



Contents lists available at ScienceDirect

Environmental Research

journal homepage: www.elsevier.com/locate/envres

Exposure to moulds and actinomycetes in Alpine farms: A nested environmental study of the PASTURE cohort[☆]

S. Roussel^{a,b,*}, B. Sudre^{a,b}, G. Reboux^{a,b}, M. Waser^c, G. Buchele^c, M. Vacheyrou^{a,b}, J.C. Dalphin^{a,b},
L. Millon^{a,b}, C. Braun-Fahrlander^c, E. von Mutius^d, R. Piarroux^e

^a UMR/CNRS Chrono-Environnement 6249, University of Franche-Comté, 25000 Besançon, France

^b University Hospital of Besançon, Parasitology-Mycology Department, 25000 Besançon, France

^c Swiss Tropical and Public Health Institute, University of Basel, Basel, Switzerland

^d University Children's Hospital of Munich, Germany

^e Department of Parasitology and Mycology, Assistance Publique-Hôpitaux de Marseille, France

ARTICLE INFO

Article history:

Received 24 March 2010

Received in revised form

18 April 2011

Accepted 4 May 2011

Keywords:

Microbiology

Exposure assessment

Allergy

Farms

Indoor air

ABSTRACT

Several studies have suggested that children exposed to a farm environment are protected against allergies and asthma. The present work is an environmental study nested within the PASTURE cohort and includes 97 farmers and 74 non-farmers in three regions of the Alpine Arc (Switzerland, France and Germany).

The objectives were to determine and compare the fungi and actinomycetes present in farming and non-farming environments (children's bedrooms and cowsheds), and to identify the agricultural practices associated with an increase in airborne fungi and actinomycetes in cowsheds. Air samples were collected by air pump and were analysed by culture and by direct counting of spores on membranes.

During their stay in bedrooms, children living on farms were exposed to significantly greater amounts of *Absidia* spp., *Eurotium* spp., *Cladosporium* spp., *Penicillium* spp. and mesophilic actinomycetes than children who did not live on farms. Depending on the season, the levels of moulds, yeasts and actinomycetes were from 14 to 82 times higher in cowsheds before feeding the cattle than in children's bedrooms, and from 12 to 464 times higher in cowsheds after feeding than in children's bedrooms. Feeding cattle in cowsheds was associated with a significant peak in airborne moulds and actinomycetes, and this peak was higher in winter than in summer. Silage distribution was associated with low amounts of moulds and actinomycetes. Other significant agricultural factors were the type of cowshed, cowshed volume, method of food distribution to cattle and use of fresh grass.

An assessment of the microbiological diversity on farms and in children's rooms may help to determine the factors protecting children from asthma and atopic diseases.

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1. Introduction

Several studies have suggested that children living in rural environments are protected against allergy and asthma (Braun-Fahrlander et al., 1999; Ernst and Cormier, 2000; Kilpelainen et al., 2000; Riedler et al., 2000; Von Ehrenstein et al., 2000). The European ALEX study showed that exposure to Alpine farm

environments during pregnancy and the first year of life conferred marked protection against these diseases (Riedler et al., 2001). More precisely, it was shown that exposure to a cowshed environment for more than 20 min a day and consumption of raw milk were associated with a significantly lower prevalence of asthma (1% versus 11%), hay fever (3% versus 13%) and atopic sensitization (12% versus 29%) than in non-exposed children. These results were confirmed by subsequent European studies (Waser et al., 2007), but they have not been replicated in all types of agricultural work (Downs et al., 2001) or in all regions of the globe (Wickens et al., 2002; Zekveld et al., 2006), suggesting there are specific factors on European farms.

Several epidemiological and experimental studies have been performed to assess the role of bacteria in conferring protection against asthma and atopy in rural settings. Most of these studies focused on exposure to endotoxin as a proxy for Gram negative bacteria (Braun-Fahrlander et al., 2002; Gehring et al., 2008;

[☆] PASTURE study group: von Mutius E., Kabesch M., Kraus-Etschmann S., Maier S., Schmid S., Ege M., Roiha H., Braun-Fahrlander C., Bitter S., Waser M., Riedler J., Humer C., Ublagger E., Weiss G., Pekkanen J., Hirvonen M.R., Roponen M., Huttunen K., Hyvärinen A., Nevalainen A., Remes S., Tuomainen T.P., Brunekreef B., Douwes J., Gehring U., Massoud R., Renz H., Blümer N., Herzum I., Lauener R., Steinhoff D., Frei R., Steinle J., Loeliger S., Weiland S., Büchele G., Vuitton D., Dalphin J.C., Dalphin M.L., Piarroux R., Millon L., Reboux G., Roussel S., Sudre B.

* Corresponding author. Fax: +33 363082232.

E-mail address: sroussel@univ-fcomte.fr (S. Roussel).

von Mutius et al., 2000). Overall, these studies reported an inverse association between endotoxin exposure and atopy. In the PAR-SIFAL study the association between endotoxin in mattresses and floor dust and atopy was no longer significant when simultaneously adjusted for fungal beta(1,3)-glucans and fungal extracellular polysaccharides (EPS) (Schram-Bijkerk et al., 2005), indicating that other components of the microbial environment were also important. In a review, von Mutius thus suggested that components other than endotoxin such as moulds might modulate immune responses and thereby protect against allergic diseases (von Mutius, 2007). Indeed, endotoxin and the few other molecules that have been studied to date reflect only a small part of the total microbial biodiversity of the cowshed environment. In particular, air in cowsheds is known to be highly contaminated by numerous species of bacteria, including high levels of actinomycetes, as well as by moulds (Hanhela et al., 1995; Kotimaa et al., 1991, 1987; Lange et al., 1997; Reboux et al., 2001) and various other antigenic compounds (Radon et al., 2002), such as grass pollen (Sudre et al., 2009).

The ongoing birth cohort study PASTURE aims to determine the factors involved in protection against allergy in five rural European regions (1133 children exposed or non-exposed to agricultural environments) (von Mutius and Schmid, 2006). The project aims to assess the role of indoor exposure to various microbial products and to investigate the immunological and genetic mechanisms involved in individual responses to these environmental influences. The present work is an environmental exposure assessment study nested within the PASTURE cohort and includes bedrooms from 97 farmers' children and 74 non-farmers' children in three regions of the Alpine Arc (Switzerland, France and Germany). The objectives of this study were (1) to determine the fungal and actinomycetes species present in cowsheds, (2) to compare the levels of airborne fungi and actinomycetes in farming and non-farming indoor environments (children's rooms and cowsheds) and (3) to identify agricultural practices associated with an increase in airborne fungi and actinomycetes.

2. Materials and methods

2.1. Farms, population

2.1.1. PASTURE study

This study was nested with the multi-centre PASTURE study, an ongoing longitudinal birth cohort study designed to evaluate risk factors and preventive factors for atopic diseases. The cohort has been described elsewhere (von Mutius and Schmid, 2006). Pregnant women living in rural areas in Austria, Finland, France, Germany and Switzerland were recruited in the third trimester of pregnancy. Women who lived on farms where any kind of livestock was kept were assigned to the farm group. For the reference group, women from the same rural areas who did not live on farms were recruited. The two groups resulted in a birth cohort of 1133 children.

2.1.2. Populations

The first 106 farming families included in the PASTURE study in France, Germany and Switzerland were invited to participate in the present exposure assessment study. No exclusion criteria were applied other than those of the PASTURE study. Ninety-seven farmers (17 in the St. Galle area, Switzerland; 31 in the Franche-Comté district, France and 49 in the Bavaria district, Germany) agreed to participate. Farms were visited twice, once during the winter fodder season and once again in summer. For the winter visit, 76 were visited between January and March 2004, and 21 between January and April 2005. In summer, between August and October 2004, only farms in which food was distributed in cowsheds were visited resulting in 63 included farms (12 in Switzerland, 12 in France and 39 in Germany).

In the same way, the first 100 non-farming families (reference group) included in the PASTURE study in France, Germany and Switzerland were invited to participate. Of these, 74 accepted (12 from Switzerland, 26 from France and 36 from Germany) and were visited between January and April 2004.

2.2. Sampling methods

Airborne particles were collected by a cassette containing a filter membrane and connected to a Gil-Air 3 pump (Gil-Air 3, Sensidyne[®], Clearwater, USA) operating at a flow rate of 3 L/min. Regular control of the pump calibration with the Giliator-2 Diagnosis Calibration System (Sensidyne[®], Clearwater, USA) allowed us to check for flow variations smaller than 5%. In children's bedrooms, air samples were pumped through a Teflon filter (37-mm diameter, 0.45- μ m pores) (Environmental Express[®], South Carolina, USA) during 90 min (final volume: 270 L). Air pumps were located in a central place in each child's bedroom at a height of 75 cm. Windows were closed during sampling. In cowsheds, two air samples of 20 min (final volume: 60 L) were obtained: one before the feeding session and one starting 10 min after the end of the feeding session. The feeding session was defined as the period during fodder distribution (hay, silage, fresh grass and/or flour) to animals in the cowshed. Pumps were located in a central place in each cowshed and fixed on a tripod at a height of 75 cm. In cowsheds, additional air samples were obtained before and during feeding on a polypropylene membrane covered with a mixed petroleum/toluene solution (final sample volume: 60 L). All filter cassettes were sent within 48 h to the laboratory of Besancon for analyses.

2.3. Identification methods

2.3.1. Microscopy

As previously described (Sudre et al., 2009), the polypropylene membranes were transferred and fixed on glass slides, and fungal spores were identified and counted according to their morphology (Grant Schmid, 1986). Most spores were from 2- to 5- μ m-diameter round or ovoid spores, hyaline and mainly belonged to *Aspergillus* and *Penicillium* genera (PenAsp spores). Because the identification criteria of *Aspergillus* and *Penicillium* are based on characteristics of the spores' production on culture media or molecular biological investigations, the spores could not be separated or identified at the species level. Some spores were larger, and most of these belonged to the genera *Cladosporium* and *Ustilago*. The results were expressed in spore counts per membrane and further converted to spore counts per cubic metre.

2.3.2. Culture

Teflon filters were rinsed with 10 ml of sterile water with 0.1% Tween 80 (Sigma-Aldrich, Steinheim, Germany), shaken for 10 min in a Stomacher apparatus (AES Laboratoire, Combours, France) and cultured on Petri dishes. Samples were cultured on four different culture media as follows: Dichloran-Glycerol (Oxoid LTD, Basingstoke, England) with 0.5% chloramphenicol (Sigma-Aldrich, Steinheim, Germany) at 30 °C for mesophilic mould isolation; 3% malt-agar (AES, Bruz, France) with 10% salt and 0.5% chloramphenicol at 20 °C for osmophilic fungal species; Difco actinomycetes isolation agar (Becton Dickinson, le Pont de Claix, France) at 30 °C for mesophilic actinomycetes and R8 medium according to Amner et al. (1989) at 52 °C to detect thermophilic actinomycetes. Moulds were identified via macroscopic and microscopic observations. When the number of colonies on culture media was higher than 300, additional dilutions were done. Actinomycetes were classified by macro- and micro-scopic observations, hydrolysis of casein and temperature tests. The number of CFUs (colony-forming units) per plate was counted after 7 days of incubation. The results were expressed in CFUs per plate and further converted to CFU per cubic metre.

2.4. Questionnaire

Data on farming methods and cowshed characteristics were collected during interviews using a standardised questionnaire, which included the type of farm (tie-stalls or loose housing system), cowshed volume (in cubic metres), whether the farm used an open or closed cowshed, hay harvesting method (loose only, loose and bales only), feed distribution method (manually/using a mixer wagon versus through a hole in the cowshed ceiling) and the quantities of fodder (kilograms of hay, silage, fresh grass and supplemental food for milk production) distributed per day.

2.5. Statistics

Numbers of spores counted and concentrations of micro-organisms were first tested for normality (skewness and kurtosis tests) and were found to be skewed. Therefore non-parametric tests were used. We performed a Mann-Whitney two-sample statistic test to compare concentrations of airborne micro-organisms in farmers' and non-farmers bedrooms, Chi-square tests to compare isolation frequencies of micro-organisms and Wilcoxon matched-pairs signed-ranks tests to compare cultivable micro-organisms before and after feeding in winter and summer. Medians and interquartile range (IQR), which is the difference between the third and the first quartiles, were used to describe data. To quantify the differences in micro-organisms between bedrooms and cowsheds, ratios were

calculated. For each farm and for each microorganism or grouped micro-organisms of interest, the number of CFU/m³ in cowsheds before feeding was divided by the number of CFU/m³ in children's bedrooms (=ratio cowshed before feeding/bedrooms), and the number of CFU/m³ in cowsheds after feeding was divided by the number of CFU/m³ in children's bedrooms (=ratio cowshed after feeding/bedrooms). These ratios were summarised by geometric means and their 95% confidence intervals.

The concentrations of total moulds and actinomycetes after feeding in the winter were also tested for normality, and were found to show a non-normal distribution. To test for associations between these variables and farm characteristics and feeding practices, we used the ratio of geometric means (Geometric Mean Ratio: GMR) and its 95% confidence intervals, which represent the increase (when GMR ratio is positive) or the decrease (when GMR ratio is negative) for concentrations between two categories. A GMR equal to one, means that concentrations are equal in the two categories. A 95% confidence interval excluding the value 1 indicates that there is significant difference between the two categories. Continuous variables were divided into tertiles (volume) and into quartiles for the quantity of fodder distributed per day (hay, grass and/or maize silage).

Data were captured from questionnaires and microbiological counts using EpiData software (Conté de Funen, Denmark & Brixton Health, UK), and STATA 10 SE (Stata Corporation, College Station, Texas, USA) was used for statistical analyses. The statistical significance was set at 5% level.

3. Results

3.1. Distribution of spores in cowsheds

Among the 97 farms that were visited during the winter season, 89 pairs (before and after feeding) of polypropylene membranes were analysed (17 in Switzerland, 30 in France and 42 in Germany) due to technical problems in sampling and the shipping of samples. In the summer, only a part of farmers distributed food to cattle (12 Swiss, 12 French and 39 German farms) in a location where samples for culture could be obtained before and after the feeding session. For these farms, only 51 pairs of polypropylene membranes were analysed. Within these samples, the median number of spores/m³ was 9.5×10^3 (IQR= 33.5×10^3) before feeding and increased to 44.8×10^3 (IQR= 208.2×10^3) during feed distribution in winter ($p < 0.001$). During the summer season, these medians were 1.9×10^3 (IQR= 6.4×10^3) before feeding and increased to 4.2×10^3 (IQR= 16.0×10^3) during feed distribution ($p = 0.004$). The three most frequent fungi were PenAsp spores, *Cladosporium* and *Ustilago*, which accounted for 75%, 12% and 4% of the overall total, respectively. Other identifiable spores were *Alternaria*, *Epicoccum*, *Dreschlera*, *Fusarium*, *Fusicladium*, *Torula*, *Pythomyces* and *Coprinus*.

3.2. Culture of micro-organisms in cowsheds

Aspergillus (*Eurotium* spp., *A. fumigatus* and *A. niger*), *Absidia* spp., *Wallemia sebi*, *Cladosporium* spp., *Penicillium* spp. and yeasts represented 95% and 97% of fungi isolated from cowshed air in the winter and in the summer. Other cultivable species isolated from cowshed air in the winter and summer were *Mucor* sp., other *Aspergillus* species (*A. nidulans*, *A. versicolor*, *A. flavus*, *A. ochraceus*, *A. terreus*, *A. wentii*), *Alternaria* spp., *Aureobasidium pullulans*, *Acremonium* sp., *Phialophora* sp., *Scopulariopsis fusca* and *Scopulariopsis brevicaulis*, *Rhizopus* sp., *Syncephalastrum* sp., *Fusarium* sp., *Epicoccum* sp., *Blastobotrys nivea*, *Trichoderma* sp. and *Trichoderma viride*, *Trichosporon* sp., *Saccharopolyspora rectivirgula* and mycelia sterilia. Actinomycetes species were classified as mesophilic and thermophilic actinomycetes. *Saccharomonospora viridis* and *S. rectivirgula* were identified at species level.

In winter, the median level of moulds was 3.3 times higher after feeding than before feeding ($p = 0.0001$), the median level of yeasts was not changed ($p = 0.87$), and the median level of actinomycetes was 3 times higher ($p = 0.0001$) (Fig. 1). In summer, the median level of moulds was 2.2 times higher after feeding ($p = 0.0001$), the median level of yeasts was not changed ($p = 0.89$), and the median level of actinomycetes was 4 times

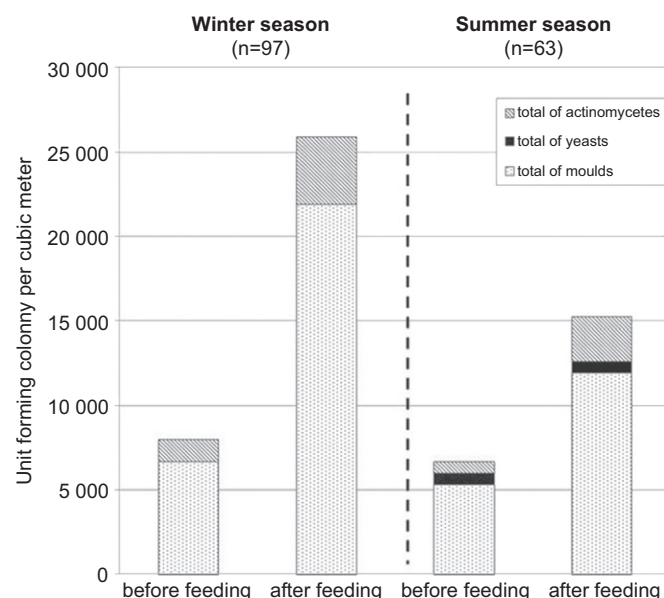


Fig. 1. Medians for moulds, yeasts and actinomycetes (in CFU/m³) in cowsheds according to season and feeding session.

higher ($p = 0.005$). Table 1 shows results of non-parametric statistical tests regarding isolation frequencies and rank sums for the main micro-organisms. In winter, *Eurotium* spp., *Cladosporium* spp., *W. sebi*, mesophilic and thermophilic actinomycetes were significantly higher in frequency and quantity after feeding. In summer, *Eurotium* spp. and *W. sebi* were significantly higher after feeding for both parameters.

3.3. Culture of micro-organisms in bedrooms

The main micro-organisms observed in the cowsheds were also found in children's bedrooms. Among them, 13 were present in more than 97% of the samples: *W. sebi*, *Absidia* spp., *Aspergillus* spp. (*Eurotium* spp., *A. fumigatus*, *A. niger*, *A. versicolor*, *A. nidulans*), *Cladosporium* spp., *Penicillium* spp., yeasts, *S. viridis* and mesophilic and thermophilic actinomycetes. Other micro-organisms were (in decreasing order): *Aspergillus wentii*, *Mucor* sp., *Alternaria* spp., *A. ochraceus* and *S. rectivirgula*. In terms of isolation frequencies in samples, *Absidia* spp., *Eurotium* spp., *Cladosporium* spp., *Penicillium* spp. and mesophilic actinomycetes were significantly less present in non-farmers' bedrooms than in farmers' bedrooms (Table 2). Medians were equal to zero for all micro-organisms because of the small number of positive samples. However, the five micro-organisms cited above were significantly lower in quantity too.

For each farm, two ratios (cowshed before feeding/bedroom and cowshed after feeding/Bedroom ratios) were computed by dividing the number of micro-organisms in the cowshed before feeding or after feeding by the number of micro-organisms in the bedroom (Table 3). These ratios indicate the level of exposures differ between a cowshed and a bedroom. In summer, children would have been 33 times more exposed to moulds and 162 times more exposed to actinomycetes during a stay in the cowshed after feeding than when they were in their bedrooms. In winter, children would have been 66 times more exposed to moulds and 464 times more exposed to actinomycetes in cowshed after feeding than in their bedrooms. For yeasts, ratios were similar in summer and in winter. Details regarding four fungi and mesophilic and thermophilic actinomycetes are shown in Table 4. In winter, *Eurotium* spp. exhibited the largest differences between bedrooms and cowsheds with a level of *Eurotium* 46 times higher in cowshed before feeding than in bedrooms, and

Table 1
Isolation frequencies and medians (in CFU/m3) for airborne micro-organisms in cowsheds.

	Winter (n=97)						Summer (n=63)					
	Before feeding		After feeding		p ¹	p ²	Before feeding		After feeding		p ¹	p ²
	PS (%)	Median (IQR)	PS (%)	Median (IQR)			PS (%)	Median (IQR)	PS (%)	Median (IQR)		
Moulds												
<i>Absidia</i> spp.	28	0 (664)	40	0 (1328)	0.06	0.01	14	0 (0)	18	0 (0)	0.53	0.03
<i>Eurotium</i> spp.	67	1328 (7968)	90	8632 (37848)	< 0.001	< 0.001	38	664 (1992)	57	2656 (664)	< 0.001	< 0.001
<i>Aspergillus fumigatus</i>	46	0 (1328)	55	664 (2656)	0.24	0.12	21	0 (664)	23	0 (0)	0.7	0.80
<i>Aspergillus niger</i>	19	0 (0)	22	0 (0)	0.59	0.10	26	0 (1328)	30	0 (0)	0.36	0.93
<i>Cladosporium</i> spp.	32	0 (664)	48	0 (2656)	0.01	< 0.001	43	664 (1992)	43	1328 (3320)	1	0.13
<i>Penicillium</i> spp.	29	0 (664)	41	0 (664)	0.76	0.84	29	0 (1328)	29	0 (1328)	1	0.38
<i>Wallemia sebi</i>	36	0 (1328)	51	664 (5976)	0.04	< 0.001	12	0 (0)	25	0 (0)	0.02	0.003
Total moulds	95	6640 (25896)	99	21,912 (73040)	0.02	< 0.001	63	5312 (9960)	64	11,952 (28552)	0.55	< 0.001
Yeasts												
Total yeasts	38	0 (664)	34	0 (664)	0.54	0.87	39	664 (3984)	36	664 (9960)	0.58	0.89
Actinomycetes												
<i>Saccharomonospora viridis</i>	29	0 (664)	37	0 (664)	0.16	0.02	9	0 (0)	8	0 (0)	0.79	0.29
<i>Mesophilic actinomycetes</i>	54	664 (2656)	72	1992 (13944)	0.007	< 0.001	31	0 (3320)	43	1328 (7968)	0.03	0.23
<i>Thermophilic actinomycetes</i>	31	0 (664)	52	664 (2656)	0.003	< 0.001	13	0 (0)	21	0 (664)	0.15	0.57
Total actinomycetes	69	1328 (3984)	79	3984 (26560)	0.10	< 0.001	34	664 (5312)	47	2656 (8632)	0.01	0.13
Overall total	98	11,952 (35192)	100	44,488 (116864)	0.15	< 0.001	64	12,616 (33864)	65	27,224 (47144)	0.31	0.005

P^1 : Chi² test for comparison of isolation frequencies; P^2 : non-parametric Wilcoxon test for comparison of medians; IQR: interquartile range; PS: number of positive samples.

Table 2
Isolation frequencies and medians of airborne micro-organisms in farmers' and non-farmers' bedrooms in winter.

	Positive samples (%)			Medians (IQR)		
	Non-farmers n=74	Farmers n=97	p Value (χ^2 test)	Non-farmers n=74	Farmers n=97	p Value (Wilc. test)
Moulds						
<i>Absidia</i> spp.	5	18	0.01	0 (0)	0 (0)	0.01
<i>Eurotium</i> spp.	16	37	0.003	0 (0)	0 (148)	0.001
<i>Aspergillus fumigatus</i>	41	42	0.09	0 (0)	0 (148)	0.68
<i>Aspergillus niger</i>	5	10	0.93	0 (0)	0 (0)	0.29
<i>Aspergillus versicolor</i>	1	7	0.14	0 (0)	0 (0)	0.07
<i>Aspergillus nidulans</i>	3	4	0.7	0 (0)	0 (0)	0.61
<i>Cladosporium</i> spp.	3	21	0.001	0 (0)	0 (0)	0.0003
<i>Penicillium</i> spp.	26	43	0.02	0 (148)	0 (148)	0.03
<i>Wallemia sebi</i>	1	5	0.23	0 (0)	0 (0)	0.18
Total moulds	70	92	0.001	296 (592)	592 (1152)	0.0005
Yeasts						
Total yeasts	3	11	0.04	0 (0)	0 (0)	0.03
Actinomycetes						
<i>Saccharomonospora viridis</i>	1	5	0.23	0 (0)	0 (0)	0.18
<i>Mesophilic actinomycetes</i>	8	18	0.05	0 (0)	0 (0)	0.05
<i>Thermophilic actinomycetes</i>	4	11	0.10	0 (0)	0 (0)	0.07
Total actinomycetes	12	26	0.03	0 (0)	0 (148)	0.02
Overall total	100	100	< 0.001	296 (888)	592 (1480)	0.0001

IQR: interquartile range.

458 times higher in cowshed after feeding than in bedrooms. In summer, the corresponding ratios were 57 and 113, respectively.

3.4. Agricultural practices

The 97 farms were in the Alpine region of Europe at a mean of 638 m above sea level (standard deviation=170). All were breeding farms. One breeds sheep only, and the others breed cows. Among them, 78 farms breed cows and horses, 6 breed cows only and 12 farms breed cows and other animals (poultry, sheep, pigs, and rabbits). Twenty-eight farms use milking stations. Quantities of hay

distributed per day were 0–119 kg/day in 24 farms, 120–280 kg/day in 24 farms, 281–584 kg/day in 22 farms, up to 585 kg/day in 22 farms and data for five farms were missing. No grass and maize silage were used as feed in 39 farms and data were missing for five farms. In other farms, quantities of silage distributed per day were 26–160 kg/day in 7 farms, 180–990 kg/day in 23 farms and up to 1000 kg/day in 23 farms. Fresh grass was distributed as feed in 15 farms.

Associations between agricultural practices and the total of moulds and actinomycetes after the feeding sessions in winter were analysed by geometric mean ratios and their 95% confidence intervals.

Geometric means of total moulds was significantly higher (1) when cowsheds were tie-stalls compared with loose housing, (2) when the volume of the cowshed was between 70 and 170 m³ compared to those from 750 to 1470 m³, (3) when hay was distributed through a hole in the ceiling compared to manually or with a wagon, (4) when no silage was used compared to when more than 1000 kg/day were used and (5) when no fresh grass was fed to the animals (Table 5).

4. Discussion

The aim of this study was to assess the airborne exposure of children to moulds and actinomycetes in an environment of Alpine farms. Such environments are associated with a lower prevalence of asthma and atopic diseases. Our results show that exposure to some moulds and actinomycetes in cowsheds is massive and peaks during cattle feeding, especially in winter. We found that farm children were also exposed to significantly greater amounts of *Eurotium* spp., *Absidia* spp., *Cladosporium* spp., *Penicillium* spp. and mesophilic actinomycetes in their bedrooms than the non-farm controls. Exposure to moulds was 14–66 times higher in cowshed than in bedrooms. Actinomycetes led to the highest differences in exposure. Results also showed that the use of grass and silage for feeding decreased the concentrations of airborne moulds and actinomycetes in cowsheds. Airborne contamination by moulds and actinomycetes was also influenced by the type and volume of cowshed and the feed distribution method.

These results complement previously ones showing that the same cohort of farm children was exposed to a high amount of pollen and Gram-positive bacteria (mainly *Staphylococcus*, *Bacillus*

and *Clostridium* genera) during their time in cowsheds (Sudre et al., 2009), and comparing exposure in bedrooms with other methods of sampling (Ege et al., 2011).

Our assessment was performed by collecting air samples instead of settled dust. We chose to study airborne samples because it was the only way to estimate the peaks of exposure during cattle feeding. This approach also provided the advantage of focusing on micro-organisms that are contaminating the air inhaled by children during their stay in cowsheds and in rooms. It minimises the distortions due to growth and destruction of viable airborne micro-organism colonies after they settle in the dust. To be as close as possible to the real exposure of young children, we put the air-sampling device in a central location in the cowshed, at a place where children are more likely to be during their time there.

However, we acknowledge that using airborne samples has also some disadvantages. As stated by Chew et al. (2003) ideal assessment of fungal exposure should include information about the biologically relevant inhaled dose of fungal spores and associated agents during an individual's lifetime. Indeed, bio-aerosol emissions are known to be highly variable from one measure to another even during short periods of time (Douwes et al., 2003). This is particularly true in the farm environment, where bio-aerosol increases drastically under various situations, such as handling of feed, storage conditions of hay and removal of manure (Hanhela et al., 1995; Kotimaa et al., 1991). The medians of cultivable micro-organisms in our study were within the exposure range previously established in breeding farms (10⁴–10⁸ CFU/m³) (Eduard, 2009). Here, a long-term assessment of airborne exposure could not be planned for a large number of subjects and only short-duration air samples could be collected on each farm. Consequently, we expect that only large differences in airborne micro-flora could be measured between groups of subjects.

The analyses of cultivable moulds and actinomycetes by a culture media panel is likely to provide more accurate data on biodiversity than the use of dosing constituents or metabolites of micro-organisms to estimate microbial exposure. However, it is essential to note that only a portion of the biodiversity can be assessed by culture methods and that other micro-organisms of interest may not be detected in this way. Quantitative PCR techniques bypass the culture steps and could be useful for assessing bacterial and fungal exposure, but these methods are not easy to apply to a large number of samples for logistical and financial reasons. Both approaches, viable and non-viable samplings, are complementary.

This study also provided information about the circumstances of children's exposure to airborne farm micro-flora. From the questionnaire, 57% of farm children were habitually present during feed distribution (hay and/or silage), for a mean duration

Table 3
Increase in level of exposure between cowsheds and farmer's bedrooms.

	Ratio cowshed before feeding/bedroom Geometric means (95%CI)	Ratio cowshed after feeding/bedroom Geometric means (95%CI)
Winter season		
Total moulds	16 (9–27)	66 (41–108)
Total yeasts	14 (7–29)	12 (6–26)
Total actinomycetes	82 (37–182)	464 (202–1065)
Overall total	24 (14–42)	78 (46–132)
Summer season		
Total moulds	14 (9–22)	33 (17–61)
Total yeasts	16 (7–36)	18 (8–45)
Total actinomycetes	53 (23–124)	162 (61–427)
Overall total	16 (10–27)	35 (19–64)

95% CI: 95% confidence interval.

Table 4
Increase in level of exposure between cowshed and farmer's bedrooms for the main micro-organisms.

	Winter		Summer	
	Cowshed before feeding/bedroom Geometric means (95%CI)	Cowshed after feeding/bedroom Geometric means (95%CI)	Cowshed before feeding/bedroom Geometric means (95%CI)	Cowshed after feeding/bedroom Geometric means (95%CI)
Moulds				
<i>Eurotium</i> spp.	46 (21–99)	458 (222–943)	57 (27–121)	113 (46–282)
<i>Aspergillus fumigatus</i>	9 (5–18)	13 (6–26)	7 (4–12)	9 (4–21)
<i>Cladosporium</i> spp.	7 (4–13)	28 (13–63)	9 (5–16)	9 (4–19)
<i>Aspergillus nidulans</i>	3 (2–4)	5 (3–9)	2 (1–3)	2 (1–3)
Actinomycetes				
<i>Mesophilic actinomycetes</i>	32 (14–71)	294 (124–697)	37 (16–84)	100 (37–268)
<i>Thermophilic actinomycetes</i>	9 (5–19)	50 (22–113)	5 (2–9)	11 (5–26)

95% CI: 95% confidence interval.

Table 5

Associations between airborne moulds and actinomycetes after feeding to cattle and farm characteristics and feeding practices.

Predictor variables	Moulds		Actinomycetes	
	Geometric means (CFU/m ³)	GMR (CI)	Geometric means (CFU/m ³)	GMR (CI)
Cowshed characteristics				
Country				
Germany	24,936	1	4026	1
Switzerland	31,734	1.27 (0.50–3.24)	4350	1.08 (0.33–3.56)
France	29,622	1.19 (0.55–2.55)	6509	1.62 (0.61–4.27)
Type of cowshed				
Loose housing system	16,557	1	4319	1
Tie-stalls	35,197	2.13 (1.03–4.40)	4990	1.16 (0.45–2.98)
Volume (m ³) ^a				
1st (70–710)	40,716	1	5096	
2nd (750–1470)	17,451	0.43 (0.19–0.95)	3775	0.74 (0.26–2.09)
3rd (1471–18900)	29,847	0.73 (0.32–1.66)	5734	1.13 (0.39–3.28)
Door(s) and window(s)				
Open	13,564	1	2470	1
Close	30,747	2.27 (0.82–6.23)	5227	2.12 (0.57–7.79)
Feeding practices				
Distribution method				
Manually or with a wagon	21,385	1	3704	
Hole on ceiling	69,504	3.25 (1.43–7.36)	11,688	3.16 (1.07–9.30)
Hay harvesting method				
Loose only	21,986	1	6065	1
Loose and bales	33,248	1.51 (0.65–3.51)	3036	0.50 (0.17–1.45)
Bales only	27,908	1.27 (0.54–2.96)	6204	1.02 (0.35–3.00)
Hay (kg/day) ^b				
1st (0–119)	26,909	1	3100	1
2nd (120–280)	29,918	1.11 (0.42–2.96)	4623	1.49 (0.44–5.04)
3rd (281–584)	26,629	0.99 (0.36–2.69)	3457	1.12 (0.32–3.87)
4th (585–4000)	26,989	1 (0.37–2.73)	10,083	3.25 (0.94–11.30)
Grass and/or maize silage (kg/day) ^b				
1st (none)	42,088	1	8713	1
2nd (26–160)	36,879	0.88 (0.23–3.35)	5089	0.58 (0.11–3.23)
3rd (180–990)	23,456	0.56 (0.24–1.32)	3191	0.37 (0.12–1.09)
4th (1000–6750)	14,568	0.35 (0.15–0.82)	2336	0.27 (0.09–0.80)
Fresh grass distribution				
Yes	11,701	1	1555	1
No	33,271	2.84 (1.14–7.08)	5879	3.78 (1.19–12.04)

GMR: geometric mean ratio.

^a In tertile of distribution.^b In quartile of distribution, CI: 95% confidence interval.

of 23 min. Thus in the cowshed, children inhale a high concentration of moulds and actinomycetes, particularly during and just after feeding. However, because stays in the cowshed are relatively short compared to those in the bedroom, the exposure to moulds and actinomycetes must also be considered. Here we showed that exposures to *Eurotium* spp., *Absidia* spp., *Cladosporium* spp., *Penicillium* spp. and mesophilic actinomycetes were higher in farm bedroom than in controls. These findings suggest that a part of the cowshed micro-flora is transported to the house indoor environment, leading to a moderate but continuous level of exposure to the cowshed bio-aerosol. Both the peaks encountered during the short stays in the cowshed and the moderate but continuous airborne micro-flora exposure in the bedrooms should be taken into account when assessing microbial exposure assessment in the farming context.

Farm exposure to moulds and actinomycetes plays a role in the modulation of immunity in humans. It is noteworthy that *Eurotium* spp., as well as several thermophilic actinomycetes, are known to be involved in immunological disorders such as hypersensitivity pneumonitis. This includes farmer's lung disease, an occupational disease due to an inappropriate immune response in some farmers exposed to high levels of moulds and

actinomycetes during hay handling (Reboux et al., 2001; Roussel et al., 2004). Immunological disorders related to this condition are not fully understood, but they differ from atopic response, and mainly result in extrinsic allergic alveolitis, corresponding to alveolitis with granuloma formation and diffuse interstitial pneumonitis (Bogaert et al., 2009; Di Stefano et al., 2007; Greenberger, 2008).

Our study also showed that the factors associated with high fungal and actinomycetes concentrations in the air were linked to specific agricultural practices, such as the lack of silage use, and the old-fashioned distribution method by a hole in the ceiling. Interestingly, some studies have already shown that not all farming environments are protective against asthma and wheezing in children. For example, in the PARSIFAL cross-sectional study, animal sheds and the presence of pigs on the farms were associated with lower adjusted odd ratios for atopic asthma, whereas the presence of sheep and the use of pressed hay were associated with higher atopic asthma risks (Ege et al., 2007). An assessment of the microbial diversity in several of these “protective” and “non-protective” farm environments may therefore help determine the factors involved in protecting children from asthma and atopy.

Acknowledgments

We grateful Gary Burkhart for editorial assistance.

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