Exposure to Environmental Microorganisms and Childhood Asthma

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ABSTRACT

BACKGROUND

Children who grow up in environments that afford them a wide range of microbial exposures, such as traditional farms, are protected from childhood asthma and atopy. In previous studies, markers of microbial exposure have been inversely related to these conditions.

METHODS

In two cross-sectional studies, we compared children living on farms with those in a reference group with respect to the prevalence of asthma and atopy and to the diversity of microbial exposure. In one study — PARSIFAL (Prevention of Allergy — Risk Factors for Sensitization in Children Related to Farming and Anthroposophic Lifestyle) — samples of mattress dust were screened for bacterial DNA with the use of single-strand conformation polymorphism (SSCP) analyses to detect environmental bacteria that cannot be measured by means of culture techniques. In the other study — GABRIELA (Multidisciplinary Study to Identify the Genetic and Environmental Causes of Asthma in the European Community [GABRIEL] Advanced Study) — samples of settled dust from children's rooms were evaluated for bacterial and fungal taxa with the use of culture techniques.

RESULTS

In both studies, children who lived on farms had lower prevalences of asthma and atopy and were exposed to a greater variety of environmental microorganisms than the children in the reference group. In turn, diversity of microbial exposure was inversely related to the risk of asthma (odds ratio for PARSIFAL, 0.62; 95% confidence interval [CI], 0.44 to 0.89; odds ratio for GABRIELA, 0.86; 95% CI, 0.75 to 0.99). In addition, the presence of certain more circumscribed exposures was also inversely related to the risk of asthma; this included exposure to species in the fungal taxon eurotium (adjusted odds ratio, 0.37; 95% CI, 0.18 to 0.76) and to a variety of bacterial species, including *Listeria monocytogenes*, bacillus species, corynebacterium species, and others (adjusted odds ratio, 0.57; 95% CI, 0.38 to 0.86).

CONCLUSIONS

Children living on farms were exposed to a wider range of microbes than were children in the reference group, and this exposure explains a substantial fraction of the inverse relation between asthma and growing up on a farm. (Funded by the Deutsche Forschungsgemeinschaft and the European Commission.)

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ENVIRONMENTAL EXPOSURE TO MICRO-organisms has repeatedly been found to be inversely related to the manifestation of atopic diseases such as asthma and hay fever. This observation has been made in various contexts, including the studies conducted in the Republic of Karelia (Russia) and North Karelia (Finland), in which two populations in geographically adjacent areas live under different environmental conditions. In the population with higher bacterial exposures, the prevalence of asthma and atopy was substantially lower.[1] Another example supporting this notion is the lower prevalence of asthma and atopy among children raised on a farm.[2] Many studies using microbial products, such as endotoxin[3] or muramic acid,[4] as simple markers of microbial exposure have corroborated these observations.[5]

In the present epidemiologic study, we characterized farming-associated microbial exposure beyond the simple markers noted above. We used data from two large-scale observational studies of schoolchildren living in predominantly rural areas of Central Europe: the German population of the PARSIFAL (Prevention of Allergy — Risk Factors for Sensitization Related to Farming and Anthroposophic Lifestyle) study and the Bavarian population of GABRIELA (Multidisciplinary Study to Identify the Genetic and Environmental Causes of Asthma in the European Community [GABRIEL]

Advanced Study). We assessed the prevalence of asthma and atopy among children living on farms and among other children living in the same areas (the reference group), measured the diversity of the microbial exposure in both groups, and related the diversity of exposure to asthma and atopy. The different methods used in the two studies — single-strand conformation polymorphism (SSCP) analysis in the PARSIFAL study and culture techniques in GABRIELA — are complementary in that each method extends and refines the microbial spectra covered by its counterpart.

METHODS

STUDY DESIGN AND POPULATIONS

Characteristics of the two study populations, the samples analyzed, and the types of analysis performed are summarized in Table 1. The PARSIFAL study was a cross-sectional survey including the children of farmers, children attending Rudolf Steiner schools (i.e., anthroposophic schools), and their respective reference groups.[6] In Bavaria, Germany, 6963 school-age children (6 to 13 years) from rural or suburban areas participated. In a randomly selected subsample of children, analyses of blood and dust samples were performed. For that sample, all children whose parents or guardians consented were eligible (55% of children living on farms and 48%

Table 1. Study Populations, Samples Analyzed, and Types of Analysis Performed in the PARSIFAL Study and GABRIELA.*

Characteristic	PARSIFAL	GABRIELA
Study population (no.)	6843	9668
Origin	Elementary schools in rural and suburban areas of Bavaria (South Germany)	Elementary schools in rural areas of Austria, South Germany, and Switzerland
Recruitment period	October 2000–May 2002	November 2006–May 2007
Samples selected for analysis (no.)	489	444
Origin	Bavaria (South Germany)	Bavaria (South Germany)
Selection	Within farm-exposure strata	Within farm-exposure and disease strata
Children living on farms (%)	52	16
Prevalence of asthma in study populations (%)	8	11
Dust analysis		
Source of dust	Mattress dust, collected with vacuum cleaner	Settled dust in children's rooms, collected with electrostatic dust collectors
Type of microbial analyses	SSCP targeting of bacterial DNA	Culture, microscopy, and Gram's staining
Coverage	Viable and nonviable bacteria	Viable fungi and bacteria

* Calculations of prevalence in GABRIELA were weighted on the basis of the total number of children who were eligible for inclusion in the study (34,491). SSCP denotes single-strand conformation polymorphism.

of children not living on farms). Among the 801 children for whom dust samples were collected, the samples for 489 children contained an amount of dust that was sufficient for SSCP analysis (Table 1 in the Supplementary Appendix, available with the full text of this article at NEJM.org).

GABRIELA was a cross-sectional study conducted at elementary schools in five rural areas in southern Germany, Switzerland, Austria, and Poland; because of differences in study design, the Polish data are not reported here. Of the 34,491 children between the ages of 6 and 12 years who were recruited, a stratified random sample of 9668 children was selected. The three strata in the sample were defined by different levels of farm exposure, from no exposure to intermediate exposure to living on a farm. Stratified, random subsampling was then performed in Bavaria, Germany, with environmental sampling performed for 444 children and measurement of lung function performed in 895 children.

Written informed consent was obtained from the parents or guardians of all participating children. For both studies, the ethics committees of the participating universities and the regional data protection authorities approved both studies.

ASSESSMENT OF PARTICIPANTS

In both studies, questionnaires were used to assess respiratory and allergic symptoms and diagnoses, farm-related exposures at various ages, and potential confounders. Children living full-time on family-run farms were classified as members of the farm group, whereas all other children were classified as members of the reference group. Asthma was defined as a diagnosis of asthma established by a doctor on at least one occasion or a diagnosis of wheezy bronchitis established on more than one occasion. Atopy was defined by the presence of specific IgE antibodies to *Dermatophagoides pteronyssinus* (dust mites), cat antigen, tree mix (in the PARSIFAL study), or birch (in GABRIELA) of at least 0.7 kU per liter or a positive reaction to a grass mix of at least 0.35 kU per liter. IgE antibodies were detected by means of the Pharmacia CAP and UNICAP 1000 systems, Phadia AB, Uppsala, Sweden.

DUST ANALYSES

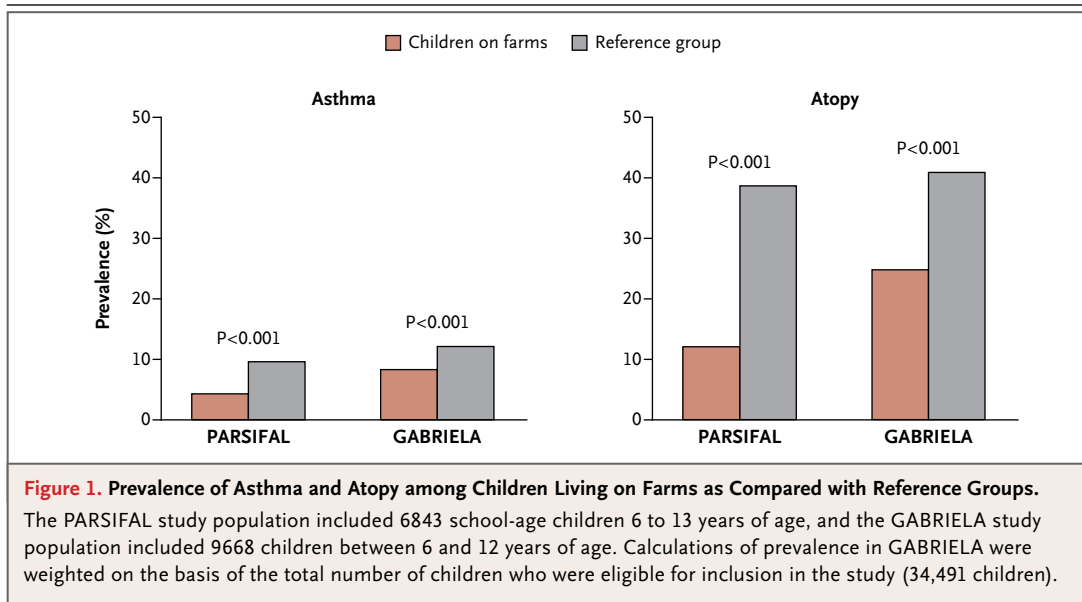In the PARSIFAL study, dust from children's mattresses was collected as described previously.[7] The dust samples were dissolved in phosphate-buffered saline, and DNA extraction[8] and SSCP analysis[9] were performed as previously reported. A fragment of the variable regions 4 and 5 of the 16S ribosomal DNA was amplified and analyzed on SSCP gels. Analysis of the SSCP profiles was performed with GelCompar II software, version 4.6 (Applied Maths). The SSCP gels were normalized with the use of an added standard present in two or three tracks on every gel. Bands of interest were excised from at least three individual tracks, amplified, and sequenced.[9] The DNA sequences were analyzed for similarities of at least 98% with the use of the Ribosomal Database Project II for phylogenetic analysis (http://rdp.cme.msu.edu/seqmatch/seqmatch_intro.jsp).

In GABRIELA, airborne dust samples were collected with the use of electrostatic dust collectors.[10,11] These collectors are plastic sample holders equipped with an electrostatic cloth with the potential to capture dust from the air. The collectors were placed in children's bedrooms by fieldworkers and left there for 14 days. The cloths were washed with polysorbate 80, and dilutions of the washing solution were systematically plated on five different growth mediums. After incubation for 7 days, the colonies were counted and identified on the basis of gross and microscopical assessment. Bacterial colonies were also treated with Gram's stain. Bacterial and fungal results were expressed as colony-forming units per dust collector.

STATISTICAL ANALYSIS

In the PARSIFAL study, bacterial exposure was modeled with the use of dichotomous variables, with a cutoff point of 5 density units corresponding to the threshold for visual detectability on the gel. For the factor analysis, continuous variables representing gel-density values for the 76 bands were used. Because of a skewed distribution and several zero values, the band-density values, ranging from 0 to 160, were augmented by 1 and log-transformed.

In GABRIELA, microbial exposure was represented by dichotomized variables for six bacterial taxa and nine fungal taxa (detectable vs. nondetectable). Additional taxa were found in less than 10% of all children and were excluded from further analyses. Because of the stratified sampling design in GABRIELA, weighted statistical methods were applied with the use of the Taylor series method for variance estimation.

Figure 1. Prevalence of Asthma and Atopy among Children Living on Farms as Compared with Reference Groups.

The PARSIFAL study population included 6843 school-age children 6 to 13 years of age, and the GABRIELA study population included 9668 children between 6 and 12 years of age. Calculations of prevalence in GABRIELA were weighted on the basis of the total number of children who were eligible for inclusion in the study (34,491 children).

For the assessment of microbial diversity, scores were generated by summing up all detectable bands (PARSIFAL) and all fungal taxa (GABRIELA). This approach was not feasible for bacterial taxa in GABRIELA, however, since they were classified mainly on the basis of rough criteria such as Gram's staining. The probability of asthma or living on a farm was calculated as predictive values from a logistic regression for asthma or living on a farm, with the respective diversity scores as the independent variable.

In the PARSIFAL study, data reduction for the 76 continuous band-density variables was achieved by performing a factor analysis with varimax rotation.[12] Factors with Eigen values of 1.5 or more were extracted. Logistic regression for asthma and atopy was performed for all extracted factors or taxa, with adjustment for study group (i.e., living or not living on a farm). P values, at an effective alpha level of 0.05, were corrected for multiple comparisons by applying the Bonferroni method (i.e., an alpha level of $0.05 \div 10$ for the 10 factors in the PARSIFAL study and an alpha level of $0.05 \div 15$ for the 15 taxa in GABRIELA). Finally, the regression models for asthma and atopy were mutually adjusted for the specific exposures or factors and the diversity scores.

RESULTS

PREVALENCE OF ASTHMA AND ATOPY

As shown in Figure 1, children living on farms had a lower prevalence of asthma than children in the reference groups in both the PARSIFAL study (adjusted odds ratio, 0.49; 95% confidence interval [CI], 0.35 to 0.69) and GABRIELA (adjusted odds ratio, 0.76; 95% CI, 0.65 to 0.89). In both studies, the differences between the two groups of children were greater for the prevalence of atopy (adjusted odds ratio in the PARSIFAL study, 0.24; 95% CI, 0.18 to 0.34; adjusted odds ratio in GABRIELA, 0.51; 95% CI, 0.46 to 0.57). In both subpopulations for which dust samples were available, similar associations between living or not living on a farm and the prevalences of asthma and atopy were found (Table 2 in the Supplementary Appendix). In GABRIELA, the diagnosis of asthma was validated with the use of spirometry. As compared with children who did not have asthma, those classified as having asthma had slightly but significantly lower mean (±SE) values for the ratio of forced expiratory volume in 1 second (FEV_1) to forced vital capacity (FVC) (91.6±0.8% of the predicted value vs. 95.3±0.4%, P=0.001) and for a forced expiratory flow between 25% and 75% of FVC (76.9±2.3% of the predicted value vs. 88.2±1.3%, P=0.001).

DIVERSITY OF MICROBIAL EXPOSURE

In the PARSIFAL study, the percentage of samples of mattress dust that were positive for bacteria was higher among the children living on farms than among those in the reference group (Fig. 2A). In GABRIELA, all bacterial (Fig. 2B) and fungal (Fig. 2C) taxa cultured in the settled mattress dust were more prevalent among chil-
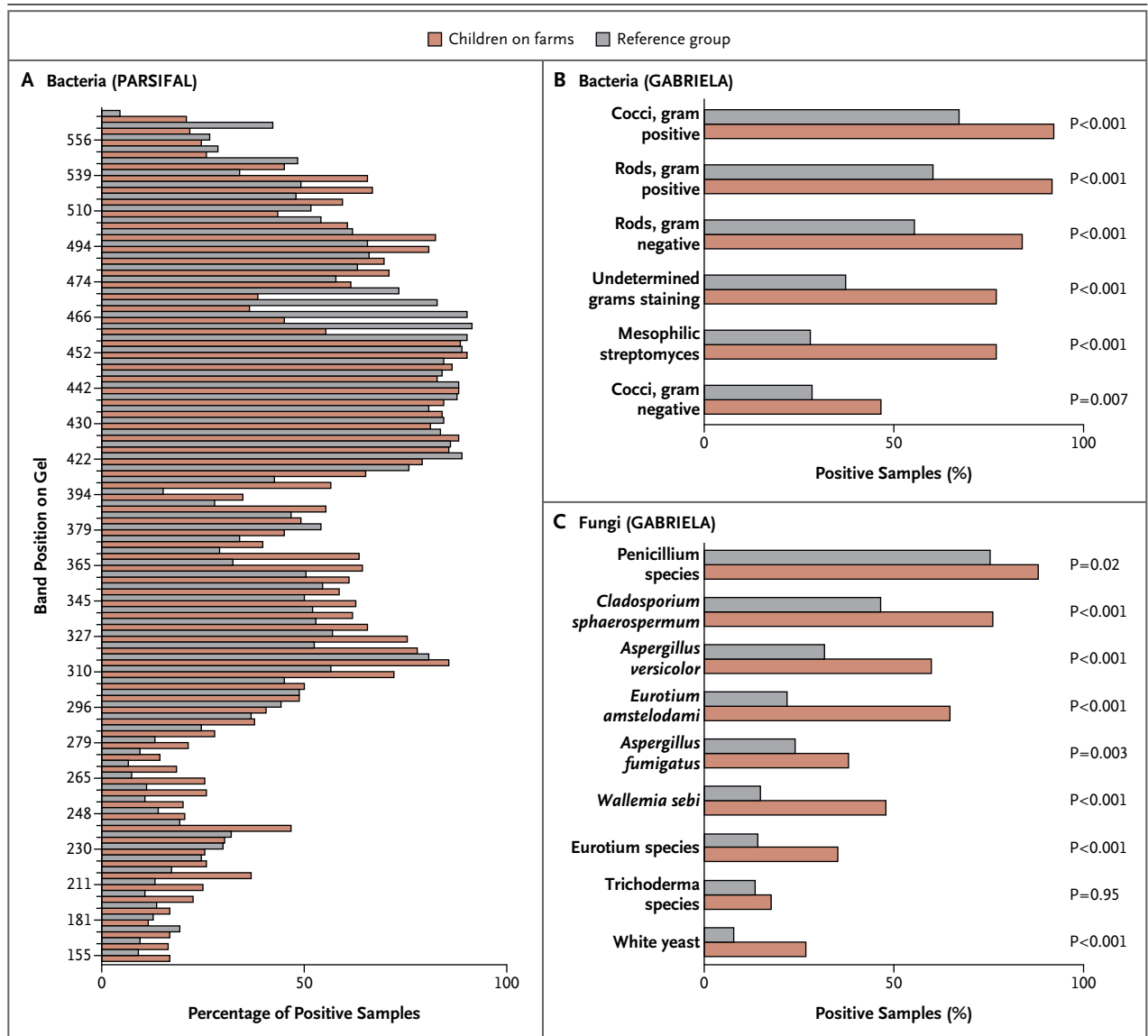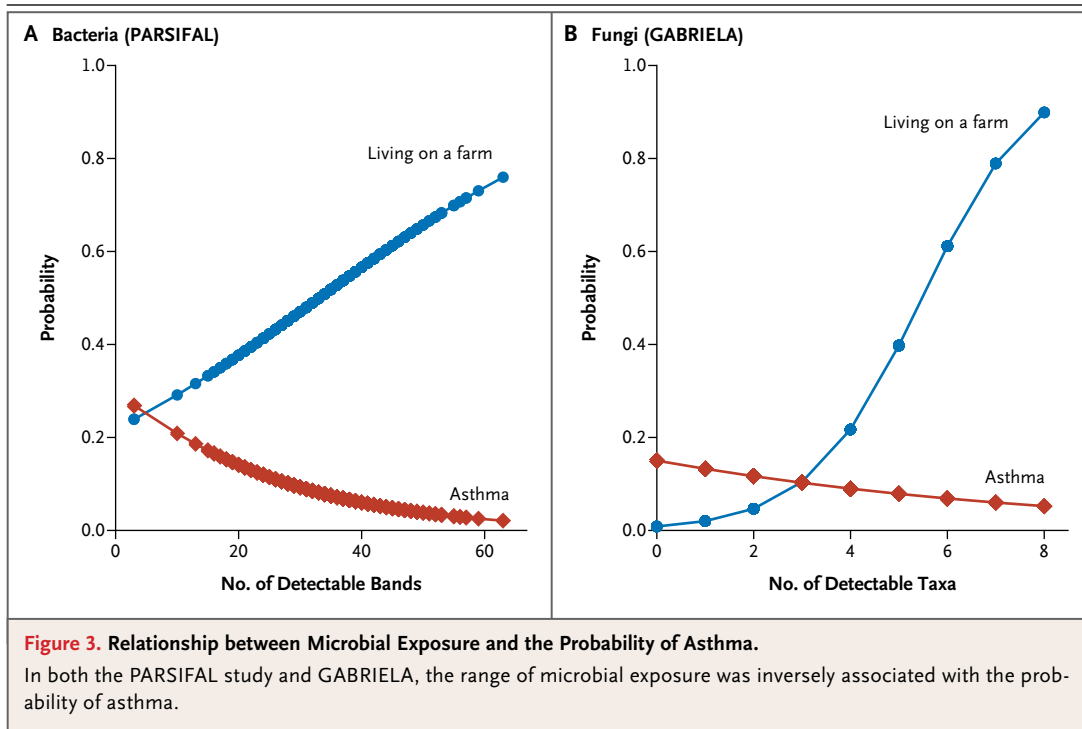
Figure 2. Detection of Environmental Microorganisms in Dust Samples in the PARSIFAL Study and GABRIELA.

In the PARSIFAL study (Panel A), samples of mattress dust were screened for bacterial origin with the use of single-strand conformation polymorphism (SSCP) analysis, with positive samples defined as those with detectable SSCP bands. In GABRIELA (Panels B and C), settled dust from children's rooms was evaluated for bacterial and fungal taxa with the use of culture techniques. The listed microbes were present in at least 10% of all samples.

dren living on farms than among the children in the reference group.

The findings indicate that indoor microbial exposure is much more common and diverse in the farming environment than in the nonfarming environment of the reference group. As illustrated in Figure 3, living on a farm was positively associated with the number of detectable bands (odds ratio for each additional 10 bands, 1.47; 95% CI, 1.20 to 1.80; P<0.001) and the number of fungal taxa (odds ratio for each additional taxon, 2.38; 95% CI, 1.89 to 3.00; P<0.001). The risk of asthma decreased significantly with the increase in the number of detectable bands in the PARSIFAL study and with the number of fungal taxa in GABRIELA (Fig. 3 and Table 2). The inverse associations of the diversity scores with asthma were not confounded by status with respect to living on a farm because adjustment did not change the respective point estimates for

Figure 3. Relationship between Microbial Exposure and the Probability of Asthma.
In both the PARSIFAL study and GABRIELA, the range of microbial exposure was inversely associated with the probability of asthma.

asthma (Table 2), although the associations became nonsignificant. In turn, the diversity scores shifted the point estimates for the effect of farm residence on asthma toward unity by 26% in the PARSIFAL study and by 98% in GABRIELA, thereby explaining the effect of the farming environment on asthma partially in the PARSIFAL study and almost completely in GABRIELA. In contrast, atopy was only weakly associated with the diversity scores (Table 2) and the association was confounded by living on a farm (Table 2); the diversity score itself accounted for only 1% of the estimated association between living on a farm and atopy in the PARSIFAL study and for only 19% in GABRIELA.

HOT SPOTS OF MICROBIAL EXPOSURE

In an attempt to reduce the complexity of the diversity scores, a factor analysis was performed with the PARSIFAL data set. Of the 10 extracted factors (Table 3 in the Supplementary Appendix), 2 showed a significant inverse association with asthma after adjustment for living on a farm (Table 2), although only the association between factor 5 and asthma withstood correction for multiple comparisons. Both factors were positively related to living on a farm and explained 22% of the effect of the farming environment on the prevalence of asthma. The bands with the highest loadings on factor 5 contained the sequences of *Listeria monocytogenes*, *Bacillus licheniformis* and other bacillus species, corynebacterium species, methylobacterium species, xanthomonas species, enterobacter species, pantoea species, and others. The highest loadings on factor 4 were in bands coding for *Staphylococcus sciuri* and other staphylococcus species, salinicoccus species, macrococcus species, bacillus species, jeotgalicoccus species, and others. No association with atopy was detected for any factor in the PARSIFAL study (Table 3 in the Supplementary Appendix).

Of 15 taxa in GABRIELA (Table 4 in the Supplementary Appendix), only eurotium and penicillium species were inversely related to asthma after adjustment for living or not living on a farm (Table 2); penicillium species, however, did not withstand correction for multiple comparisons. The only significant determinant for atopy was the presence of gram-negative rods; this finding explained 30% of the effect of the farming environment on atopy. Adjustment for the diversity scores did not alter the effects of the factors or taxa, whereas the estimates of the diversity scores shifted toward unity (Table 2).

Table 2. Associations of Asthma and Atopy with Measures of Microbial Diversity and with Specific Microbial Exposures.

Microbial Exposure	Unadjusted Odds Ratio (95% CI)	P Value	Odds Ratio Adjusted for Living on a Farm (95% CI)	P Value	Mutually Adjusted Odds Ratio (95% CI)*	P Value
Asthma						
PARSIFAL						
Diversity score†	0.62 (0.44–0.89)	0.01	0.65 (0.45–0.94)	0.02	0.83 (0.55–1.24)	0.36
Factor 4‡	—		0.62 (0.42–0.91)	0.01	0.67 (0.44–1.01)	0.06
Factor 5‡	—		0.53 (0.35–0.81)	0.003§	0.57 (0.38–0.86)	0.007
GABRIELA						
Diversity score¶	0.86 (0.75–0.99)	0.04	0.87 (0.73–1.03)	0.09	1.01 (0.86–1.19)	0.93
Eurotium species	—		0.37 (0.19–0.71)	0.003§	0.37 (0.18–0.76)	0.007
Penicillium species	—		0.56 (0.32–0.99)	0.04	0.57 (0.31–1.05)	0.07
Atopy						
PARSIFAL						
Diversity score†	0.79 (0.60–1.04)	0.09	0.86 (0.65–1.15)	0.309	—	
GABRIELA						
Diversity score¶	0.88 (0.77–1.01)	0.07	0.93 (0.79–1.11)	0.435	0.97 (0.81–1.15)	0.723
Gram-negative rods	—	—	0.45 (0.27–0.76)	0.003§	0.46 (0.27–0.78)	0.004

* Mutual adjustment means that all the exposure variables were entered into the same model for the analysis of asthma (the diversity score and factors 4 and 5 in the PARSIFAL study and the diversity score and eurotium and penicillium species in GABRIELA) and the analysis of atopy (the diversity score and gram-negative rods in GABRIELA).
† The odds ratios are for each increase of 10 bands.
‡ Factors 4 and 5 are from a factor analysis of band densities.
§ P<0.05 if Bonferroni's correction was applied. In the PARSIFAL study, 10 factors were tested simultaneously; in GABRIELA, 15 independent taxa were tested simultaneously.
¶ The odds ratios are for each increase of one taxon.

DISCUSSION

Children growing up on farms in Central Europe were protected from asthma and atopy. These children were exposed to a greater variety of environmental fungi and bacteria as compared with children in the reference group who lived in the same regions. The greater diversity of environmental microbial exposure was inversely related to asthma, but not to atopy, independently of farming. These data support the idea that the greater diversity of microbial exposure among children who live on farms is associated with protection from the development of asthma. Within the spectrum of microbial diversity detected, several focal zones were associated with a greater protective potential than others. On a species level, however, the identification of protective microorganisms was not possible.

The transport of environmental microorganisms from animal sheds and barns to the indoor environment has been reported.[13] The relationship between microbial exposures and health outcomes has been assessed in school-age children, although exposures may be more relevant when children are younger.[2] However, dairy farming, a known source of microbial exposures, has been constant over time, indicating that our findings probably reflect the effects of both current and long-term exposures with considerable accuracy.

Even when indoors, children living on farms were exposed to a greater variety of microbes than children who did not live on farms, as indicated by the distribution of the frequencies of detectable bands or taxa and the summation scores for microbial diversity. The central finding of this analysis was the inverse association of the diversity scores with asthma, which was not confounded by living on a farm. Moreover, the diversity scores explained a substantial proportion of the effect of the farming environment

on asthma. Obviously, summation scores are imperfect measures of diversity, since they do not account for cross-correlation patterns and might be influenced by specific effects. In the PARSIFAL data set, however, it was possible to conduct a sensitivity analysis with the use of the results from the factor analysis before rotation. By definition, the first unrotated factor explained most of the variance of all bacterial bands. Because 76% of all variables loaded substantially on this factor (loadings >0.3), this factor can be interpreted as a diversity score that accounts for the cross-correlation matrix of all bacterial bands. This modified diversity score showed the same strong association with asthma and farm residence as did the summation score. It was not driven by specific effects, since the loadings of all band variables were below 0.6.

We can speculate about how the diversity of microbial stimuli may be protective against asthma. Microorganisms trigger the innate immune system through pattern-recognition receptors, such as the toll-like receptors. Activation of several toll-like receptors has been found in children exposed to farming environments.[14,15] Combinations of microbial exposures may activate several signaling pathways downstream of these receptors, with subsequent induction of regulatory T cells.[16] Type 1 helper T cells may be activated and may counterbalance the predominance of type 2 helper T cells that is characteristic of asthma.[17] Mucosal immunity may also play a specific role in the abrogation of response by type 2 helper T cells.[18]

An alternative interpretation of diversity may be found in the counterbalancing of specific detrimental exposures. Environmental exposure to a broad range of microorganisms may prevent colonization of the lower airways with harmful bacteria, which has been associated with an increased risk of asthma among children and adults.[19,20] Balanced colonization of the airways may parallel the beneficial effects of a diverse microbiome at other surfaces, such as the gut and skin.[21,22]

The notion of diversity as a summation of stimuli, however, is constrained by the facts that the number of pattern-recognition receptors is quite limited and the pathways of the innate immune system are redundant. Consequently, diversity in itself may not explain the protective effects against asthma mediated by the innate immune system, since small numbers of microbial exposures may be sufficient to stimulate all pattern-recognition receptors. Furthermore, overgrowth of harmful bacteria might be achieved by a limited number of bacterial species. Consequently, we sought to narrow the spectrum of microbial exposures to several focal zones. This approach led to the identification of two factors in the PARSIFAL study, each of them accounting for a number of correlated bands in our SSCP analysis, with each band representing several bacterial species with synonymous 16S rRNA sequences. The bacteria covered by factor 4 belong to the staphylococcaceae family, which has been reported to be the predominant bacterial family in samples of house dust from the Karelian region that has the lowest prevalences of atopic diseases.[23] One of the bacterial species related to factor 5, *L. monocytogenes,* had preventive effects on airway hyperresponsiveness and inflammation in a murine model of asthma.[24]

The analysis of microbial diversity and its relation to asthma in GABRIELA brought two fungal genera into focus: eurotium and penicillium. At first glance, these findings may challenge previous observations suggesting that molds may account for the increased risk of asthma ascribed to dampness.[25] However, molds are very heterogeneous, and different genera or species within very large taxa, such as penicillium species, may exert diverse effects. Eurotium species are the sexual form of certain aspergillus species. The identification of penicillium and eurotium as protective factors against asthma is supported by previously published data from the PARSIFAL study in which exposure to extracellular polysaccharides, a generic marker for exposure to aspergillus and penicillium species, was inversely related to the risk of childhood asthma.[15]

The assessment of bacterial diversity in GABRIELA was based mainly on Gram's staining and morphologic assessment. These broad categories did not allow a further refinement of the bacterial exposure. However, gram-negative rods were found to be protective against atopy. This finding parallels the inverse association between atopy and endotoxin, a cell-wall component of gram-negative bacteria, which has been reported in many previous studies.[3,26] An association of atopy with the diversity scores, however, was not found. This contrast between the findings for asthma and those for atopy may indicate that

microbial exposures affect the risks of asthma and atopy through different mechanisms; the contrast parallels that between the different genetic determinants of the two conditions.[27]

In conclusion, the results of both the PARSIFAL study and GABRIELA showed that children living on farms had a wider range of microbial exposures than children in the reference groups, which largely explained the protective effect of the farming environment on the development of asthma in children. Our methods do not allow us to identify specific microbes that may confer protection, but they have allowed us to identify broad families of species within microbial taxa that could be responsible for the effect of the farming environment. The challenge will be to identify these species with the precision needed to allow specific tests of the relationship between microbial exposure and protection against asthma.

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