

Original article

High levels of grass pollen inside European dairy farms: a role for the allergy-protective effects of environment?

Background: There is evidence of an allergy protective effect in children raised on farm. It has been assumed that microbial exposure may confer this protection. However in farm, little attention has been given to the pollen level and to concomitant microbiological exposure, and indoor pollen concentrations have never been precisely quantified.

Methods: The kinetics of pollen in dairy farms have been studied in a pilot study ($n = 9$), and exposure in a sub-sample of the ongoing European birth cohort PASTURE ($n = 106$). Measurements of viable microorganisms and pollen were performed in air samples. To identify factors that modulate the pollen concentration multivariate regression analyses were run.

Results: Indoor pollen (95% of *Poaceae* fragments and grains) were significantly higher in winter than in summer ($P = 0.001$) and ranged between 858 to 11 265 counts/m³ during feeding in winter, thus exceeding typical outdoor levels during the pollen season. Geometric mean in French farms was significantly higher than in German and Swiss farms (7 534, 992 and 1 079 count/m³, respectively). The presence of a ventilation system and loose housing systems significantly reduced indoor pollen levels. This pollen concentration rise after feeding was accompanied by an increase in fungal and actinomycetal levels, whereas the concentration of bacteria was not associated with feeding.

Conclusions: Farmers and their children who attend cowsheds during the feeding sessions are exposed perennially to high pollen concentrations. It might be speculated that the combined permanent exposure to microbes from livestock and grass pollen may initiate tolerance in children living on a farm.

B. Sudre¹, M. Vacheyrou¹, C. Braun-Fahrlander², A.-C. Normand¹, M. Waser², G. Reboux¹, P. Ruffaldi¹, E. von Mutius³, R. Piarroux^{1,4} and the PASTURE study group*

¹CNRS-Université de Franche-Comté/UMR 6249

Laboratoire Chrono-environnement, France;

²Institute of Social and Preventive Medicine, University of Basel, Switzerland; ³University of Munich, University Children's Hospital, Germany;

⁴University hospital de la Timone, Parasitology and mycology laboratory, Marseille, France

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Sudre Bertrand

CNRS-University of Franche-Comté/UMR 6249

Laboratoire Chrono-environnement

Parasitology-Mycology departement

University hospital of Besançon

France

*The PASTURE study group: G. Weiß, E. Üblagger, C. Humer, M. Rußegger, J. Riedler (Austria); R. Juntunen, R. Tiitonen, P. Tiittanen, M. R. Hirvonen, K. Huttunen, S. Virtanen, T. Kauppila, A. Nevalainen, A. Hyvärinen, T. P. Tuomainen, A. Karvonen, M. Roponen, S. Remes, J. Pekkanen (Finland); D. A. Vuitton, J. C. Dalphin, M. L. Dalphin, S. Roussel (France); M. J. Ege, G. Büchele, S. Schmid, S. Illi, N. Korherr, J. Genuneit, R. Peter, Serdar Sel, N. Blümer, P. Pfefferle, I. Herzum, S. Krauss-Etschmann, H. Renz (Germany); U. Gehring, B. Brunekreef (the Netherlands); S. Bitter, F. H. Sennhauser, S. Loeliger, J. Steinle, R. Frei, R. P. Lauener (Switzerland).

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Charles Harrison Blackley observed that 'farmers and other individuals who were chronically exposed to high levels of pollen appeared to be unaffected by this exposure' (Blackley, 1873). From the turn of the 21st century, the 'farmer paradox' has received new support from epidemiological studies, which gave evidence for a protection against allergic diseases by farm environments (1, 2). These studies have become part of the 'hygiene hypothesis', assuming that microbial exposure in farm environments confers this protection (3–5). Whereas the

protective influence of massive inhalation of cat and/or dog allergens has been shown (6–8), raising the hypothesis of tolerance induction by antigen exposure in early life (9), pollen exposure on a farm has not received much attention and has never been precisely quantified.

The interest in measuring pollen exposures within farms in addition to microbial compounds has been further stimulated by recent findings of the ongoing birth cohort PASTURE including 500 farm children and 500 nonfarm children from rural areas of five European

countries (10). The study provided evidence of an inverse relationship between maternal exposure to animal sheds during pregnancy and infants' cord blood immunoglobulin E (IgE) against seasonal allergens which was enhanced by maternal contact to hay during pregnancy (11).

We hypothesized that subjects attending cowsheds might (1) be exposed not only to high levels of microbes but also to high levels of grass pollen and (2) that different farm characteristics might influence the indoor pollen concentrations. To evaluate these hypotheses, we measured levels of pollen and bacteria in air samples of stables of a subgroup of PASTURE farms.

Materials and methods

Study design

A pilot study was performed in nine French farms located in the Franche-Comté region to establish the kinetics of pollen settlement after hay distribution for cattle feeding. Measurements were performed in winter before starting the feeding session (T1), 5 min after starting the feeding session (T2) and 5 min (T3), 30 min (T4) and 55 min after completing the feeding session (T5) respectively (Fig. 1). Each farm has been sampled two times in winter season for pollen kinetic assessment.

Among the PASTURE cohort families (10), a random sample of 106 farmer's families were selected to participate in the exposure study. Farms were visited once in winter (2004/2005) and once in summer 2005. Pollen measurements were performed 20 min before feeding (T1) and 5 min after the beginning of fodder distribution

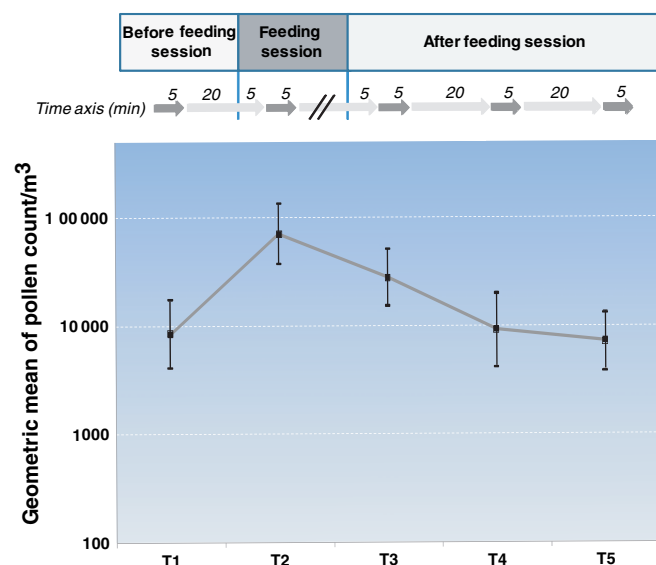


Figure 1. Indoor pollen counts/m³ in winter before, during and after the feeding session in nine French cowsheds (geometric means and 95% confidence intervals, $n = 18$ per time point). Measurements were taken during 5 min intervals each. (T1) 25 min before the start of the feeding session, (T2) 5 min after the start of the feeding session, (T3) 5 min, (T4) 30 min and (T5) 55 min after the end of the feeding session.

(T2). During summer, T2 samples could only be measured in 63% of the farms, because cattle was not fed in the cowsheds.

For a sub-sample of 70 farms, additional measurements of bacteria and fungi in air samples were available. These samples were collected at (T1) and immediately following (T3) during 20 min each. The samples were collected by fieldworkers and analysed in the Parasitology and Mycology Laboratory of the University of Franche-Comté, the Swiss and German samples being sent by express mail. Information about farm characteristics and agricultural practices was obtained by the main PASTURE questionnaires (10) and an additional short questionnaire completed during the field visit.

Pollen measurements

Collection of pollen was based on active impaction of air particles (with a 3 L/min flow) on a membrane during a 5-minute pumping session using an Gil-Air 3 pump (Sensidyne®, Clearwater, FL, USA), fixed on a tripod at a fixed height of 75 cm. The device was located at a central place of the cowshed. The pumps were equipped with a removable plastic cassette (37 mm; Sensidyne) that contained a polypropylene membrane covered with a mixed petrolatum/toluene solution. In the laboratory, the membrane was transferred and fixed on a glass slide, then coloured with fuchsin for counting. Microscopic examination was performed by direct reading, covering 11 adjacent fields (CF) of 0.48 mm in diameter from centre (CF1) to periphery (CF10 and CF11). Counting included pollen grains, folded pollen and fragments of pollen grains which were summed up to total pollen counts. To estimate the pollen count (PC) of the central part of the membrane (5.28 mm²), the following formula was used:

$$PC = 1.5[CF1 + 4(CF2 + CF3) + 8(CF4 + CF5) + 12(CF6 + CF7) + 16(CF8 + CF9) + 20(CF10 + CF11)]$$

Identification of pollen was performed by palynologists according to reference manuals (12). Results were expressed in pollen counts per membrane and further converted to pollen counts/m³.

Bacterial measurements

Bacterial samples were collected on the same device using a cassette (37 mm; Sensidyne) containing a Teflon® filter (pore diameter of 0.45 µm; Millipore Sensidyne). Filters were rinsed with 10 ml of 0.1% Tween 80 sterile water (Sigma-Aldrich, Steinheim, Germany) and shaken for 10 min in Stomacher (AES Laboratoire, Combourg, France). Two-hundred and fifty microliters of 10-fold dilutions was spread on Petri dishes with Müller-Hinton medium (MH; Becton Dickinson, Cockeysville, MD, USA) incubated at 30°C for culture of thermotolerant aerobic bacteria species, with Difco actinomycetes isolation agar (Becton Dickinson, le Pont de Claix, France) at 30°C for mesophilic actinomycetes and with R8 medium according to Amner and colleagues at 52°C to recognize thermophilic actinomycetes (13). Actinomycetes colonies were identified on macroscopic and microscopic criteria and hydrolysis of casein and temperature test. Results were expressed in Colony Forming Unit per cubic meter (CFU/m³) after 48 h of incubation for thermotolerant aerobic bacteria species and after 7 days of incubation period for actinomycetes. The five most frequent phenotypes of thermotolerant aerobic bacteria species were isolated based on macroscopic characteristics, stained by Gram procedure and conserved at -80°C in glycerol-heart-brain medium.

Phenotypes from before work ($n = 33$) and after work ($n = 30$) samples were identified by sequencing polymerase chain reaction (PCR) at species level. Bacterial DNA of given phenotypes was extracted as described by Drancourt et al. (14) and 100 μ l of the supernatant was collected. fD1 and rD1 were used as primers for 16S rDNA PCR (15). DNA amplification was performed with 0.5 μ M of primers fD1 and rD1, 200 μ M of dNTP, 1 mM of MgCl₂, 1 $\times$ of redTaq buffer, 0.01 U of redTaq, and 1 μ l of DNA sample. The reaction mixture was subjected to the PCR program as reported by Paster et al. (16). The amplification products of each reaction were analysed by electrophoresis for 60 min at 80 V in agarose (1.5%) gels. DNA bands were visualized by UV transillumination. For sequencing, the mix contained DNA polymerase, pyrophosphatase, buffer, dNTPs, dye terminators, DNA templates, water and internal reverse primer P-519 (5'-GTA TTA CCG CGG CTG CTG GCA C-3'), that allowed to sequence a 497-bp fragment. The sequencing mixture was subjected to 35 cycles of 20 s denaturation at 96°C, 20 s primer annealing at 50°C and 2 min primer extension at 60°C. The reading was performed with Ceq8000 (Beckman Coulter, Fullerton, CA, USA) and compared to the NCBI genbank database using Blast (<http://www.ncbi.nlm.nih.gov/blast>). Molecular identification at genus level was defined by sequence similarity higher than 97% (17, 18).

Fungal measurements

Two-hundred and fifty microliters rinsing solution from filter were spread on two different Petri dishes: Dichloran-Glycerol (Oxoid LTD, Basingstoke, England) with 0.5% of chloramphenicol (Sigma-Aldrich, Steinheim, Germany) at 30°C for mesophilic mould isolation and 3% malt-agar (AES, Bruz, France) with 10% salt and 0.5% chloramphenicol for osmophilic fungal species. Fungal colonies were identified on macroscopic and microscopic criteria and results were expressed in CFU/m³ after 7 days of incubation period.

Statistical analysis

Pollen and microbial counts were log transformed and expressed as geometric mean counts/m³ for different sampling time points. Zero values were replaced by half of the detection limit. Comparison of viable species count was performed with Wilcoxon paired test. To evaluate the association between pollen concentrations and farm characteristics taking into account the repeated measurements, a generalized linear latent and mixed model (GLLAMM in STATA) was run with farm as random effect. Variables associated with pollen counts in univariate analyses ($P < 0.25$) were included in the final multivariate model. Results were expressed as (adjusted) geometric means ratio and 95% confidence interval (CI). The analyses were restricted to winter pollen measurements. To evaluate factors associated with an increase of pollen concentration between T1 and T2 a separate multivariate regression model was run with the difference between T1 and T2 (log transformed) as the dependent variable. Statistical analyses were performed using Stata 10.0 (Stata Corporation, College Station, TX, USA).

Results

Microscopic examination of the pollen glass slides indicated that more than 95% of the pollen grains, folded grains and fragments originated from Poaceae (grass

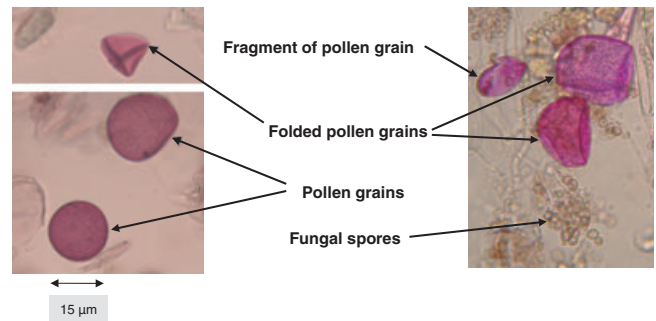


Figure 2. Microscopic examination of a pollen sampling membrane. Microscopic examination of pollen slide preparation at magnification $\times 400$.

family); the remaining 5% included Apiaceae, Pinaceae, Betulaceae and Asteraceae (Fig. 2). The size of the pollen grains (60% of total counts) ranged between 15 and 25 μ m; pollen fragments were < 15 μ m in diameter and accounted for 15% of total counts.

Figure 1 illustrates that pollen counts/m³ were the highest during the feeding session (T2), persisted after the feeding session at T3 and then dropped rapidly. Country-specific geometric means of pollen counts/m³ at T1 and T2 in winter are shown in Fig. 3. During feeding, mean pollen counts/m³ increased by a factor of 17.2, 10.2, and 3.0 in France, Switzerland and Germany respectively and ranged between 858 and 11 265 counts/m³. In the nine French pilot farms, the observed values exceeded 15 000 counts/m³.

Mean indoor pollen counts in winter were significantly higher than in summer [pollen counts/m³ T1 winter 345 (95% CI 231–516), T2 winter 2 214 (95% CI 1368–3581) T1 summer 120 (95% CI 78–183), T2 summer 167 (95% CI 93–300)]. Geometric means of thermotolerant aerobic

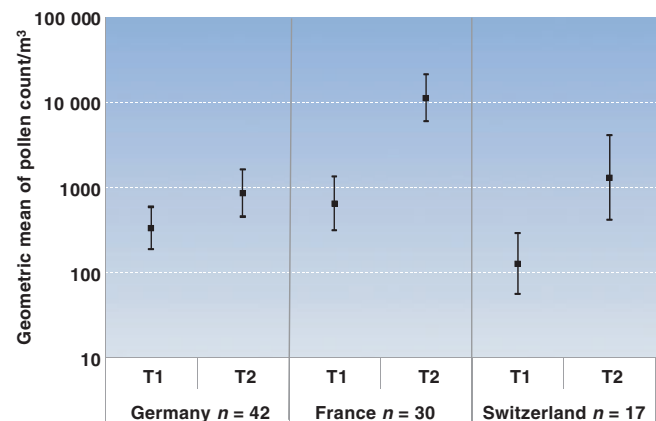


Figure 3. Country-specific indoor pollen counts/m³ in winter before (T1) and during (T2) feeding session (geometric means and 95% CI; $n = 89$ cowsheds. (T1) 25 min before the start of the feeding session and (T2) 5 min after the start of the feeding session.

Table 1. Farm characteristics, stratified by country

		Total, (<i>n</i> = 106) (%)†	Germany, (<i>n</i> = 57) (%)	France, (<i>n</i> = 32) (%)	Switzerland, <i>n</i> = 17 (%)	<i>P</i> -value*
Cowshed characteristics						
Altitude meters above sea level, arithmetic mean (95% CI)		639 (607–671)	686 (667–706)	592 (499–684)	577 (520–633)	0.007
Type of cowshed	Tie-stalls	67	75	55	60	0.129
	Loose housing systems	33	25	45	40	
Number of cows	Geometric mean (95% CI)	42.4 (36.3–49.5)	39.3 (30.8–50.1)	58.9 (48.1–72.0)	26.9 (18.9–38.3)	0.010
Cowshed volume (m ³)	Geometric mean (95% CI)	1015 (822–1253)	795 (595–1063)	2084 (1524–2850)	594 (392–899)	0.002
Ventilation system	Yes	23	28	19	12	0.304
	No	77	72	81	88	
Agricultural practices						
Hay storage system	Loose hay only	35	43	13	49	<0.001
	Storage of hay balls	31	16	68	13	
	Both systems	34	41	19	38	
Fodder type	Hay only	35	4	74	67	<0.001
	Hay and silage (grass and/or maize)	65	96	26	33	
Transport of hay to stable	Manually	78	70	81	100	0.036
	Hole in the ceiling	22	30	19	0	
Feeding system	By hand	90	85	93	100	0.191
	Mechanical	10	15	7	0	
Quantity of fodder (kg/day)						
Quantity of hay	Geometric mean (95% CI)	277 (223–343)	147 (118–183)	573 (414–794)	413 (270–634)	<0.001
Quantity of silage (hay and/or maize)	Geometric mean (95% CI)	810 (584–1123)	952 (694–1305)	1118 (364–3436)	142 (29–703)	0.002
	No silage	39	12	69	71	

CI, confidence interval.

†Computed among those answering a specific question. Internal nonresponse/missing rate was <15% for all characteristics.

*P-value from chi-squared test (categorical variables) or ANOVA test (continuous variables) to assess differences between countries.

bacteria species were 2.82×10^4 CFU/m³ and 3.62×10^4 CFU/m³ before (T1) and after work (T3) respectively, corresponding to a nonsignificant increase by a factor of 1.3 ($P = 0.28$). About 75% of the bacteria were gram positive and 25% gram negative. The three genera representing more than 70% of species identified by PCR were *Staphylococcus* spp. (46.5% and 47.8% before and after work, respectively), *Corynebacterium* spp. (16.3% and 5.4%) and *Bacillus* spp. (15.3% and 18.3%).

Geometric mean of total of fungi spp. significantly increased between T1 (7.8×10^3 CFU/m³) and T3 (27.4×10^3 CFU/m³) by a factor of 3.51 ($P < 0.001$). The same trend was found for actinomycetes spp. comparing T1 (1.8×10^3 CFU/m³) and T3 (4.7×10^3 CFU/m³) leading to an increase of a factor 2.7 ($P < 0.001$). The results will be presented in detail in a separate paper.

Farm characteristics differed in many aspects between the countries (Table 1). The associations between these farm characteristics and winter indoor pollen counts/m³ were evaluated in multivariate regression models (Table 2). In cowsheds with loose housing systems, indoor pollen counts were significantly lower than in tie-stalls and the use of mechanical ventilation system significantly lowered indoor pollen levels. In French farms, significantly higher indoor pollen levels were

measured than in German and Swiss farms and none of the tested farm characteristics could explain this difference.

To evaluate factors associated with an increase in pollen counts/m³ between T1 and T2, multivariate models were run indicating that the increase was somewhat higher in French farms compared with German farms [adjusted odd ratio (OR) (95% CI) 5.66 (0.90–35.5), $P = 0.06$], when only hay was fed compared to silage and hay [adjusted OR (95% CI) 4.78 (0.87–26.2), $P = 0.07$], when loose hay was stored compared to storage in balls [adjusted OR (95% CI) 3.44 (0.99–11.9), $P = 0.05$], but lower in loose housing systems compared with tie-stalls [adjusted OR (95% CI) 0.13 (0.03–0.62), $P = 0.01$]. The quantity of fodder did not further explain the increase in pollen counts/m³.

Discussion

This study demonstrates that exposure to grass pollen in cowsheds is massive and peaks during cattle feeding. Its level is recorded all year round, exceeds outdoor concentrations by a large amount (19, 20) and is particularly high in winter. Farmers and their children who attend stables during the feeding sessions are thus perennially exposed to high pollen concentrations.

Table 2. Crude and adjusted association between farm characteristics and indoor pollen counts/m³ GMR and 95% CI, *n* = 89 cowsheds

Predictor variables	Geometric mean pollen count/m ³	Crude models		Mutually adjusted model	
		GMR (95% CI)	P-value	GMR (95% CI)	P-value
Time point*	T1	1		1	
	T2	6.50 (3.71–11.40)	<0.001	5.87 (3.36–10.26)	<0.001
Country	Germany	1		1	
	France	4.79 (2.37–9.68)	<0.001	4.73 (1.83–12.20)	0.001
	Switzerland	0.77 (0.33–1.81)	0.554	0.75 (0.28–1.99)	0.563
Type of cowshed	Tie-stalls	1		1	
	Loose housing systems	0.65 (0.31–1.34)	0.239	0.30 (0.14–0.66)	0.003
Cowshed volume (m ³)	1st tertile (70–710)	1		1	
	2nd tertile (750–1470)	1.28 (0.58–2.83)	0.534	0.99 (0.49–2.02)	0.982
	3rd tertile (1471–18 900)	1.96 (0.86–4.48)	0.111	1.41 (0.56–3.52)	0.463
Ventilation system	Yes	1		1	
	No	2.04 (0.92–4.51)	0.079	3.17 (1.53–6.56)	0.002
Hay storage system	Loose hay only	1		1	
	Balles with/without loose hay	2.16 (1.07–4.35)	0.031	1.18 (0.63–2.20)	0.601
Fodder type	Hay only	1		1	
	Hay and silage	0.35 (0.18–0.70)	0.003	0.82 (0.35–1.93)	0.647

GMR, geometric means ratios; CI, confidence interval.

* (T1) 25 min before the start of the feeding session and (T2) 5 min after the start of the feeding session.

The presence of a ventilation system and loose housing systems significantly reduced indoor pollen levels. Yet, in French farms, significantly higher pollen levels were measured compared with German or Swiss farms and this difference could not be explained by taking into account farm characteristics, which significantly differed between the countries. The increase in pollen concentrations during a feeding session was most notably related to feeding hay only and not feeding silage. On some of the French farms (especially in Franche-Comté), feeding silage is not allowed because of cheese production regulations and this might partly explain the high pollen levels measured in French farms, notably in those of the pilot study.

Concomitant to the rise in pollen concentrations associated with feeding the cattle, levels of total fungi also increased, whereas the airborne levels of thermotolerant aerobic bacteria were not significantly related to the feeding process. This is most likely because hay is not the predominant source of bacteria in cowsheds. In addition, only a limited part of the bacterial biodiversity was measured in the present study. In line with previous research on the bacterial composition of animal shed dust, the present study of stable air samples found *Bacillus* spp. to be one of the most prevalent bacterial species. *In vitro*, *Bacillus licheniformis* spores have been found to activate a Th1-cytokine expression profile (21). Other bacterial species, abundant in cowshed microflora, namely *Lactococcus lactis* (gram-positive) and *Acinetobacter lwoffii* (gram-negative), have recently been shown to protect mice against the development of an allergic airway inflammation that might be because of the Th1 promoting effect of these bacteria (22).

The present study showed that in addition to microbial exposure in stable air high levels of grass pollen could be

measured. One might therefore speculate that differential pollen exposure patterns contribute to the 'protective farming effect' because of tolerance induction in subjects exposed to repeated and massive inhalation of pollen. Although specific immunotherapy leading to tolerance uses 'low doses' of allergens, 'high dose' induction of tolerance against allergic sensitization is suggested by the epidemiological studies, which show a negative relationship between cat allergen exposure in infancy and risk of sensitization to cat and more generally atopic sensitization (7, 23). Animal models suggest that continuous exposure is likely to be crucial. In mice models of allergenic sensitization, acute or discontinuous inhalation of the allergen induced IgE sensitization, bronchial hyperresponsiveness and inflammation, but chronic exposure to the same allergen led to tolerance. Interestingly, inhalational tolerance induced by continuous ovalbumin exposure demonstrated bystander suppression of cockroach allergen-mediated airway eosinophilia (24). Such a 'bystander effect' could explain why children living on a farm are not only protected against allergy to seasonal allergens but also, more globally, to other environmental allergens. However, farming exposure could lead to tolerance in a more complex way (25) and a possible role for an 'allergen-induced tolerance', mainly driven by continuous exposure to pollen, does not preclude the additional and decisive influence of microbial components of the farm environment. The most recent results of the PASTURE study suggest that combined permanent exposure to livestock and grass pollen through their mothers may initiate tolerance in children living on a farm in their foetal life (11). But more research is needed to determine how these exposures interact with the developing immune system. Yet, high levels of allergen

exposure together with high levels of microbial (bacterial and fungal) exposure may result in the allergy protective

effect of childhood animal shed exposure as seen in numerous farm studies.

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