ORIGINAL ARTICLE

Quantity and diversity of environmental microbial exposure and development of asthma: a birth cohort study

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Abstract

Background: Early-life exposure to environmental microbial agents may be associated with the development of allergies. The aim of the study was to identify better ways to characterize microbial exposure as a predictor of respiratory symptoms and allergies.

Methods: A birth cohort of 410 children was followed up until 6 years of age. Bacterial endotoxin, 3-hydroxy fatty acids, *N*-acetyl-muramic acid, fungal extracellular polysaccharides (EPS) from *Penicillium* and *Aspergillus* spp., β-D-glucan, ergosterol, and bacterial or fungal quantitative polymerase chain reactions (qPCRs) were analyzed from dust samples collected at 2 months of age. Asthma, wheezing, cough, and atopic dermatitis were assessed using repeated questionnaires. Specific IgEs were determined at the age of 1 and 6 years.

Results: Only few associations were found between single microbial markers and the studied outcomes. In contrast, a score for the total quantity of microbial exposure, that is, sum of indicators for fungi (ergosterol), Gram-positive (muramic acid) bacteria, and Gram-negative (endotoxin) bacteria, was significantly (inverted-U shape) associated with asthma incidence ($P < 0.001$): the highest risk was found at medium levels (adjusted odds ratio (aOR) 2.24, 95% confidence interval (95% CI) 0.87–5.75 for 3rd quintile) and the lowest risk at the highest level (aOR 0.34, 95% CI 0.09–1.36 for 5th quintile). The microbial diversity score, that is, sum of detected qPCRs, was inversely associated with risk of wheezing and was significantly (inverted-U shape) associated with sensitization to inhalant allergens.

Conclusion: Score for quantity of microbial exposure predicted asthma better than single microbial markers independently of microbial diversity and amount of dust. Better indicators of total quantity and diversity of microbial exposure are needed in studies on the development of asthma.

Microbial exposure may contribute to the development of allergic diseases, but the role of different agents and mechanisms are still unknown (1). Most epidemiological studies with microbial exposure assessment among children have been restricted to studies on endotoxin (2) or have used only a limited set of microbial markers (3–7). These microbial markers reflect typically only a narrow part of the total microbial exposure. Recently, some studies have applied combined indicators of microbial exposure (8, 9), like diversity of microbes, which appears to have a protective effect on

the development of asthma (8). Thus, there is a clear need to use combined indicators and other new approaches to describe exposure to environmental microbes.

The objective of this study was therefore to study prospectively the associations between several markers of environmental microbial exposure—including their combinations—and the development of doctor-diagnosed asthma, respiratory symptoms, atopic dermatitis, and sensitization up to the age of 6 years.

Methods

The study population consisted of children born in Eastern and Middle Finland: the first half of the study population ($N = 214$) belongs to a European birth cohort (PASTURE) (10) among farmers and nonfarmers, while the second half of the cohort is an extension to unselected children ($N = 228$) (11). Pregnant women were recruited, whose children were born between September 2002 and May 2005. The study protocol was approved by a local ethics committee in Finland (11). A written informed consent was obtained from the parents.

Follow-up

The first questionnaire was administered during the third trimester of pregnancy (11). The follow-up questionnaires (at the age of 2, 12, 18, and 24 months, and thereafter annually), except the questionnaire at 2 months, enquired about any wheezing, other respiratory symptoms apart from cold (wheezing, cough, nocturnal cough), and confounders for the time period after the preceding questionnaire. 'Asthma ever' was defined as first parent-reported doctor-diagnosed asthma and/or second diagnoses of asthmatic (or obstructive) bronchitis. 'Current asthma' was defined as those asthma ever cases who also reported the use of asthma medication and/or wheezing in past 12 months in the 6-year follow-up. 'Atopic dermatitis ever' was defined as first parent-reported doctor-diagnosed atopic dermatitis during the follow-up.

House dust samples

The protocols for dust collection are explained in detail in the Supplementary material. Briefly, in the Finnish PASTURE study, fieldworkers took two samples from living room floors for the present report. The first samples were processed without sieving at Utrecht University, where they were extracted and analyzed for endotoxin (as a marker of Gram-negative bacteria) and EPS as described previously

(12), and the stored residue after extraction was later analyzed for $\beta(1,3)$-glucans (as a marker of fungal biomass) by heat extraction and inhibition enzyme immunoassay (EIA) (13). The second samples were processed and analyzed at National Institute for Health and Welfare (THL), where they were sieved to remove larger particles, dried, split and then stored, and finally analyzed for muramic acid (as a marker of Gram-positive bacteria), ergosterol (as a marker of fungal biomass), and lipopolysaccharide ($LPS_{10:0-16:0}$) (as a marker of Gram-negative bacteria) by gas chromatography tandem mass spectrometry (GC-MS–MS) (14) and by quantitative polymerase chain reaction (qPCR) for microbial DNA (15–17).

In the extended cohort, results from only one dust sample from the living room floor collected by the families are used in the present report. The sample was sieved and dried at THL, then split in aliquots for various analyses: One aliquot was sent to Utrecht where it was extracted and analyzed for endotoxin, EPS, and $\beta(1,3)$-glucans (13), and other aliquots were analyzed for the chemical markers (muramic acid, ergosterol, $LPS_{10:0-16:0}$) (14) and microbial DNA (15–17).

Quantitative polymerase chain reaction (qPCR) (15–17) was used to determine microbes that have been suggested to be common in moisture-damaged buildings: two genera of Gram-positive bacteria, *Mycobacterium* spp. and *Streptomyces* spp., and six fungal species, genera, or groups: the assay group for *Aspergillus fumigatus/Neosartorya fischeri* (AspNeo group)*; Cladosporium* spp.; the combined assay group for *Penicillium* spp.*, Aspergillus* spp.*,* and *Paecilomyces variotii* (PenAsp group); *Stachybotrys chartarum; Trichoderma viride/atroviride/koningii* (*Trichoderma viride* group); and *Wallemia sebi*.

Exposure to all markers was expressed as loads, that is, the amount per m^2 sampling surface. As the laboratory procedures determine concentrations (agents per mg dust), loads are calculated by multiplying the concentrations with the measured dust weight per m^2. However, as endotoxin, EPS, and glucans in the PASTURE samples were determined in nonsieved dust, while all other analyses were performed with sieved dust, we calculated loads for the former parameters by multiplying with the load of sieved dust in the parallel sample taken for the other analyses. Weights for these parallel sieved and nonsieved dust samples in the Finnish PASTURE study showed a fair correlation (Spearman $r = 0.7$), but with a systematic difference: as expected, sieved dust samples contained on average 60% (95% CI 57, 64) less dust. Mean concentrations in sieved and nonsieved dust from nonfarm homes were, however, very similar, which suggests that endotoxin, EPS, or glucans are not preferentially bound to the smaller (sieved) or larger (removed by sieving) particle fractions.

Abbreviations

EPS, fungal extracellular polysaccharide antigens of *Penicillium* and *Aspergillus* species; GC-MS–MS, gas chromatography tandem mass spectrometry; LPS, lipopolysaccharide; $LPS_{10:0-:16:0:0}$, amount (mol) of LPS in each sample. The number of mol of 3-hydroxy fatty acids of $C_{10:0}$ to $C_{16:0}$ carbon chain length divided by 4; qPCR, quantitative polymerase chain reaction; PASTURE, Protection against Allergy STUdy in Rural Environments; OR, odds ratio; aOR, adjusted odds ratio; sIgE, specific immunoglobulin E; kU/L, kilo units per liter; GEE, general estimating equations; EIA, inhibition enzyme immunoassay.

Blood sampling

Venous blood samples collected at one ($N = 374$) and at 6 years of age ($N = 304$) were analyzed for specific immunoglobulin E (sIgE) to 13 inhalant (*Dermatophagoides pteronyssinus, D. farinae*, alder, birch, European hazel, grass pollen mixture, rye, mugwort, plantain, cat, horse and dog dander, and *Alternaria alternata*) and 6 food allergens (hen's egg, cow's milk, peanut, hazelnut, carrot, and wheat) (Mediwiss Analytic, Moers, Germany) (18). The cutoff levels for sIgE concentrations were 0.35 kU/l at one and 0.70 kU/l at 6 years of age. The higher cutoff was used at the age of 6 years due to high prevalence of sensitization (Table 1).

Statistical analyses

Generalized estimating equations (GEE) with an exchangeable correlation structure to account for correlation between repeated measures within subjects were used to determine associations between microbial exposure and repeated measures of parent-reported wheezing and cough at different ages. Survival analyses (discrete-time hazard models) were used in analyzing asthma ever, current asthma, and atopic dermatitis ever. Logistic regression was used to analyze sensitization to inhalant allergens. The results are presented as adjusted odds ratios (aORs) and their 95% confidence intervals (95% CI).

In the analysis of single microbial markers, the amounts of dust and microbial markers were generally divided into three categories using tertiles as cutoffs. For *Wallemia sebi*, the lowest category consisted of levels below the limit of detection (37.4%) and the rest of the values were evenly divided into two groups (medium and high). *Stachybotrys chartarum* and AspNeo group were dichotomized at the detection level due to high numbers of nondetected values. Microbial diversity score was defined as the sum of all the detected qPCR markers (range 0–8). Due to a low number of observations with low and high diversity, the variable was categorized into four classes: 0–4, 5, 6, and 7–8.

Total microbial quantity score was calculated as the sum of three markers for the three different microbial groups, that is, for fungi, Gram-negative bacteria, and Gram-positive bacteria (one marker per group). Single markers were first divided into five groups using quintiles as cutoffs with scores 0, 1, 2, 3, and 4 from lowest to highest and then summed.

Table 1 Incidence of doctor-diagnosed asthma and atopic dermatitis and the point prevalence of respiratory symptoms during the first 6 years and prevalence of sensitization to inhalant allergens at the age of 1 and 6 years

	At 1 year			At 1.5 years			At 2 years			At 3 years			At 4 years			At 5 years			At 6 years		
	N	n	%	N	n	%	N	n	%	N	n	%	N	n	%	N	n	%	N	n	%
Asthma ever*	389	6	1.5	353	9	2.6	332	14	4.2	291	15	5.2	271	9	3.3	257	6	2.3	247	3	1.2
Current asthma†	353	3	0.9	331	7	2.1	315	8	2.5	290	7	2.4	281	3	1.1	274	4	1.5	270	3	1.1
Wheezing																					
Apart from cold	391	41	10.5	358	24	6.7	358	25	7.0	338	23	6.8	358	29	8.1	346	22	6.4	357	25	7.0
Any	391	91	23.3	358	58	16.2	358	67	18.7	339	65	19.2	358	68	19.0	348	46	13.2	357	51	14.3
Cough																					
Apart from cold	391	98	25.1	358	66	18.4	358	85	23.7	338	80	23.7	357	91	25.5	348	87	25.0	357	94	26.3
Nocturnal	386	18	4.7	358	47	13.1	358	76	21.2	338	63	18.6	357	78	21.9	348	59	17.0	357	79	22.1
Atopic dermatitis ever‡	390	61	15.6	300	24	8.0	265	12	4.5	230	14	6.1	211	9	4.3	199	7	3.5	188	2	1.1
Sensitization to inhalant allergens§	374	78	20.9																304	115	37.8

N = in respirtory symptoms: total number of children with information on given symptom at each follow-up; in sensitization: total number of children with IgE results at a given time point; in asthma ever: total number of children at each follow-up who has information on asthma ever at each follow-up and has not censored from the follow-up due to onset of asthma ever; in current asthma: total number of children at each follow-up who has information on asthma ever and asthma medication/any wheezing at each follow-up and has not censored from the follow-up due to onset of current asthma; in atopic dermatitis ever: total number of children at each follow-up who has information on atopic dermatitis at each follow-up and has not censored from the follow-up due to onset of atopic dermatitis. n = the number of children with the presence of wheezing/cough symptom or with positive IgE result, or the number of newly diagnosed asthma/current asthma/atopic dermatitis cases at each follow-up.

*Doctor-diagnosed asthma ever is defined as doctor-diagnosed asthma at least once and/or doctor-diagnosed asthmatic bronchitis more than once during the 6-year follow-up.

†Current asthma is defined as asthma ever with asthma medication and/or wheezing symptom past 12 months at the age of 6 years.

‡Atopic dermatitis ever is defined as docotor-diagnosed astopic dermatitis during the follow-up.

§Atopic sensitization is defined as any inhalant sIgE ≥0.35 kU/l at the age of 1 year and ≥0.70 kU/l at the age of 6 years.

All possible combinations with measured microbial markers were used to create four different total quantity scores for microbial exposure: (1) ergosterol, endotoxin, and muramic acid; (2) β-D-glucan, endotoxin, and muramic acid; (3) ergosterol, $LPS_{10:0-16:0}$, and muramic acid; and (4) β-D-glucan, $LPS_{10:0-16:0}$, and muramic acid. The created variables, with ranges from 0 to 12, were divided into five groups using quintiles as cutoffs for statistical analyses. Linear and quadratic trends in the associations between microbial diversity or total quantity scores and health outcomes were tested using polynomial contrasts [19].

All models were adjusted for study cohort, farming, and well-known risk factors for asthma (maternal history of allergic diseases, gender, number of older siblings, smoking during pregnancy). The models of total microbial quantity score (ergosterol, endotoxin, and muramic acid) and asthma ever, atopic dermatitis ever, and sensitization to inhalant allergen were carefully tested for 20 additional confounding factors, which have been described earlier [12]. If a confounder changed the estimates of the total microbial quantity score by more than 10%, all the models of the outcome were adjusted for the factor. All the models of respiratory symptoms and current asthma were adjusted for the same confounding factors as in the asthma ever models. Given an exposure prevalence of 25%, power calculations (β = 80%, α = 0.05) show that with these data, we can detect an odds ratio of 2.52 for asthma ever with a population prevalence of 14%. The data were analyzed using SAS 9.2 for Windows (SAS lnc., Cary, NC, USA).

Results

The present analyses included all those 410 children with data on at least one microbial marker available in any of the quantity scores. Of those, 341 (83%) had the results of all microbial determinations. During the 6-year follow-up, 62 of 389 children with data on asthma developed asthma, and of those children, 35 still had current asthma at the age of 6 years (Table 1). Distributions of all microbial markers are shown in Table 2.

Most correlations between the amount of house dust and single microbial markers as well as intercorrelations between two microbial markers together were between 0.30 and 0.85 (Table S1). Total microbial quantity scores were correlated

Table 2 Levels of microbial markers

				Percentiles			
	N	<LOD*	(%)	25th	Median	75th	95th
Amount of dust (mg/m²)†	410	0		173	311	587	1332
Gram-positive bacteria or marker of Gram-positive bacteria							
Mycobacterium (cells/m²)	399	21	(5.3)	186 857	641 427	1 753 136	7 041 045
Streptomyces (cells/m²)	399	7	(1.8)	3923	13 089	45 939	462 289
Muramic acid (ng/m²)	386	7	(1.8)	3122	6616	13 713	34 852
Marker of Gram-negative bacteria							
LPS $_{10:0-16:0}$ (nmol/m²)	398	2	(0.5)	3	8	15	46
Endotoxin (EU/m²)	372	0		2668	6253	13 485	66 569
Fungi or marker of fungi							
PenAsp group (cells/m²)	399	3	(0.8)	13 580	59 405	233 616	1 282 236
Trichoderma viride group (cells/m²)	400	62	(15.5)	340	2028	9906	153 816
Wallemia sebi (cells/m²)	400	152	(38.0)	0	883	11 541	152 041
Cladosporium (cells/m²)	399	50	(12.5)	3652	25 683	104 690	982 138
AspNeo group (cells/m²)	400	320	(80.0)	0	0	0	14 254
Stachybotrys chartarum (cells/m²)	400	390	(97.5)	0	0	0	0
EPS (EPSU/m²)	374	15	(2.1)	7079	15 312	38 025	126 107
β-D-glucan (μg/m²)	376	0		511	970	1818	4066
Ergosterol (ng/m²)	401	0		1473	3015	7040	21 978
Quantity scores							
Ergosterol, endotoxin, and muramic acid	347			3	6	9	12
β-D-glucan, endotoxin, and muramic acid	352			3	6	9	12
Ergosterol, $LPS_{10:0-16:0}$, and muramic acid	380			3	6	9	12
β-D-glucan, $LPS_{10:0-16:0}$, and muramic acid	354			3	7	9	12
Diversity score‡	399			5	6	6	7

*LOD = the level of detection, percentage of samples under detection level from analyzed samples (%).
†The amount of dust refers to dust samples used for analyses of qPCRs, ergosterol, muramic acid, and LPS (sieved).
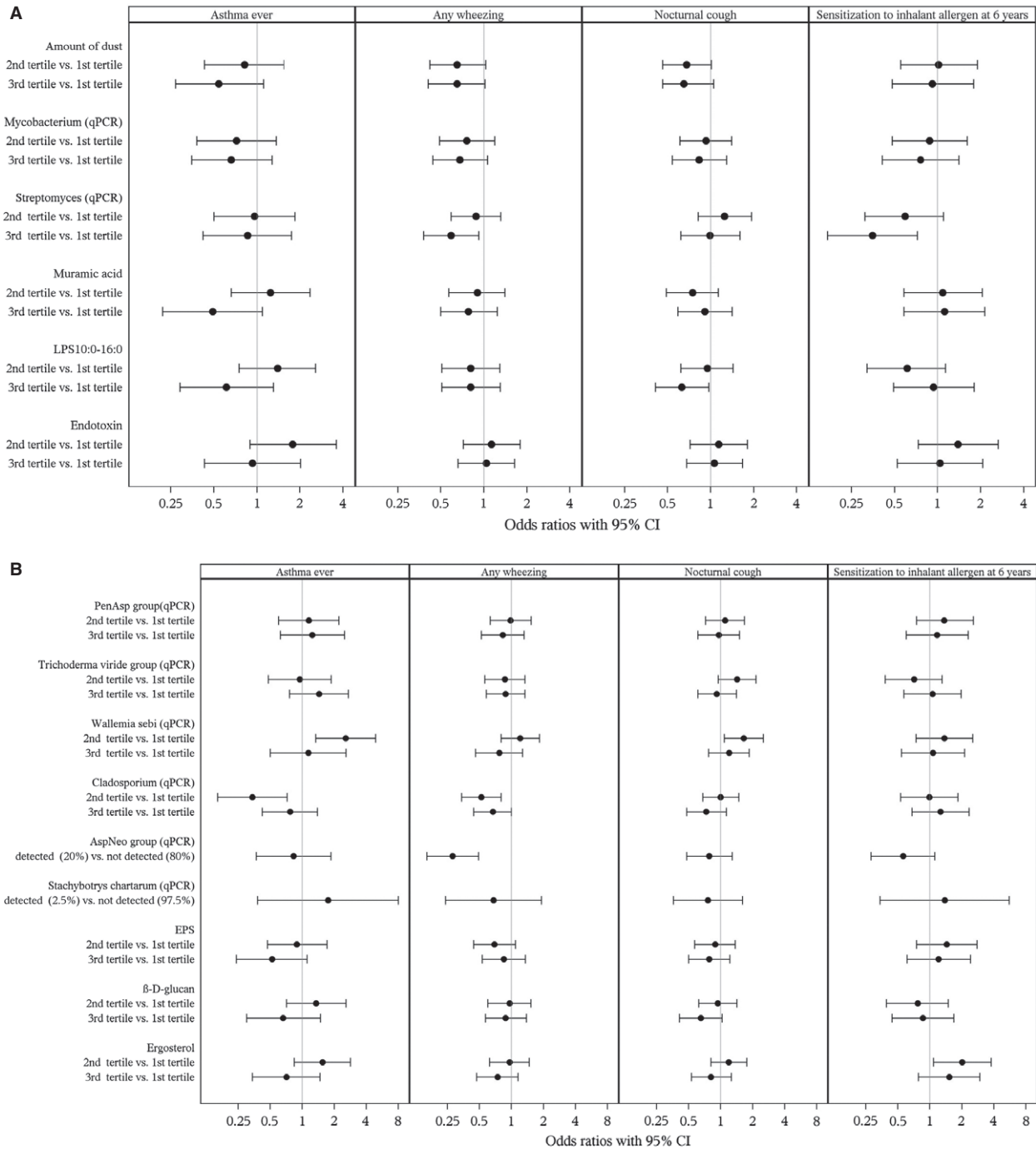‡The number of detected 6 fungal or 2 bacterial qPCRs.

Figure 1 Adjusted associations between amount of dust and bacterial (A) and fungal (B) exposure at the age of 2 months and asthma and respiratory symptoms during the first 6 years of life and sensitization to inhalant allergen at the age of 6 years. Models are adjusted for study cohort, farming status, gender, maternal history of allergic diseases (hay fever, atopic dermatitis, and/or asthma), smoking during pregnancy, the number of older siblings, and living area of the home. Models of sensitization to inhalant allergens (cut point ≥0.70 kU/l) are additionally adjusted for floor type of dust sampling.

with each other (around $r = 0.90$) and also with amount of dust (around $r = 0.85$), but correlations between total microbial quantity scores and qPCR results were lower (around $r = 0.30$), except for *Mycobacterium* and the PenAsp group

(around $r = 0.50$). Diversity score had low correlations with amount of dust, all microbial markers, and total microbial quantity scores (around $r = 0.20$), but higher with *Wallemia sebi* ($r = 0.66$).

In general, the associations of single microbial markers with asthma ever and respiratory symptoms were mostly nonsignificant, while the shape of the association varied (Fig. 1). Total microbial quantity score had a significant inverted-U-shaped association with asthma ever: the highest risk for asthma ever was found with the medium level of total microbial quantity score and the lowest risk with the highest quantity score (Fig. 2, Table 3). The association was independent of the diversity score. The associations were quite similar to current asthma (Table 3) and when using other microbial markers than ergosterol, endotoxin, and muramic acid to create the total microbial quantity score (Table S2) as well as when using EPS as a marker of fungi (data not shown). Total microbial quantity scores tended to be inversely associated with respiratory symptoms (Table S2). The inverted-U-shaped association between total microbial quantity score and asthma ever tended to be stronger after adjusting for the amount of dust (data not shown). Adjustment for dust also strengthened the associations between asthma, and *Streptomyces*, endotoxin, PenAsp group, β-D-glucan and ergosterol, but not the other single microbial markers (data not shown).

The associations between total microbial quantity score and any wheezing were similar among children who were sensitized against at least one of the tested inhalant or food allergens at the age of 6 years and who were not sensitized (*P*-value = 0.66 for interaction term). In contrast, in the stratified analyses, the inverted-U-shaped associations between the quantity scores and asthma ever were always stronger among nonatopic than among atopic children, but the *P*-values for interaction ranged between 0.08 and 0.28 (data not shown). There were no interactions with birth cohorts (*P*-values ≥ 0.27 for interaction terms). Associations between total microbial quantity score and any wheezing were also similar in farms and in rural/suburban areas (*P*-value = 0.58 for interaction term). Due to

low numbers of children with asthma ever among farmers, interaction tests between total microbial quantity score and farming on asthma ever could not be performed.

Diversity score was associated with a decreased risk of any wheezing, and a similar tendency was seen with asthma ever and current asthma (Table 3). The association with asthma ever was, however, fairly sensitive to different adjustments, especially adjustment for farming. The association with any wheezing was similar among children from farms and from rural/suburban areas as well as children who were sensitized against at least one of the tested inhalant or food allergens at the age of 6 years and who were not sensitized (*P*-values ≥ 0.4 for interaction terms) (data not shown).

Most of the associations between single microbial markers and atopic dermatitis and sensitization to inhalant allergens at the age of 1 (Table S3) and 6 years (Fig. 1) were nonsignificant. Total microbial quantity score was not associated with atopic dermatitis or sensitization to inhalant allergen at the age of either 1 year (data not shown) or 6 years (Table 3). Diversity score was significantly (inverted-U shape) associated with sensitization to inhalant allergen at the age of 6 years (Table 3), while similar tendency, though nonsignificant, was seen with sensitization to inhalant allergen at 1 year (Table S4). No association was found between diversity score and atopic dermatitis (Table S4).

Discussion

The present study suggests that an index of total quantity of environmental microbial exposure may predict asthma better than single microbial markers, independently of microbial diversity score and amount of dust. The diversity score decreased the risk of wheezing and was significantly (inverted-U shape) associated with sensitization to inhalant allergens. Only a few associations were observed between single microbial markers and the outcomes studied.

This is the first study using an index of total microbial quantity score when studying the risk of asthma. Previously, one cross-sectional study found a tendency of inverse association between microbial quantity score ($LPS_{10:0-16:0}$, ergosterol, and muramic acid) and allergic sensitization (9). Typically, epidemiological studies have used only one microbial marker as a surrogate for total microbial exposure. However, it is clear that such assessments provide an incomplete picture of the total quantity and quality of human microbial exposure. Due to high correlations, a major difficulty of earlier studies has been to separate the effects of single microbial markers from each other and from the effect of the amount of dust (5, 20). In the present study, total microbial quantity score predicted the risk of asthma independently of microbial diversity and amount of dust.

The reason behind the observed association between total microbial quantity score and asthma is unclear. A potential explanation is that different components of microbial groups or genera share common antigen or molecule structures on their cell wall surface, which are detected by pattern recognition receptors in innate immunity cells and have a profound influences on the immune system (21). If total exposure to
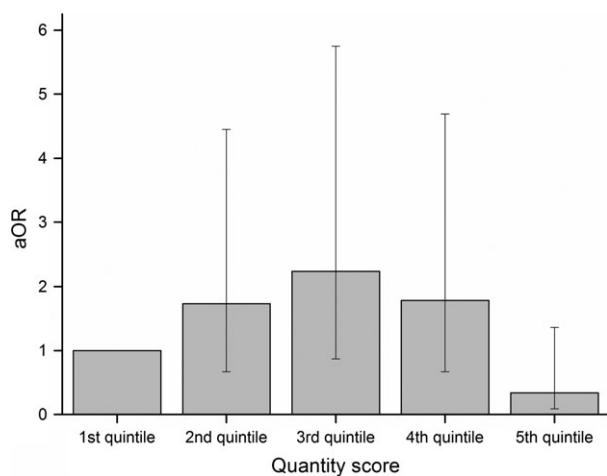

Figure 2 Risk of asthma in relation to total microbial quantity score in quintiles (sum of ergosterol, endotoxin, and muramic acid) (aOR). Model is adjusted for study cohort, farming status, gender, maternal history of allergic diseases (hay fever, atopic dermatitis, and/or asthma), smoking during pregnancy, the number of older siblings, living area of the home, and diversity score.

Table 3 Adjusted associations between microbial quantity or diversity scores and asthma or respiratory symptoms up to the age of 6 years and current asthma and sensitization to inhalant allergen at 6 years of age

	Asthma ever				Current asthma				Any wheezing¥	Nocturnal cough¥	Sensitization to inhalant allergens at 6 years¤		
	N#	n	N‡	aOR (95% CI)	N#	n	N‡	aOR (95% CI)	aOR (95% CI)	aOR (95% CI)	N	n	aOR (95% CI)
Quantity score£													
Reference (1st quintile)	63	8	354	1	56	5	352	1	1	1	53	19	1
2nd quintile	73	12	390	1.73 (0.67–4.45)	63	6	380	1.32 (0.38–4.61)	0.65 (0.35–1.22)	0.68 (0.39–1.21)	57	26	2.19 (0.92–5.23)
3rd quintile	62	13	300	2.24 (0.87–5.75)^	57	7	297	2.01 (0.59–6.79)	0.89 (0.49–1.62)	0.86 (0.48–1.55)	44	17	1.42 (0.55–3.64)
4th quintile	56	11	311	1.78 (0.67–4.69)	53	7	318	1.86 (0.55–6.32)	0.98 (0.55–1.75)	0.70 (0.40–1.24)	47	17	1.32 (0.51–3.40)
5th quintile	75	3	438	0.34 (0.09–1.36)	67	2	409	0.39 (0.07–2.11)	0.73 (0.43–1.24)	0.68 (0.38–1.20)	57	20	1.50 (0.61–3.70)
P-value				<0.001¶				0.03¶	0.95¶	0.74¶			0.46¶
Diversity score§													
Reference (0–4)	45	9	232	1	37	8	215	1	1	1	33	11	1
5	108	13	566	0.69 (0.27–1.78)	95	7	557	0.30 (0.10–0.89)*	0.68 (0.38–1.20)	0.90 (0.50–1.62)	81	36	2.10 (0.84–5.27)
6	133	22	736	1.03 (0.43–2.49)	122	10	731	0.35 (0.12–0.94)*	0.54 (0.30–0.97)*	1.23 (0.71–2.15)	108	46	2.16 (0.86–5.45)
7–8	43	3	259	0.64 (0.15–2.76)	42	2	253	0.40 (0.07–2.29)	0.14 (0.06–0.32)**	1.21 (0.58–2.50)	36	6	0.42 (0.12–1.50)
P-value				0.67†				0.31†	<0.001†	0.45†			0.001¶

£Sum of quintiles of loads of ergosterol, endotoxin, and muramic acid.
§Number of detected qPCRs.
Models are adjusted for time, study cohort, farming status, gender, maternal history of allergic diseases (hay fever, atopic dermatitis, and/or asthma), smoking during pregnancy, the number of older siblings, living area of the home, and either diversity score or quantity score.
N# Total number of children in the beginning of follow-up, n = number of cases in each category.
N‡ Subjects contributed up to seven repeated observations to this survival analysis using discrete-time hazard models (DTH).
¥Subjects contributed up to seven repeated observations to this analysis using generalized estimating equations.
¤Models are additionally adjusted for floor type of dust sampling.
P-value ^<0.1, *<0.05, **<0.01, †P-value for linear trend test, ¶P-value for quadratic trend test.

such structures is the main causal factor explaining the association between microbial exposure and lower risk of allergic diseases, then total microbial quantity score is likely to better reflect this exposure than single microbial markers. Varying correlations of single microbial markers with total microbial exposure would then explain the often weak and conflicting findings from earlier studies. In addition, some studies have shown synergistic interactions between different exposing agents (22), and an index of total microbial quantity score may capture such synergistic effects. Data from deep sequencing of microbial exposure (23) will give opportunities to explore further the above potential mechanisms.

Total microbial quantity score had an inverted-U-shaped association with asthma. Previous studies have suggested that the immune system may react first with enhanced response and subsequently with tolerance (24). Associations of similar shape have also been reported earlier between allergen levels and β-D-glucan, and risk of asthma, wheezing, or atopic sensitization (25–27). The shape of the association in the present study may also relate to some unknown characteristics of the present study, given that some single microbial markers also showed an association of a similar shape. Further studies in larger cohorts and in different settings are needed to better define the shape of the association between total microbial quantity score and risk of asthma.

We found a similar inverse association between measured microbial diversity score and wheezing, as has been reported with asthma in two recent cross-sectional studies (8). The association with asthma was nonsignificant, though in the same direction. There were differences in the microbiological methods used between the present study and previous studies. We used qPCR, a method that quantitatively describes the presence of specific viable and nonviable organisms of prior chosen microbes (28). The previous studies either cultivated viable microbes (GABRIELA study) or used single-strand conformation polymorphism analyses, which is a qualitative rather than quantitative method (PARSIFAL study) (8). We calculated a diversity score based on 6 fungal and 2 bacterial qPCR assays representing either microbial species, genera, or groups, which hence only provides a suggestive and rough estimate of the overall microbial diversity. Nonetheless, the results are in line with previous studies (8) targeting a broader spectrum of microbes. Taken together, the studies suggest that microbial diversity score may predict asthma and may be a useful marker to describe the quality of microbial exposure.

The observed associations between studied health outcomes and single microbial markers should be interpreted with caution, as no adjustments for multiple testing were done. The most interesting findings were maybe the protective associations in the case of Gram-positive bacteria (*Mycobacterium* spp., *Streptomyces* spp., and muramic acid), which have been reported earlier for muramic acid (6, 29).

The main strengths of the present study are the prospective birth cohort design with high participation rate (varied between 80% and 95% in each follow-up) and a good variety of measured markers of exposure to environmental microbes. The small size of our study cohort is the main limitation of the study, and the results need to be confirmed in further stud-

ies. The microbial markers and combinations of those used to build total microbial quantity scores in the present study do not measure total microbial exposure without error, for example using muramic acid together with endotoxin overestimates the amount of Gram-negative bacteria, because muramic acid is found also in Gram-negative bacteria, although to a lesser extent (30). These weaknesses do not, however, appear to be a major problem, as the different quantity scores used in the present study showed very similar associations.

In conclusion, our indicator for total quantity of microbial exposure predicted asthma better than single microbial markers. Detected associations were independent of the measured microbial diversity and amount of dust. Better indicators of total quantity and diversity of microbial exposure are needed in studies on the development of asthma.

Author contributions

All authors approved the submitted version. Karvonen involved in statistical data analyses, data collecting, manuscript preparation, and drafting. Hyvärinen and Rintala involved in dust sampling assessment, microbial analyses, and manuscript preparation. Korppi and Remes involved in authority on pediatric issues and manuscript preparation. Tàubel, Keski-Nisula, and Lampi involved in manuscript preparation. Doekes and Gehring involved in microbial analyses and manuscript preparation. Renz and Pfefferle involved in IgE measurements and manuscript preparation. Genuneit involved in data management and manuscript preparation. von Mutius and Pekkanen involved in study concept and design, and manuscript preparation.

Conflicts of interest

The authors declare that they have no conflicts of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

   Table S1. Spearman correlations of microbial markers per sampled area ($/m^2$).

Table S2. Adjusted associations between quantity or diversity scores and asthma or respiratory symptoms.

Table S3. Adjusted associations between microbial exposure and atopic dermatitis up to the age of 6 years and sensitization to inhalant allergen at the age of 1 year.

Table S4. Associations between microbial diversity and atopic dermatitis ever until the age of 6 years and sensitization to inhalant allergen at the age of 1 and 6 years.

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