

Original article

Bacterial and fungal components in house dust of farm children, Rudolf Steiner school children and reference children – the PARSIFAL Study

Background: Growing up on a farm and an anthroposophic lifestyle are associated with a lower prevalence of allergic diseases in childhood. It has been suggested that the enhanced exposure to endotoxin is an important protective factor of farm environments. Little is known about exposure to other microbial components on farms and exposure in anthroposophic families.

Objective: To assess the levels and determinants of bacterial endotoxin, mould $\beta(1,3)$ -glucans and fungal extracellular polysaccharides (EPS) in house dust of farm children, Steiner school children and reference children.

Methods: Mattress and living room dust was collected in the homes of 229 farm children, 122 Steiner children and 60 and 67 of their respective reference children in five European countries. Stable dust was collected as well. All samples were analysed in one central laboratory. Determinants were assessed by questionnaire.

Results: Levels of endotoxin, EPS and glucans per gram of house dust in farm homes were 1.2- to 3.2-fold higher than levels in reference homes. For Steiner children, 1.1- to 1.6-fold higher levels were observed compared with their reference children. These differences were consistently found across countries, although mean levels varied considerably. Differences between groups and between countries were also significant after adjustment for home and family characteristics.

Conclusion: Farm children are not only consistently exposed to higher levels of endotoxin, but also to higher levels of mould components. Steiner school children may also be exposed to higher levels of microbial agents, but differences with reference children are much less pronounced than for farm children. Further analyses are, however, required to assess the association between exposure to these various microbial agents and allergic and airway diseases in the PARSIFAL population.

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Several studies have shown that growing up on a farm protects against the development of atopic diseases (1–6). Contact to livestock has been suggested to account for this association (1, 2). It has been speculated that farm children are exposed to higher levels of microbial compounds, which may stimulate innate immunity and suppress atopic sensitization. Indeed, elevated levels of endotoxin, an intrinsic part of the outer membrane of Gram-negative bacteria, have been found in mattresses of farm children when compared with nonfarm children in Germany, Austria and Switzerland. Also nonfarm children with regular contact to farm animals were exposed to elevated endotoxin levels (7).

The same study showed that the endotoxin levels in mattress dust were inversely related to the prevalence of hay fever, atopic asthma, and atopic sensitization (8). An increased expression of receptors for microbial compounds (Toll-like receptor 2 and CD14) in farm children was also observed, and thought to be due to the higher endotoxin exposure (9). However, the Toll-like receptors are not specific for endotoxin (9), and the observed changes may thus also be due to other microbial components, like $\beta(1 \rightarrow 3)$ -glucans, cell wall constituents of most fungi, with known immunomodulatory effects (10–15).

Occupational studies have shown that farmers are exposed to high levels of both bacteria and fungi in animal houses (16, 17) but little is known of their domestic exposure. We aimed to investigate whether the levels of fungal components in house dust are, like endotoxin, higher in farm children when compared with reference children. We therefore assessed levels of house dust-associated $\beta(1 \rightarrow 3)$ -glucans and extracellular polysaccharides (EPS) from *Aspergillus* and *Penicillium* spp., which both have been shown to be associated with total culturable fungi in house dust, and thus may be good markers for indoor fungal exposure (18). We further studied whether differences in bacterial and fungal exposure between farm children and reference children would be similar in five European countries, with large differences in farming practices, which may influence indoor microbial agent levels differently (19, 20).

A low prevalence of atopic diseases and sensitization has also been found in children attending Rudolf Steiner schools, who predominantly come from families adhering to an anthroposophic lifestyle (21). While that study showed an inverse relation between the number of characteristic features of an anthroposophic lifestyle (like restricted use of antibiotics and immunizations) and risk of atopy (21), no attention was given thus far to possible differences in exposure to house dust-associated microbial agents in this population. We therefore also assessed levels of these agents in house dust from Rudolf Steiner school children and reference children.

Finally, we investigated whether exposure differences between the study groups and between countries could be

explained by parent-reported differences in home and family characteristics.

Methods

PARSIFAL

In 2000, the cross-sectional PARSIFAL (Prevention of Allergy – Risk factors for Sensitization In children related to Farming and Anthroposophic Lifestyle) study on the association between environmental and lifestyle factors and allergies and asthma was initiated. The goal of PARSIFAL is to explore factors characteristic of farming and anthroposophic populations that may protect against the development of atopic diseases in children. A total of 14 901 children aged 5–13 years were included among farmers, families with an anthroposophic lifestyle, and respective reference groups in five European countries: Austria, Germany, the Netherlands, Sweden, and Switzerland. Children with an anthroposophic lifestyle were recruited through Rudolf Steiner schools. The reference children were selected such that they lived in the neighbourhood of farm and Steiner school children, but did not actually live on a farm or did not attend a Rudolf Steiner school, respectively. Exposure information like home and farm characteristics was assessed through a questionnaire administered to the parents of the participating child. The study design further included collection of house dust in a subsample of 100 children per country: 50 farm children, 25 children of Rudolf Steiner schools and 12–13 children of each reference group. Farms without livestock (representing <20% of the farm children questionnaire population) were excluded from the selection of families for dust sampling. The Steiner and reference children were randomly selected from the children whose parents consented to dust sampling and blood sampling (more than 50% of the questionnaire population). Selection procedures differed slightly between countries; in Austria consent to blood sampling was not required and in Sweden and the Netherlands the selection was restricted to some geographical areas. The subpopulation, selected for dust sampling, had essentially the same distribution of determinants (like age of the home, contact to farm animals, etc.) as the total PARSIFAL population. In total, fieldworkers collected dust from the mattresses and living room floors of 229 farm children, 122 Steiner children and 60 and 67 of their respective reference children. On farms, dust from the animal stable(s) was collected as well.

Dust collection

Dust from mattresses and living room floors was collected on pre-weighed glass fibre filters using vacuum cleaners with sampling nozzles (ALK, Horsholm, Denmark) according to a standardized protocol with photo- and video-instructions. The whole area of the mattress (with under-sheets only) was vacuumed for 2 min. For living room floors, sampling time and area depended on type of floor covering; carpeted floors, 1 m², 2 min; smooth floor with ≥ 4 m² rug, 1 m² of rug, 2 min; smooth floor with no rug or smaller rug(s), 2 m² of smooth floor, 4 min. Stable dust was collected at 0.5–1.5 m above the floor from various surfaces (shelves, window sills, etc.), using a brush and a dust pan. After each sampling of mattress, living room or stable dust all sampling tools (ALK nozzle or brush and dust pan) were cleaned thoroughly with 70% ethanol. All samples (stable dust or filters with sampled house dust) were stored in tightly closed new, disposable 50 ml tubes, stored frozen at the various centres, and shipped on dry ice to one laboratory

(IRAS, Utrecht, NL), where samples were stored at -20°C for 4–19 months until extraction. To assess the risk of contamination during sampling and sample processing procedures, each centre included during the field work a number of ‘field blanks’ (total $n = 41$): preweighed filters in 50 ml tubes that were transported to field locations, opened and further treated as sample filters, except that no actual sampling was performed.

Dust extraction

For house dust samples, filters plus dust were weighed and then extracted in a volume of 5–40 ml, determined by the net dust weight (<0.5 g, 5 ml; 0.5 – 1.0 g, 10 ml; 1.0 – 2.0 g, 20 ml; >2.0 g, 40 ml). Stable dust was sieved through a 0.425 mm mesh and 150 mg of each sample was extracted in a volume of 5 ml. Endotoxin, EPS and glucans were extracted sequentially, essentially as described previously (22–24). First, 5 – 40 ml (0.05% , v/v) Tween-20 in pyrogen-free water was added, suspensions were incubated in an end-over-end roller for 1 h at room temperature, and after centrifugation (15 min, 1000 g) the upper 10% of supernatant was harvested and stored in four aliquots at -20°C for endotoxin analysis. For the second step, the removed supernatant was replaced with the same volume of $10\times$ concentrated phosphate-buffered saline (PBS), thus changing the extraction medium into PBS- 0.045% Tween-20. After re-suspension and thorough mixing of the first pellet in the new medium, incubation was continued in the end-over-end roller (1 h), followed by centrifugation (15 min, 2000 g). The supernatant was harvested and stored in six to 10 aliquots at -20°C for analysis of EPS and pet and mite allergens. The remaining dust pellets were stored at -20°C . For the extraction of glucans, the pellets were re-suspended in the original volume of PBS-Tween (0.05%), incubated in an end-over-end roller for 15 min, autoclaved for 1 h at 120°C and incubated in an end-over-end roller again for 15 min. After centrifugation (15 min, 1000 g), supernatant was harvested and stored in two aliquots at -20°C until analysis.

Analysis of microbial components

Endotoxin was analysed with the kinetic chromogenic Limulus Amebocyte Lysate (LAL) test, using the same batch of LAL reagents and standards for all analyses (BioWhittaker, Walkersville, MD, USA; LAL lysate lot no. 1L676S, LPS standard lot no. 2L0090) (22). The EPS was analysed with a specific sandwich enzyme immunoassay (EIA) for EPS of *Aspergillus* and *Penicillium* spp. (23) and glucans were measured with an inhibition EIA (24). Concentrations were expressed as endotoxin units (22), EPS units [based on an in-home standard consisting of a pooled house dust extract given an arbitrary value of 5000 EPS units/ml (22)] and micrograms of glucans per gram of dust and per square meter. For stable samples, levels of all three components were only calculated per gram of dust. The average interday/interassay coefficients of variation, as determined by testing duplicate extract aliquots of 10% of all samples on another day as the first aliquot, ranged from 14.5 to 30.5% . The 41 field blanks [which were treated exactly the same as the other filters (including transport to homes, etc.), except dust sampling] showed nondetectable (90 – 95%) or very low levels of microbial agents.

Amounts of dust lower than 0.020 g were considered undetectable and were given a value of 0.013 g. All mattress samples had a detectable amount of dust. For living room ($n = 25$) and stable ($n = 15$) samples with undetectable amounts of dust, no concentrations per gram of dust were calculated, unless the amounts of microbial components were undetectable too. Samples with non-detectable amounts of endotoxin, EPS or glucans ($n = 18$, $n = 14$

and $n = 9$ respectively) were given a value of two-thirds of the lowest observed detectable amount per gram of dust or per square meter for the specific component determined. Overall, $<5\%$ of results are missing, because of the undetectable amounts of dust or sampling or extraction failures, or because the surface area of the mattress had not been recorded.

Statistical analysis

Levels of microbial components were approximately normally distributed after natural log (ln)-transformation. The differences between groups were evaluated by performing Student's *t*-tests, with ln-transformed values. Correlations were evaluated by calculating Pearson correlation coefficients with ln-transformed values. The associations between the ln-transformed levels and self-reported home, farm and family characteristics were studied in univariate and multivariate regression analyses. The association of each dichotomous variable (set at 1 for children with that characteristic and 0 for children without that characteristic) with the level of any of the microbial agents was expressed as the ratio of covariate-adjusted geometric mean (GM) levels of that microbial agent for children with and without that characteristic (mean ratio). Characteristics that did not have a significant effect on any of the microbial component levels were left out of the final multivariate models because of power implications. To test for heterogeneity in the observed associations, the multivariate regression models were also run for each country and study group separately. SAS statistical software (version 8.2, SAS Institute, Cary, NC, USA) was used for all analyses.

Results

Differences between groups and countries

More living room and mattress dust was collected in homes of Steiner children when compared with their reference children (GM: 0.34 vs 0.17 and 0.45 vs 0.35 g respectively). For farm children, only amounts of living room dust were significantly higher when compared with farm reference children (GM: 0.27 vs 0.18 g). Figure 1 shows the distributions of microbial components per gram of dust for both sampling sites for each of the four groups. Farm children had higher levels of endotoxin (2.6 - to 3.2 -fold), EPS (2.4 - to 3.1 -fold) and glucans (1.2 - to 1.3 -fold) when compared with farm reference children. Steiner children had somewhat higher levels than their reference children (endotoxin 1.2 - to 1.3 -fold.; EPS 1.4 - to 1.6 -fold; glucans 1.1 - to 1.2 -fold) but differences were smaller and not always significant. For farm children and their reference children, the differences were 1.5 - to 4.2 -fold ($P < 0.05$) when levels were expressed per square meter, for Steiner children and their reference children, the differences were 1.6 - to 2.5 -fold ($P < 0.01$) when expressed per square meter (data not shown). The trends were consistent across countries, although mean levels in each country varied considerably (Fig. 2). For example, for mattress samples from farm children, mean levels of endotoxin, EPS, and glucans per gram of dust varied up to 2.6 -, 1.3 - and 2.2 -fold respectively, between the countries. Highest levels of

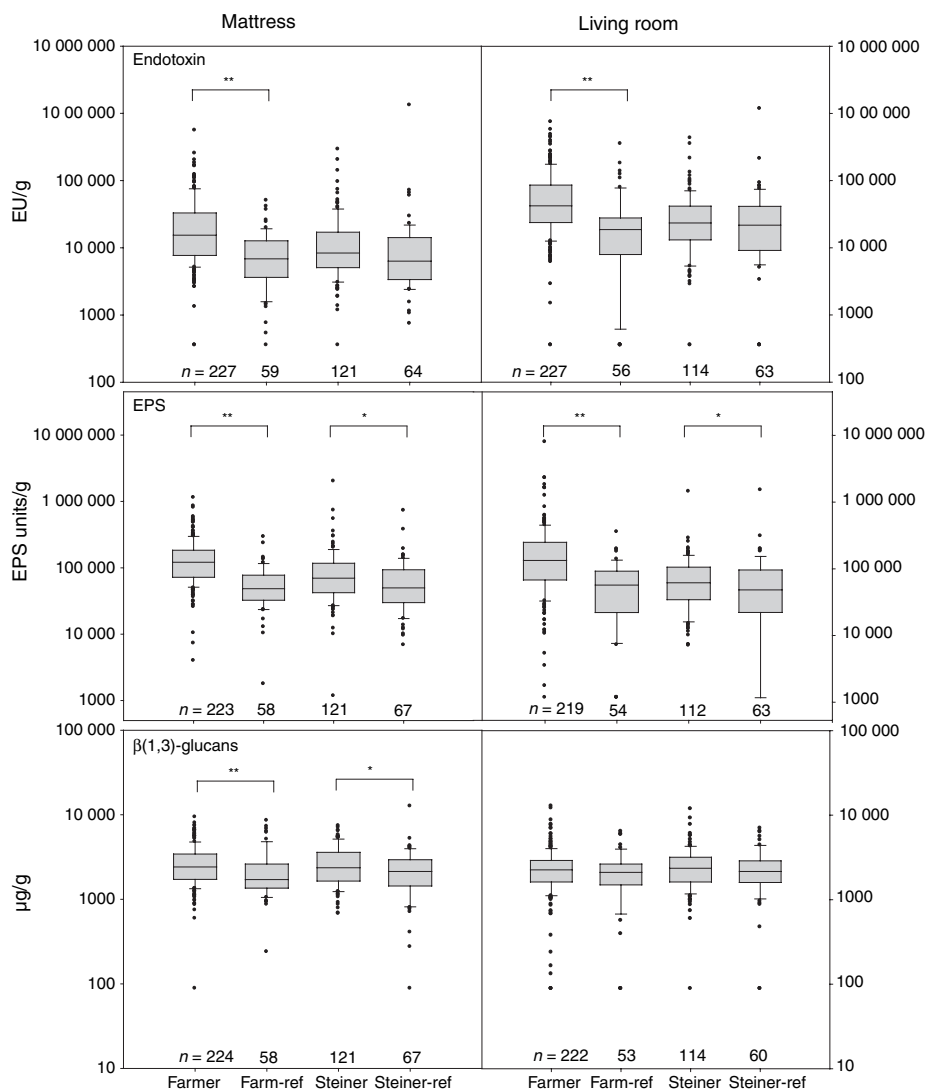


Figure 1. Box plots showing medians, 10th, 25th, 75th and 90th percentiles of the levels of microbial components per gram of dust in mattresses and living room floors. * $P < 0.05$; ** $P < 0.01$.

EPS were observed in Switzerland (mattress) and Austria (living room), whilst highest levels of endotoxin and glucans were observed in Germany and Sweden respectively.

Associations between levels of different microbial agents at the same site and between levels at different sampling sites

For both mattress and living room dust, levels of the three microbial agents per gram dust showed moderate, but significant correlations, with Pearson's r for log-transformed values ranging from 0.20 to 0.38. A comparison of levels in mattress dust with those in living room floor dust from the same home also showed moderate correlations, with r ranging from 0.26 to 0.36.

The levels of endotoxin, EPS and glucans in dust from stables were respectively five-, seven- and threefold higher

than in dust of living room floors of farm children. The EPS and glucans in dust of stables were moderately correlated with the levels of these agents in dust from mattresses and floors with carpets or rugs (r : 0.19–0.34), whilst no significant correlations were found for endotoxin (r : 0.04–0.06).

Determinants of microbial agent levels

A number of home characteristics differed between the groups; of the children included in the multivariate analyses described below, more farm children lived in houses built after 1960 (62%) compared with farm reference children (30%). The same was true for Steiner children when compared with Steiner reference children (39% vs 27% respectively). Of the farm children, 88% had frequent contact to farm animals, vs 26% of farm

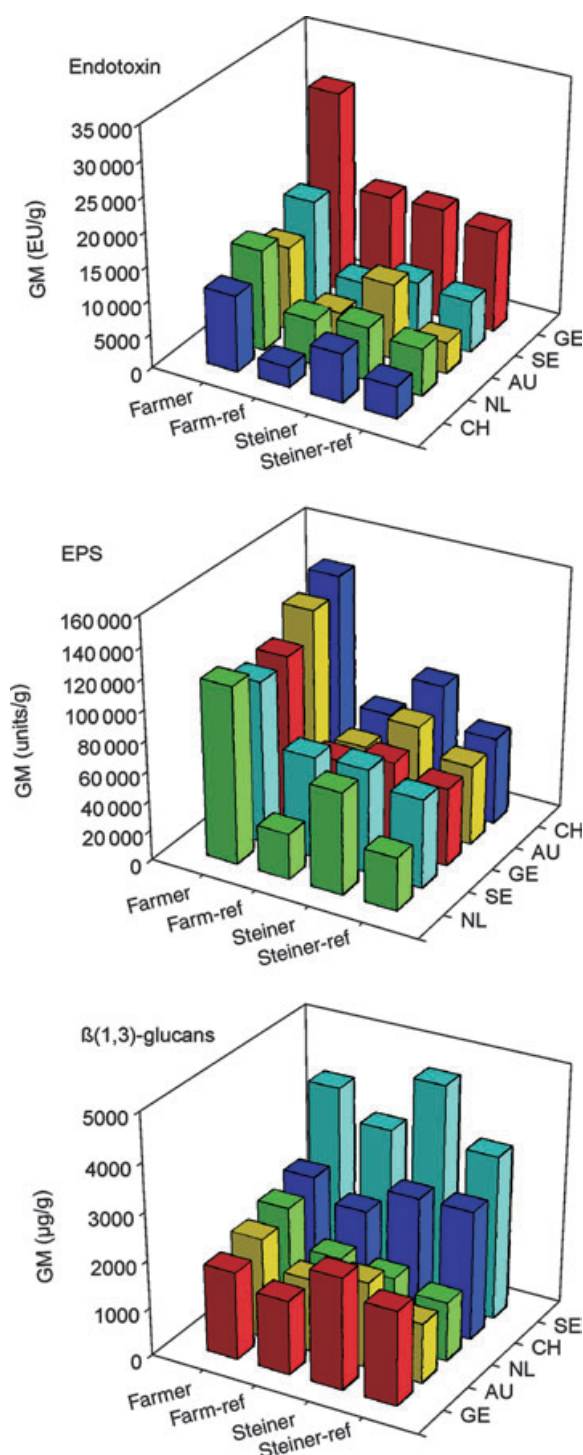


Figure 2. Levels of microbial components in mattress dust by group and by country^a (GE, Germany; SE, Sweden; AU, Austria; NL, the Netherlands; CH, Switzerland). ^aTo improve visibility, results for the different countries are given in a different order in the various graphs.

reference children, 23% of Steiner children and 11% of Steiner reference children. Of the farm children, 37% had a carpeted floor or floor with a rug, vs 45% of farm

reference children, 59% of Steiner children and 49% of Steiner reference children. We therefore also compared levels of microbial agents between groups, while adjusting for these factors and for country of residence. Table 1 shows the multivariate associations between home and family characteristics and the observed levels in mattress dust. Part of the observed differences between groups was explained by age of the home (EPS), frequent use of a gas or wood stove for heating (EPS), having both a cat and a dog (endotoxin) and contact of the index child to farm animals (EPS and endotoxin). Contact to farm animals was correlated with being a farm child ($r = 0.67$), but the analyses for all groups separately showed that in each group contact to farm animals was associated with higher levels of endotoxin and EPS in mattress dust.

The same multivariate model, including type of floor covering, was used to explain levels per gram of living room dust. Significant, positive associations were found for country, being a farm child (EPS, endotoxin) or Steiner child (EPS), parent-reported dampness or mould growth in the home (EPS), age of the home (EPS), type of floor covering (carpet or rug vs smooth) (EPS, glucans) and frequent use of a gas or wood stove for heating (endotoxin) (data not shown). Table 2 shows the associations between farm characteristics and the observed levels in mattress dust of farm children after adjustment for home and family characteristics and presence of farm animals other than cows and pigs (horses, sheep, chicken, goats). Full-time farming increased the levels of EPS in mattress dust. A direct connection between house and stable appeared to increase endotoxin and EPS levels in mattress dust, but this increase was not significant. For living rooms, full-time farming was associated with higher levels of EPS and endotoxin (not shown).

Discussion

This study demonstrates that not only endotoxin levels, but also levels of mould EPS and glucans are higher in homes of farm children when compared with homes of farm reference children. Also for Steiner children, somewhat higher levels of endotoxin, EPS and glucans were observed when compared with their reference children, but differences were smaller and not always significant.

Differences between farm children and their reference children

The results regarding endotoxin levels in homes of farm children are in line with the previously published ALEX study which also showed that endotoxin levels in homes of farm children are higher than in homes of reference children in Austria, Germany and Switzerland (7). We showed exposure differences for mould components as well: EPS and glucans. The differences were most

Table 1. Multivariate association between home and family characteristics and levels of microbial components in mattress dust: mean ratio (MR) and confidence intervals (CI)

Characteristics	N†	Endotoxin (EU/g)	EPS (EPS units/g)	β(1,3)-Glucans (μg/g)
N_{model}		435	433	434
R^2 (%)		24.3	24.8	33.2
Group		MR (CI)	MR (CI)	MR (CI)
Farmer vs Steiner-ref‡	202	1.44 (1.00–2.06)	1.43 (1.08–1.89)*	1.16 (0.97–1.37)
Steiner vs Steiner-ref	115	1.28 (0.94–1.75)	1.23 (0.97–1.57)	1.18 (1.02–1.37)*
Farm-ref vs Steiner-ref	54	0.85 (0.59–1.23)	0.88 (0.66–1.18)	0.95 (0.79–1.14)
Country				
Switzerland vs Sweden	65	0.73 (0.53–1.00)	1.20 (0.93–1.54)	0.66 (0.57–0.78)**
The Netherlands vs Sweden	77	0.99 (0.73–1.35)	0.85 (0.67–1.08)	0.46 (0.39–0.53)**
Germany vs Sweden	102	2.12 (1.59–2.83)**	1.00 (0.79–1.25)	0.49 (0.43–0.57)**
Austria vs Sweden	93	0.90 (0.67–1.22)	1.01 (0.80–1.28)	0.46 (0.40–0.53)**
Home and family characteristics§				
Dampness or moulds living-/bedroom (yes vs no)	34	1.26 (0.87–1.81)	1.18 (0.89–1.57)	0.99 (0.83–1.18)
House built ≥1960 vs <1960	204	1.06 (0.86–1.30)	1.25 (1.06–1.47)**	0.98 (0.89–1.09)
Dog vs no cat and dog	41	1.05 (0.74–1.48)	0.84 (0.64–1.10)	1.02 (0.86–1.20)
Cat vs no cat and dog	111	1.09 (0.86–1.38)	1.05 (0.87–1.27)	1.11 (0.99–1.25)
Dog and cat vs no cat and dog	65	1.52 (1.12–2.05)**	1.24 (0.97–1.57)	1.09 (0.94–1.26)
Frequent use of gas/wood stove for heating (yes vs no)	72	0.93 (0.71–1.22)	1.26 (1.01–1.56)*	1.04 (0.91–1.19)
Child's contact to farm animals (yes vs no)	226	1.49 (1.15–1.93)**	1.50 (1.22–1.84)**	1.10 (0.97–1.24)

* $P < 0.05$; ** $P < 0.01$.

†Indicates the number of children with the mentioned characteristic (e.g. 202 farm children, 41 children with a dog, etc.) of 435 children included in the endotoxin model.

‡This model included all four groups to increase the power, which implicated that one group had to be chosen as reference group for all groups. We chose Steiner-reference children as reference group. The MR for farmer vs farm-reference is $1.44/0.85 = 1.69$ for endotoxin, 1.63 for EPS and 1.22 for glucans.

§Family size, parental education level, age of the mattress, type of floor covering in bedroom and number of days since last vacuuming did not show significant effects and were excluded from the model.

Table 2. Multivariate association† between farm characteristics and levels of microbial components in mattress dust of farm children: mean ratio (MR) and confidence intervals (CI)

Farm characteristics‡	N§	Endotoxin (EU/g)	EPS (EPS units/g)	β(1,3)-glucans (μg/g)
N_{model}		178	174	175
R^2 (%)		16.8	19.7	36.4
Full time vs part time farming	118	0.98 (0.69–1.38)	1.28 (0.99–1.66)	0.88 (0.75–1.04)
House connected to stable, yes vs no	67	1.27 (0.81–2.01)	1.24 (0.88–1.76)	1.10 (0.88–1.36)
Pig(s) vs no pig	53	1.01 (0.69–1.49)	0.90 (0.67–1.21)	1.01 (0.84–1.21)
Cow(s) vs no cow	158	1.27 (0.70–2.29)	0.98 (0.63–1.53)	0.95 (0.72–1.26)

* $P < 0.05$; ** $P < 0.01$.

†Adjusted for country, home and family characteristics (listed in table 1) and the presence of horses, sheep, goats and chicken.

‡Stable visit frequency of the child and number of cows did not show significant effects and were excluded from the model.

§Of 178 children included in endotoxin model.

pronounced for EPS, which was previously shown to be a useful marker of indoor mould growth (18). Glucans are important cell wall constituents of most fungi, but have other sources too, like bacteria and many higher and lower plants. Furthermore, the variation of glucan measurements has been shown to be similar within- and between-homes, whereas for EPS within-home variation was smaller than that occurring between-homes (18). Although there have been signs that farming also influences the domestic area (25–27), this is the first study showing elevated indoor exposure to fungal components and a correlation between levels in stable dust and in house dust. It is interesting that such a

correlation was not found for endotoxin. In the ALEX study, no correlation between stable endotoxin levels and indoor levels was found either (28). It might be speculated that endotoxin levels in stables vary more than mould levels over time. This study did not include repeated measurements to assess variability of levels over time, but the ALEX study showed a high correlation ($r = 0.73$) between endotoxin levels in dust samples of stables taken on two separate occasions (28). It can also be speculated that variation in other sources of indoor levels or variation in factors determining transfer of bacterial and fungal components from stables to the indoor environment cause these

apparent discrepancies in associations between levels indoors and in stables.

One important determinant which partly explained the higher endotoxin and EPS levels in mattress dust of farm children was contact to farm animals. Contact to farm animals was also in Steiner children, Steiner reference children and farm reference children associated with higher levels of endotoxin and EPS in mattress dust, although not always significantly. This is in line with the study showing that not only farm children but also nonfarm children with regular contact to farm animals are exposed to elevated endotoxin levels (7).

Differences between Steiner children and their reference children

Exposure differences were not only found for farm children and their reference children, but also, although less clearly, for Steiner children and their reference children. More mattress and living room dust was collected in homes of Steiner children when compared with Steiner reference children, which might be related to cleaning habits. Steiner families vacuumed their living room on average 3.4 days before the home visit for the last time, whereas Steiner reference families vacuumed on average 2.4 days before the home visit for the last time ($P = 0.01$), which might indicate that Steiner families clean less often. However, even after adjustment for days since last vacuuming (not shown) and various home characteristics, differences in microbial exposure remained, although the observed differences did not always reach significance. The first studies in this group of children did not include measurements in house dust (21, 29). Future studies into the factors possibly protecting these children from atopic diseases should take microbial components in house dust into account, although it seems unlikely that the rather small differences in exposure, compared with the differences found between farm children and reference children, would substantially contribute to a presumed protective effect in the development of atopic sensitization, as hypothesized for farm children.

Differences between countries

The differences between groups were consistent across countries. Despite large differences in farming practices, the differences in microbial exposure between farm children and reference children were similar in all countries. The mean levels per country, however differed substantially, even after adjustment for differences in home characteristics. This cannot be due to differences in sampling and laboratory procedures, because we used standardized procedures and one laboratory for all analyses. Inter-fieldworker differences might have occurred, but no consistent, significant fieldworker effects on levels of microbial components were observed within countries. There were some differences in the timing of fieldwork, but studies in German homes found no effects of season (30), temperature and relative humidity (30, 31) on endotoxin levels, so this is an unlikely explanation for the observed exposure differences. For EPS and glucans, no seasonal effects have been reported previously either (18, 32). We have no explanation for the international exposure differences observed in this study.

This study assessed differences in exposure to bacterial and fungal components in house dust between farm and Steiner children and their respective reference children. Exposure to immunomodulatory components of moulds might protect against atopy and asthma, as has been suggested for exposure to endotoxin and other bacterial components (33). The association between microbial exposure and allergic and airway diseases in these children will be assessed in a separate publication.

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