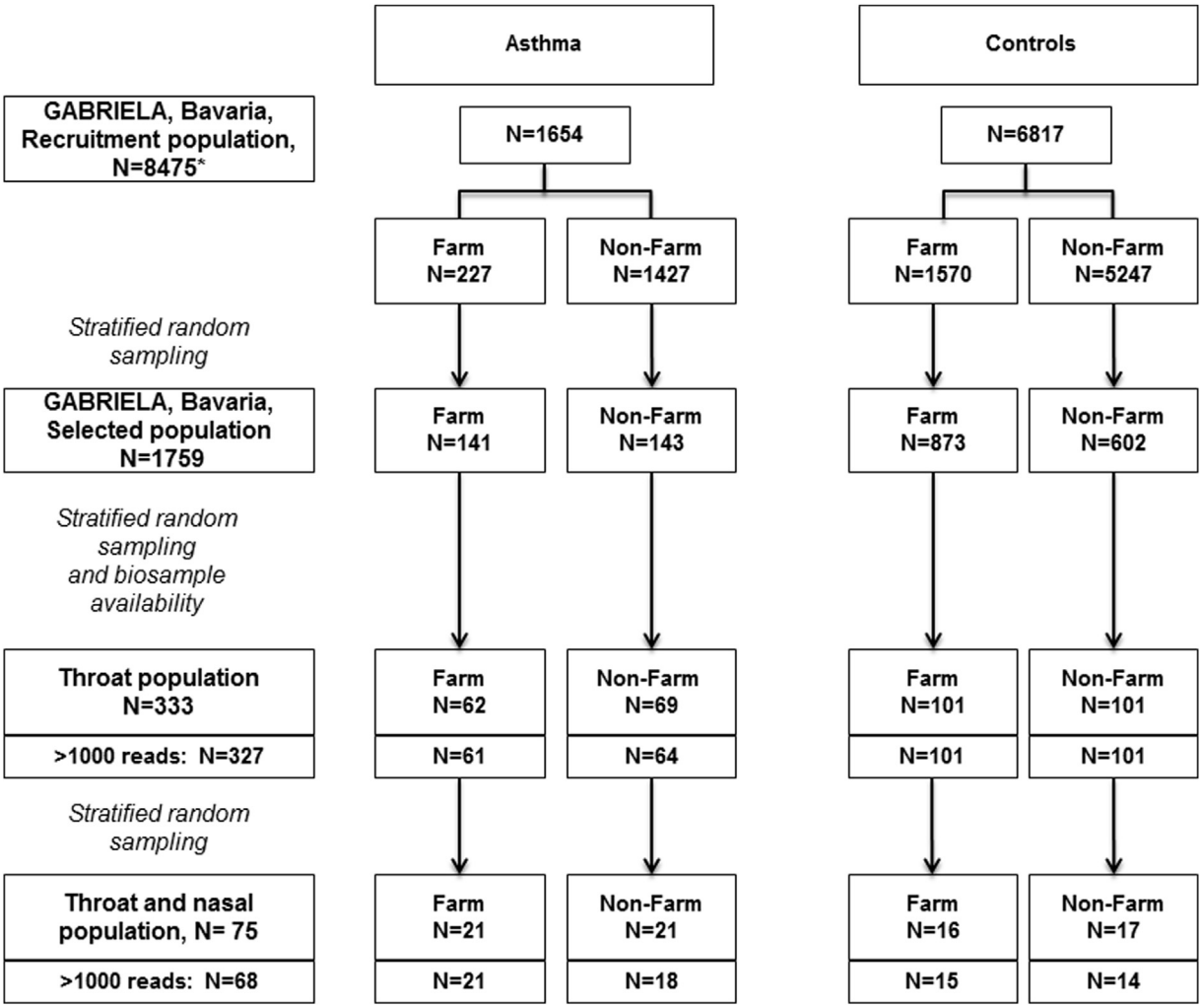

FIG E1. Study design. Shown are the final throat (N = 327) and nasal (N = 68) study populations and their derivations. The recruitment population consisting of children with asthma and control children from the Bavarian GABRIELA study population was the origin of the 2 analysis populations. For 4 children of the recruitment population, no asthma information was available (*). Stratified random sampling was used. N is the number of children for the different groups in the respective population. Note that samples with low read counts after sequencing and filtering (<1000 reads) were removed in the final study population.

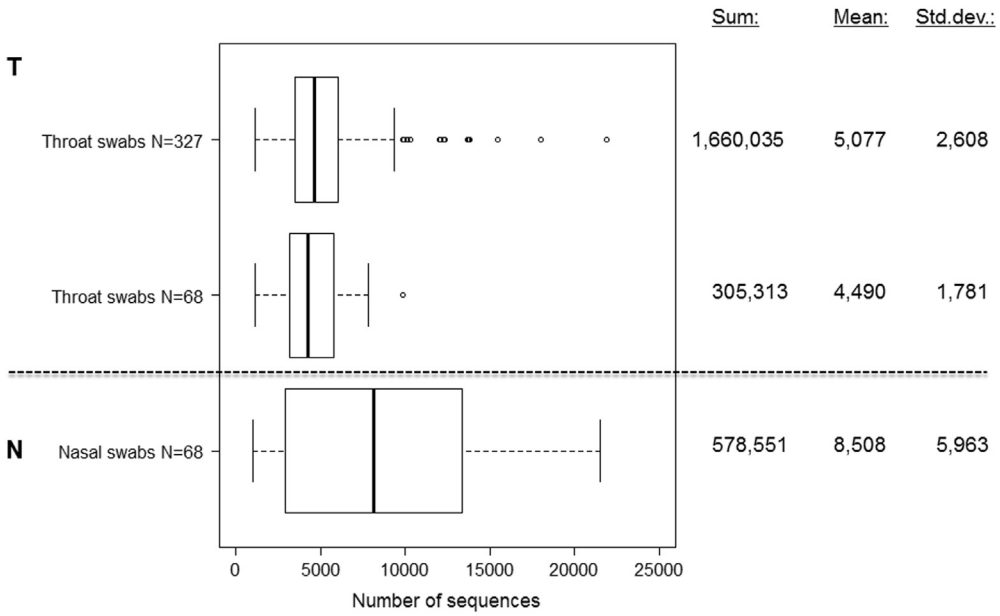

FIG E2. Number of sequences per sample for the 3 analysis populations after denoising and filtering. At the right border, sum, mean, and SD of sequences are reported. Methodical differences in sample processing preclude direct comparison of throat and nasal samples.

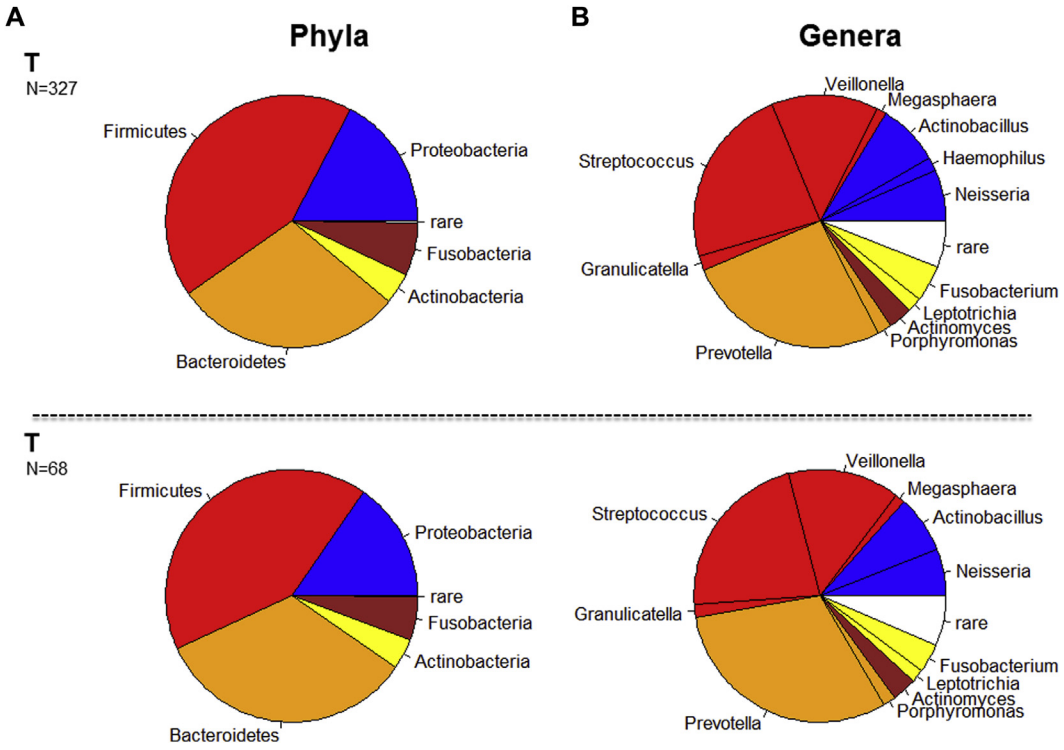

FIG E3. Taxonomic composition of the throat microbiota based on 327 and 68 children, respectively. The lower panel is restricted to the throat samples of the 68 children who also provided nasal samples. Shown is the survey-weighted relative abundance of phyla **(A)** and genera **(B)**. Genera belonging to the same phylum are represented by the color of the respective phylum. Phyla and genera representing less than 0.5% of all reads for phyla and less than 1% for genera in the combined throat and nasal sample, respectively, are grouped in "rare."

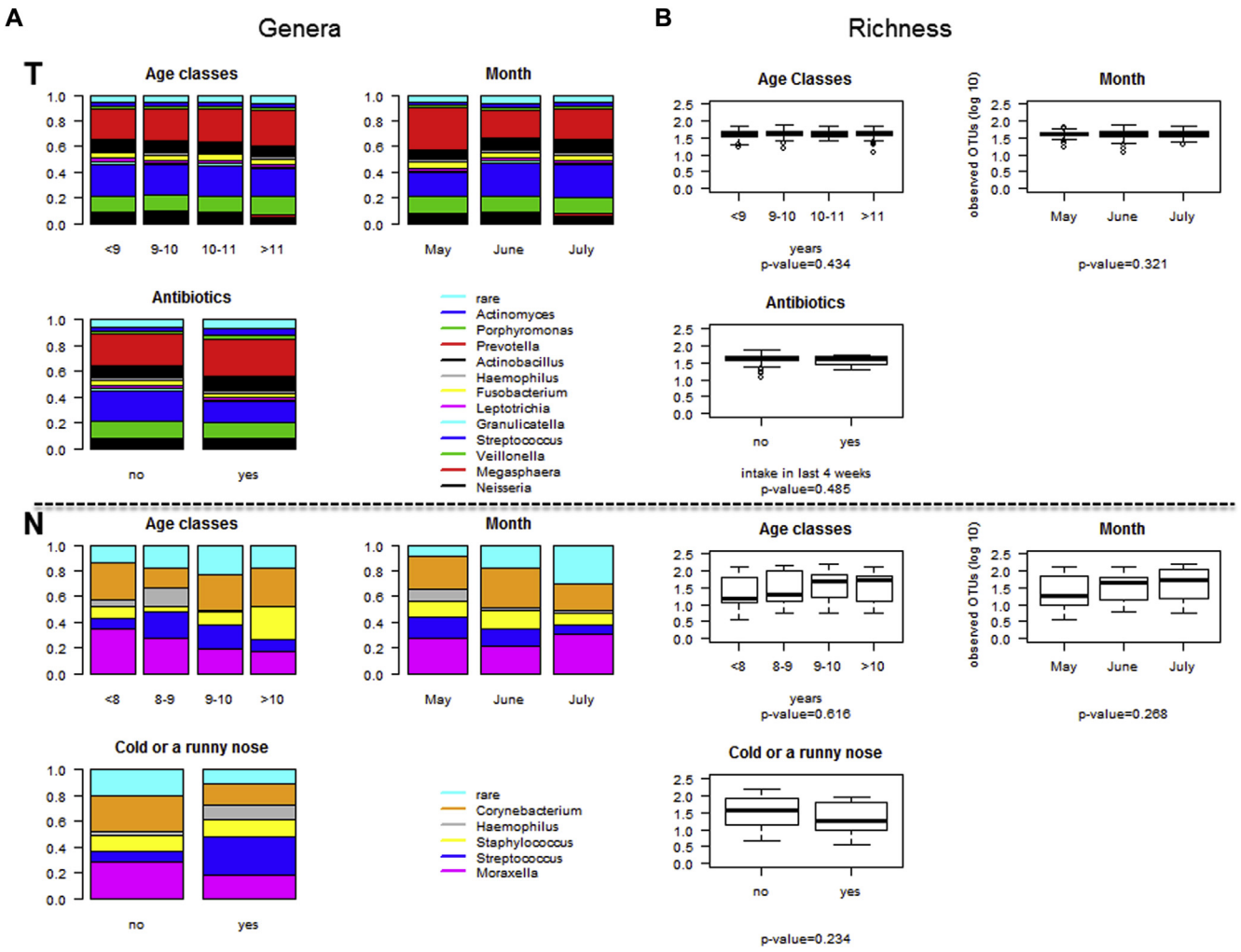

FIG E4. Taxonomic composition and richness stratified for age, sampling month, and clinical conditions. Shown is the relative abundance of genera **(A)** and the boxplots for richness **(B)** in throat (T) and nasal (N) samples, respectively. The *P* values of Wilcoxon or Kruskal-Wallis tests refer to comparisons across strata. All values refer to unweighted analyses. Methodical differences in sample processing preclude direct comparison of throat and nasal samples.

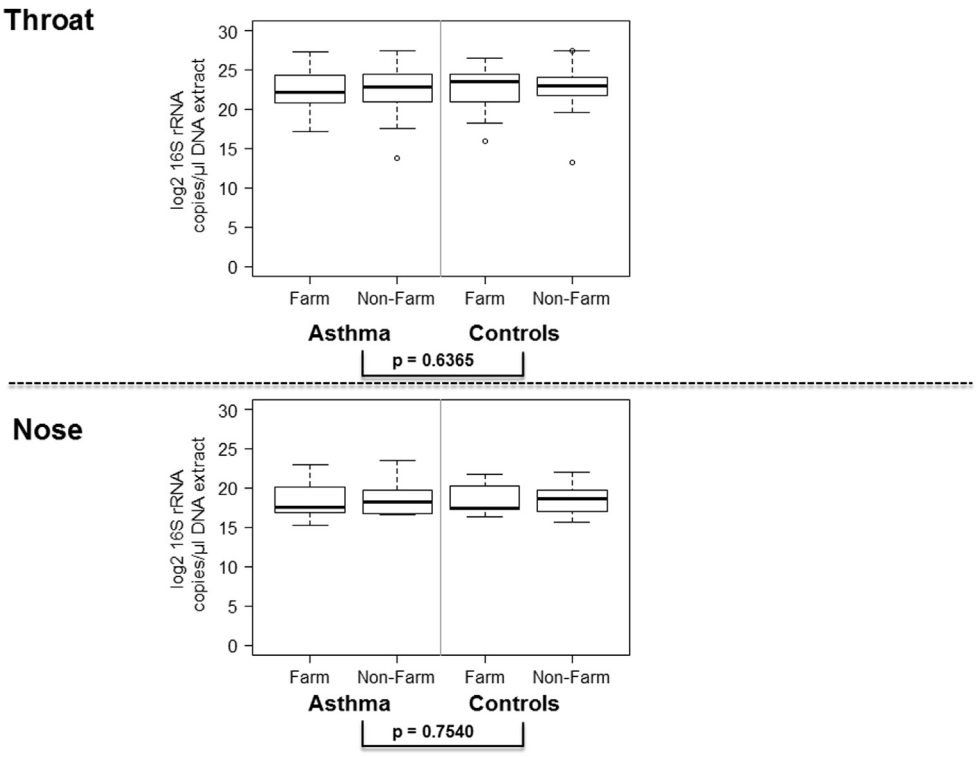

FIG E5. Number of bacterial 16S rRNA gene copies in throat and nasal samples. The analyses are based on 327 throat and 68 nasal samples, grouped by asthma occurrence and farm exposure. The *P* values of survey-weighted Wilcoxon tests refer to comparisons between asthma cases and controls.

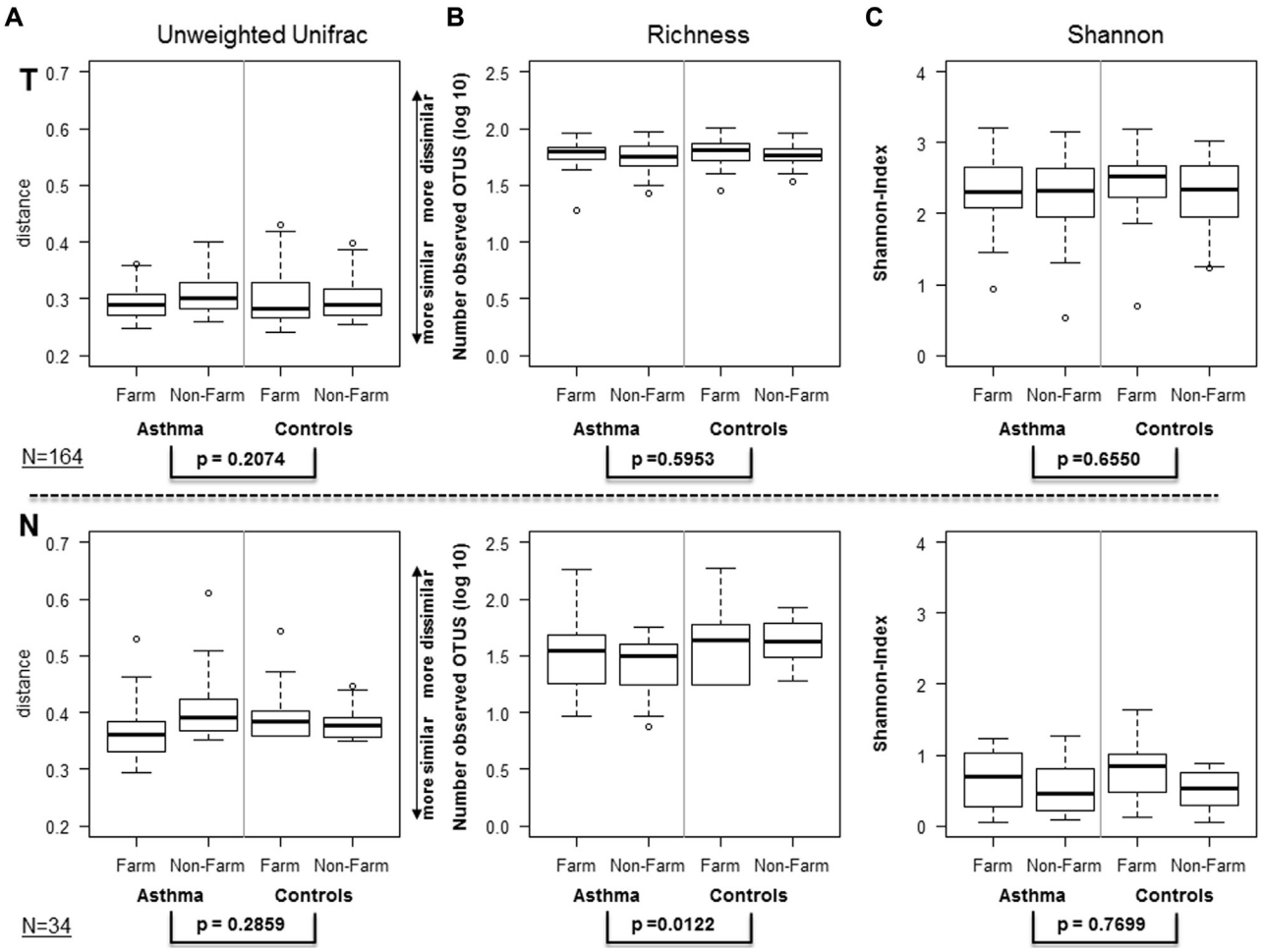

FIG E6. Bacterial diversity in throat (T) and nasal (N) samples from children with asthma and controls based on samples with adequate reads. Shown are β-diversity as unweighted UniFrac **(A)**, and α-diversity as richness **(B)** and Shannon index **(C)** for throat samples and nasal samples. Adequate read counts were defined by the median of reads, that is, 4635 reads for throat samples and 8120 reads for nasal samples. The *P* values for Wilcoxon tests comparing asthma versus control group are shown.

TABLE E1. Primer sequences

Primer	Sequence	Reference
520F	5′-AYT GGG YDT AAA GNG-3′	E1
802R	5′-TAC NVG GGT ATC TAA TCC-3′	E1
357F	5′-CCT ACG GGA GGC AGC AG-3′	E2
926R	5′-CCG TCA ATT CMT TTR AGT-3′	E2

TABLE E2. Description of the samples at sampling time point

Throat population	Asthma (N = 125)	Controls (N = 202)	*P* value*
Age of study subject (y) (%)			
7-9	28.8	31.2	
9-10	25.6	18.8	.273
10-11	19.2	24.3	.725
11-12	26.4	25.7	.876
Month of sampling (%)†			
May	23.2	27.5	
June	52.0	51.0	.954
July	24.8	21.5	.585
Health information of study subject (%)			
Antibiotic use in the last 4 wk	1.7	3.3	.892

Nasal population	Asthma (N = 39)	Controls (N = 29)	*P* value
Age of study subject (y) (%)			
6-8	33.3	34.5	
8-9	20.5	13.8	.476
9-10	17.9	24.1	.825
10-11†	28.2	27.6	.654
Month of sampling (%)			
May	35.9	34.5	
June	33.3	48.3	.342
July	30.8	17.2	.557
Health information of study subject (%)			
Cold or a runny nose at sampling day	20.5	17.9	.839

All percentages are given for the real sample and refer to the nonmissing values (no weighting is done here).

**P* value is the *P* value of a survey-weighted logistic regression model with asthma as dependent variable.

†Two throat samples taken in August are excluded from analyses.

TABLE E3. Asthma effects on richness in all children, and stratified for farm exposure

Richness	Asthma	Controls	*P* value Wilcoxon test
Throat			
All	41.07	41.39	.8675
Farmer	42.16	44.58	.2348
Nonfarmer	40.85	40.48	.8246
Nasal			
All	34.22	51.66	.0521
Farmer	41.89	65.19	.3019
Nonfarmer	32.53	47.11	.0964

TABLE E4. Relative abundance of the main phyla and genera tested for association with asthma

					Test for asthma	
Sample site	**Taxonomic level**	**Taxonomic group***	**Mean relative abundance**	**Samples with respective taxon (%)**	**OR (95% CI)†**	***P* value**
Throat	Phylum	*Firmicutes*	0.4157	100.00	0.93 (0.69-1.25)	.6328
		Bacteroidetes	0.2981	100.00	0.90 (0.68-1.21)	.4937
		Proteobacteria	0.1769	99.69	1.17 (0.91-1.51)	.2248
		Fusobacteria	0.0683	100.00	1.00 (0.77-1.30)	.9868
		Actinobacteria	0.0386	100.00	0.98 (0.76-1.27)	.9015
		rare	0.0025	56.57	1.00 (0.80-1.26)	.9975
	Genus	*Prevotella*	0.2674	100.00	0.91 (0.68-1.22)	.5312
		Streptococcus	0.2266	100.00	1.06 (0.80-1.41)	.6836
		Veillonella	0.1368	100.00	0.85 (0.65-1.10)	.2165
		Actinobacillus	0.0784	96.33	1.26 (0.97-1.64)	.0782
		Neisseria	0.0672	99.69	1.00 (0.75-1.34)	.9870
		rare	0.0607	100.00	0.99 (0.80-1.24)	.9596
		Fusobacterium	0.0461	99.69	0.99 (0.77-1.27)	.9197
		Actinomyces	0.0304	99.69	0.98 (0.75-1.26)	.8592
		Leptotrichia	0.0200	98.78	1.05 (0.81-1.37)	.7172
		Porphyromonas	0.0186	98.47	1.00 (0.77-1.29)	.9759
		Haemophilus	0.0183	90.83	1.00 (0.75-1.33)	.9961
		Granulicatella	0.0178	99.69	0.83 (0.64-1.08)	.1705
		Megasphaera	0.0117	86.85	0.81 (0.59-1.12)	.2017
Nose	Phylum	*Actinobacteria*	0.3637	100.00	0.62 (0.34-1.14)	.1309
		Firmicutes	0.3213	100.00	0.88 (0.46-1.71)	.7141
		Proteobacteria	0.2888	100.00	**2.44 (1.07-5.59)**	**.0384**
		Bacteroidetes	0.0187	94.12	0.39 (0.13-1.15)	.0918
		rare	0.0075	92.65	1.00 (0.43-2.34)	.9956
	Genus	*Corynebacterium*	0.3353	98.53	0.62 (0.34-1.11)	.1137
		rare	0.1871	100.00	0.63 (0.31-1.28)	.2087
		Moraxella	0.1737	95.59	**3.78 (2.02-7.05)**	**.0001**‡
		Streptococcus	0.1382	98.53	0.94 (0.46-1.92)	.8657
		Staphylococcus	0.1161	98.53	1.11 (0.57-2.15)	.7584
		Haemophilus	0.0497	86.76	0.69 (0.36-1.32)	.2674

Significant results are given in boldface.

*Shown are taxonomic groups with ≥1% of all reads in the throat and nasal swab samples, respectively. Survey-weighted mean of relative abundances and percentages of study children with the respective group are presented. Groups with <1% relative abundance were combined in "rare." All unclassified reads on the respective level were excluded from analysis.

†ORs are presented for z-scaled relative abundances. All models are adjusted for farming.

‡Significant after correction for multiple testing.

TABLE E5. Relative abundance of the main OTUs tested for association with asthma

					Test for asthma	
Sample site	**OTU ID***	**Genus**	**Mean relative abundance**	**Samples with respective OTU (%)**	**OR (95% CI)†**	***P* value**
Throat	OTU 445	*Streptococcus*	0.2040	100.00	1.08 (0.82-1.43)	.5888
	OTU 1475	*Prevotella*	0.1575	99.69	0.87 (0.66-1.16)	.3475
	rare OTUs		0.1474	100.00	1.03 (0.79-1.35)	.8319
	OTU 104	*Veillonella*	0.1261	100.00	0.87 (0.67-1.14)	.3234
	OTU 769	*Actinobacillus*	0.0783	96.33	1.26 (0.97-1.64)	.0783
	OTU 3256	*Neisseria*	0.0645	99.08	1.00 (0.75-1.33)	.9764
	OTU 2648	*Fusobacterium*	0.0456	99.69	0.99 (0.77-1.26)	.9277
	OTU 3271	*Prevotella*	0.0257	95.41	0.82 (0.59-1.14)	.2427
	OTU 2792	*Actinomyces*	0.0249	99.39	0.99 (0.76-1.29)	.9373
	OTU 1853	*Streptococcus*	0.0197	94.80	0.84 (0.63-1.11)	.2240
	OTU 1967	*Prevotella*	0.0194	97.55	1.01 (0.76-1.36)	.9259
	OTU 820	*Granulicatella*	0.0178	99.69	0.83 (0.64-1.08)	.1706
	OTU 2638	*Porphyromonas*	0.0134	96.33	1.01 (0.74-1.38)	.9381
	OTU 2091	*Leptotrichia*	0.0118	90.83	1.09 (0.84-1.41)	.5323
	OTU 2621	*Megasphera*	0.0116	86.85	0.81 (0.59-1.12)	.2021
	OTU 1946	*Prevotella*	0.0113	92.97	0.95 (0.73-1.23)	.6991
	OTU 2370	*Haemophilus*	0.0106	68.50	1.03 (0.77-1.37)	.8464
	OTU 1699	*Prevotella*	0.0105	66.67	0.95 (0.76-1.19)	.6371
Nose	OTU 2405	*Corynebacterium*	0.3004	89.71	0.63 (0.36-1.10)	.1095
	rare OTUs		0.2231	100.00	0.62 (0.30-1.28)	.1995
	OTU 1462	***Moraxella***	0.1686	89.71	**3.73 (2.02-6.91)**	**.0001‡**
	OTU 445	*Streptococcus*	0.1244	98.53	0.95 (0.49-1.84)	.8803
	OTU 1034	*Staphylococcus*	0.1147	98.53	1.11 (0.57-2.15)	.7587
	OTU 2370	*Haemophilus*	0.0479	64.71	0.70 (0.37-1.31)	.2675
	OTU 1301	*Corynebacterium*	0.0209	73.53	1.20 (0.64-2.26)	.5711

Significant results are given in boldface.

*Shown are taxonomic groups with ≥1% of all reads in the throat and nasal swab samples, respectively. Survey-weighted mean of relative abundances and percentages of study children with the respective group are presented. OTUs with <1% relative abundance were combined in "rare OTUs."

†ORs are presented for *z*-scaled relative abundances. All models are adjusted for farming.

‡Significant after correction for multiple testing.

TABLE E6. Relative abundance of the main phyla and genera tested for association with asthma in samples with adequate reads¶

					Test for asthma	
Sample site	**Taxonomic level**	**Taxonomic group***	**Mean relative abundance**	**Samples with respective taxon (%)**	**OR (95% CI)†**	***P* value**
Throat	Phylum	*Firmicutes*	0.4182	100.00	0.93 (0.61-1.42)	.7306
		Bacteroidetes	0.3001	100.00	0.77 (0.51-1.17)	.2237
		Proteobacteria	0.1757	100.00	1.42 (0.95-2.13)	.0900
		Fusobacteria	0.0653	100.00	1.10 (0.75-1.61)	.6346
		Actinobacteria	0.0369	100.00	0.77 (0.44-1.33)	.3487
		rare	0.0038	65.24	0.99 (0.72-1.36)	.9499
	Genus	*Prevotella*	0.2695	100.00	0.77 (0.50-1.16)	.2095
		Streptococcus	0.2278	100.00	1.12 (0.75-1.66)	.5832
		Veillonella	0.1345	100.00	0.75 (0.48-1.17)	.2059
		Actinobacillus	0.0776	96.34	1.42 (0.99-2.03)	.0611
		Neisseria	0.0715	100.00	1.13 (0.75-1.70)	.5672
		rare	0.0613	100.00	1.09 (0.73-1.61)	.6770
		Fusobacterium	0.0474	99.39	1.04 (0.72-1.49)	.8434
		Actinomyces	0.0281	99.39	0.81 (0.45-1.43)	.4627
		Leptotrichia	0.0160	98.78	1.30 (0.82-2.07)	.2703
		Porphyromonas	0.0183	97.56	1.11 (0.74-1.68)	.6083
		Haemophilus	0.0163	90.24	1.01 (0.68-1.51)	.9607
		Granulicatella	0.0198	99.39	0.82 (0.58-1.15)	.2436
		Megasphaera	0.0119	87.80	0.66 (0.38-1.15)	.1472
Nose	Phylum	*Actinobacteria*	0.4452	100.00	0.40 (0.14-1.17)	.1071
		Firmicutes	0.2677	100.00	1.05 (0.38-2.90)	.9190
		Proteobacteria	0.2856	100.00	**5.49 (1.91-15.79)**	**.0040**§
		Bacteroidetes	0.0010	88.24	0.35 (0.02-6.38)	.4877
		rare	0.0005	88.24	0.47 (0.04-5.31)	.5503
	Genus	*Corynebacterium*	0.4430	97.06	0.40 (0.14-1.17)	.1072
		rare	0.0255	100.00	NA‡	.8044
		Moraxella	0.2687	94.12	**4.52 (1.75-11.67)**	**.0044**§
		Streptococcus	0.1142	97.06	1.20 (0.46-3.18)	.7121
		Staphylococcus	0.1412	97.06	0.92 (0.36-2.35)	.8682
		Haemophilus	0.0074	73.53	2.33 (0.62-8.73)	.2216

Significant results are given in boldface.

NA, Not available/applicable.

*Shown are taxonomic groups with ≥1% of all reads in the throat and nasal swab samples, respectively. Survey-weighted mean of relative abundances and percentages of study children with the respective group are presented. Groups with <1% relative abundance were combined in "rare." All unclassified reads on the respective level were excluded from analysis.

†ORs are presented for *z*-scaled relative abundances. All models are adjusted for farming.

‡OR not available because of weighting issues.

§Significant after correction for multiple testing.

¶Adequate read counts were defined by the median of reads, that is, 4635 reads for throat samples and 8120 reads for nasal samples.