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Recurrence of *Stachybotrys chartarum* during mycological and toxicological study of bioaerosols collected in a dairy cattle shed

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Abstract

Agricultural occupations associated with animal breeding and the processing of animal materials in confinement systems could potentially lead to bioaerosol exposures. Moulds and mycotoxins could be constituents of bioaerosols and should be studied because of their possible involvement in respiratory diseases and cancers. In order to characterize the fungal contamination of the indoor air in a dairy barn, bioaerosols were collected during 20 days in a cattle farm located in Normandy (France). Mycobiota, mycotoxins and the mutagenicity of bioaerosols were studied. The toxigenic ability of *Aspergillus flavus* group and *Aspergillus fumigatus* isolates was also evaluated *in vitro*. The prevalent airborne moulds were from the following potentially toxigenic species: *Aspergillus flavus* group, *Aspergillus fumigatus*, *Penicillium chrysogenum*, *Stachybotrys chartarum*, and the allergenic species *Ulocladium chartarum*, *Cladosporium cladosporioides*. In comparison with harvesting, grain handling or broiler breeding, the concentrations of viable moulds were lower in the cattle shed. Seasonal variations in levels of several species were also observed. This study revealed that aflatoxins were detected in bioaerosols and, for the first time, showed that farmers are possibly exposed to *Stachybotrys chartarum* during routine barn work. Moreover, the finding of mutagenicity from bioaerosols needs further investigations on bioaerosol composition.

Key words

airborne moulds, cattle shed, dairy farm, mutagenicity, mycotoxins

INTRODUCTION

Normandy (France) is an agricultural area characterized by bovine breeding for the production of milk and meat [1]. On these farms, cattle are kept on loose straw in sheds for several months, and fed with corn silage and oilseed cakes.

Farmers could be exposed to dust generated during daily tasks, such as cattle feeding (preparation and distribution of feed), animal care and mulching. This dust is composed of organic and inorganic particles, gases, chemicals, biological agents and other toxicants [2]. The concept of bioaerosols can be applied to airborne agricultural dust. Bioaerosols are usually defined as aerosols or particulate matter of organic origin [3]. These organic particles include pollens, plant fragments, bedding materials, manure and microorganisms. The microbial part may consist in bacteria, endotoxins, moulds and mycotoxins.

The agricultural environment is an important source of exposure to airborne fungi. Moulds belonging to *Aspergillus*, *Penicillium*, *Cladosporium* and *Alternaria* genera were previously identified from bioaerosols generated during grain unloading and handling [4], and can also be associated with the handling of feedstuffs, e.g. corn silages and oilseed cakes [5]. Mycotoxins are toxic fungal metabolites produced by moulds in vegetal matrices, and could be potentially detected in bioaerosols because of their adsorption on spores and dust particles [6, 7].

Exposure to bioaerosols in the occupational environment is associated with a wide range of health effects including infectious diseases, acute toxic effects, allergies and cancer. Various constituents could be aerosolized during activities associated with animal breeding, including those derived from bioaerosols, such as fungi, bacteria, endotoxins, viruses and parasites. Exposure to them may result in respiratory diseases [8]. Respiratory symptoms and lung function impairments: asthma, farmer's lung disease (FLD) or rhinitis, are probably the most widely studied among organic dust associated health effects [9, 10]. Among bioaerosols constituents, moulds and mycotoxins could be responsible for several diseases. Farmers exposed to mould dust may exhibit signs of alveolitis [11], and severe toxic irritative reactions can occur after a single inhalation of high levels of spores [12]. Studies have suggested that inhalation exposure to mould spores is another cause of organic dust toxic syndrome [13]. Larsson *et al.* [11] have also shown that asymptomatic dairy farmers exposed to airborne mould dust may have signs of immunostimulation and inflammation in their alveolar space.

Exposure to mycotoxins like aflatoxins and ochratoxin can also occur by inhalation during peanut or livestock feed processing and grain handling [6, 14]. Mycotoxins represent the only clearly established non-viral biological occupational carcinogens. Several cases of pulmonary cancers were reported in workers exposed to aflatoxins *via*

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the respiratory route [15, 16]. In Denmark, an increase in the risk of liver cancer has been reported for workers exposed to aflatoxins in concerns processing livestock feed [17]. Although various toxic effects of mycotoxins (cytotoxicity, neurotoxicity, immunosuppressive effects), as well as the carcinogenicity of mycotoxins standards have been widely studied [18, 19], little is known about occupational airborne exposures to mycotoxins and the genotoxicity of bioaerosols generated during farming activities.

Because the evaluation of fungal contamination and genotoxicity of the ambient air in dairy shed has rarely been investigated, the aims of this study were: 1) to characterize fungal contamination (moulds and mycotoxins) of bioaerosols collected in a dairy shed during 20 days, 2) to study the toxigenic abilities of *Aspergillus fumigatus* and *Aspergillus flavus* group isolates, 3) to evaluate the mutagenic proprieties of bioaerosols samples using the Ames test.

MATERIALS AND METHODS

Chemicals and reagents. The 17 mycotoxin standards (aflatoxins B_1, B_2, G_1, G_2, M_1, citrinin, diacetoxyscirpenol, fumagillin, gliotoxin, HT-2 toxin, mycophenolic acid, neosolaniol, nivalenol, ochratoxin A, patulin, T-2 toxin, verruculogen) and the 2 internal standards fumonisin B_1-$^{13}C_{34}$ and deoxynivalenol-$^{13}C_{15}$, were supplied by Sigma-Aldrich (St. Louis, MO, USA), Calbiochem (Merck, VWR Prolabo, Fontenay-sous-Bois, France) and LGC Promochem (Molsheim, France). The stock solutions of 250 µg/mL were prepared in LC grade methanol from Chromanorm VWR Prolabo (Fontenay sous Bois, France) and stored at -20°C in the dark. The diluted solutions were prepared by diluting the stock solutions with mobile phase acetonitrile/water (10:90, v/v). The working solutions were prepared by combining suitable aliquots of each individual standard stock dilution to obtain each mycotoxin at 500 µg/L Milli-Q quality water (Millipore, Bedford, MA, USA). All other chemicals of LC grade were obtained from Prolabo.

Bioaerosols collection. A cattle shed located in Normandy was monitored during 2 periods of 10 days each (period 1: January-February and period 2: March-April). The selected farm was a dairy farm characterized by 372 acres of cultivated land and a total of 205 cattle, mainly fed on corn silage, oilseed cakes and hay. During the monitoring, 170 cows were kept in the barn with a surface area of 2,500 square metres. The livestock presented a permanent movement in the building because of the presence of a milking robotic system allowing voluntary and regular milking.

During the 20 days, ambient air sampling was conducted 24 hours per day in the feeding corridor, using a high volume sampler (Model DA-80, DIGITEL Elektronik AG, Hegnau, Switzerland), drawing air at a flow rate of 500 L/min. The device was equipped with a PM 10 head and of 150 mm quartz microfibre filters (CAT No. 1851-150, Whatman International Ltd., Maidstone, UK). A quarter filter was used for the analysis of mycobiota, a second quarter was used for the multimycotoxin analysis, and a third part was used for mutagenic evaluation.

Mycological analysis from bioaerosols. Quartz microfibre filters were suspended in 20 mL of sterile water containing Tween 80 (0.05%, w/v) (Sigma-Aldrich, St. Louis, MO, USA). After 30 min of magnetic shaking at 420 rpm, 3 dilutions of the suspension (10^{-1}, 10^{-2} and 10^{-3}) were made. 1 mL of each dilution (triplicate) was deposited in a Petri dish (90 mm diameter), and the culture medium constituted by malt extract (1.5%)/agar (1.5%) medium (MEA) complemented with chloramphenicol (0.05 %, w/v) was poured over it, following the dilution plates method. The plates were incubated at 25°C and 30°C and viable moulds were counted each day during the 7 days of incubation. The fungal concentration was expressed as colony-forming units per cubic meter of air (cfu/m^3). The quantification limit corresponded to the minimum number of colonies countable from the filter (80 colonies). Each colony was isolated and purified on MEA. *Aspergilli* and *Penicillia* were identified after culture on Czapek yeast autolysate agar (CYA) and 25% glycerol nitrate agar (G25N) [20], and *Fusaria* on potato dextrose agar medium (PDA) [21]. The identity of each colony was checked through macro- and microscopic examinations [20, 21, 22, 23, 24, 25, 26].

Isolates of *Aspergillus fumigatus* and *Aspergillus flavus* group were preserved on agar slants (MEA) at 4°C before their multi-mycotoxin screening by HPLC-MS/MS.

Toxigenic ability of *Aspergillus flavus* and *Aspergillus fumigatus* isolates. Twenty-five isolates of *Aspergillus flavus* and 19 isolates of *Aspergillus fumigatus* collected from the bioaerosols were purified. They were respectively tested (triplicate) *in vitro* for aflatoxins B_1, B_2, G_1, G_2, M_1 and fumagillin, gliotoxin, verruculogen production. After 2 weeks of culture on MEA, 3 agar plugs were extracted by ethyle acetate, acidified with 1% acetic acid as previously described by Garon *et al.* [27]. All mycotoxins were quantifiable by HPLC-MS/MS from 0.5 ng/g medium.

Multi-mycotoxin analysis from bioaerosols. The mycotoxins were extracted twice with 30 mL of methanol acidified with acetic acid (0.5%) from the quartz microfibre filter. The solutions were kept in an ultrasonic bath for 3 min, and then shaken for 10 min in a multi-tube vortexer. After evaporation to dryness, the final residue was dissolved in 0.5 mL of a mixture of acetonitrile/water (10:90, v/v), and then filtered through Millex HV 0.45 µm before injection into HPLC-MS/MS.

An Agilent Technologies 1200 HPLC system coupled to a spectrometer (Agilent Technologies, Santa Clara, CA, USA) was used to analyze the samples, equipped with a MassHunter B.02.00 software for data processing. The analytes were chromatographed according to 2 chromatographic methods.

Three mycotoxins (HT-2 toxin, nivalenol, patulin) were separated (Method 1) onto a Zorbax Eclipse Plus, Rapid Resolution HD-C_{18} column (1.8 µm, 50 × 2.1 mm; Agilent Technologies) at 60°C. Deoxynivalenol-$^{13}C_{15}$ was used as the internal standard. The injection volume of the samples on the analytical column was 20 µL. The mobile phase consisted of a variable mixture of methanol (solvent A) and water (solvent B) at a flow rate of 0.4 mL/min. A linear gradient was run, starting with 10% - 100% solvent A for 10 min, and stay at 100% for 1 min.

The mass spectrometer was operated in both negative and positive modes using multiple reaction monitoring (MRM). Three retention windows were defined according to the retention time and the optimized ESI mode. The nebulizer gas and desolvatation gas were nitrogen heated at 250°C at

Table 1. HPLC-MS/MS parameters for multi-mycotoxin quantification

Retention time (min)	Mycotoxins	ESI mode	Precursor ion (*m/z*)	Molecular ion	Fragmentor (V)	Product ions (*m/z*)	Collision energy (V)
Method 1: Zorbax Eclipse Plus, Rapid Resolution HD-C_{18} column							
0.90	PAT	-	153	$[M-H]^-$	40	109.1* 81.1	2 6
1.11	NIV	-	371	nd	100	281	10 5
1.73	DON-C13[a]	+	312.1	$[M+H]^+$	100	263.1* 216.1	8 12
5.90	HT2	+	442.2	$[M+NH_4]^+$	96	263.1* 215.1	10 10
Method 2: Zorbax SB, Rapid Resolution HT-C_{18} column							
1.79	NEO	+	400.2	$[M+NH_4]^+$	96	305.1* 185.1	6 14
2.61	AFM1	+	329.1	$[M+H]^+$	150	273.1* 259.1	24 24
2.86	AFG2	+	331.1	$[M+H]^+$	155	313.1* 285	24 28
3.05	GLIO	+	263	nd	130	245.1* 111.1	8 16
3.11	AFG1	+	329.1	$[M+H]^+$	150	243.1* 311	28 24
3.18	AFB2	+	315.1	$[M+H]^+$	150	287.1* 259	24 28
3.38	DAS	+	384.2	$[M+NH_4]^+$	95	307.1* 105.1	8 50
3.41	FB_1-C13[a]	+	756.4	$[M+H]^+$	182	374.1* 356.4	40 44
3.42	AFB1	+	313.1	$[M+H]^+$	170	285.1* 241	20 30
4.21	CIT	+	251.1	$[M+H]^+$	100	233.1* 91.1	16 50
4.24	MPA	+	321.1	$[M+H]^+$	99	303.1* 207.1	6 18
4.77	T2	+	484.2	$[M+NH_4]^+$	100	305.1* 185.1	8 20
5.06	OTA	+	404.1	$[M+H]^+$	110	239* 358	24 12
5.65	VER	+	494.2	$[M-H_2O]^+$	120	352* 255	10 26
5.97	FUM	+	459.2	$[M+H]^+$	120	131.1* 177.1	28 12

AFB1 – aflatoxin B_1, AFB2 – aflatoxin B_2, AFG1 – aflatoxin G_1, AFG2 – aflatoxin G_2, AFM1 – aflatoxin M_1, CIT – citrinin, DAS – diacetoxyscirpenol, FUM – fumagillin, GLIO – gliotoxin, HT2 – HT-2 toxin, MPA – mycophenolic acid, NEO – neosolaniol, NIV – nivalenol, OTA – ochratoxin A, PAT – patulin, T2 – T-2 toxin, VER – verruculogen.
nd - not determined.
[a] internal standards: fumonisin B_1-$^{13}C_{34}$ (FB1-C13) and deoxynivalenol-$^{13}C_{15}$ (DON-C13)
* most abundant production.

10 L/min (except for the third retention window, at 12 L/min), and 400°C at 12 L/min, respectively.

Fourteen mycotoxins (aflatoxins B_1, B_2, G_1, G_2, M_1, citrinin, diacetoxyscirpenol, fumagillin, gliotoxin, mycophenolic acid, neosolaniol, ochratoxin A, T-2 toxin, verruculogen) were separated (Method 2) onto Zorbax SB, Rapid Resolution HT-C_{18} column (1.8 µm, 50 × 2.1 mm; Agilent Technologies) at 60°C. Fumonisin B_1-$^{13}C_{34}$ was used as the internal standard The injection volume of the samples on the analytical column was 10 µL. The mobile phase consisted of a variable mixture of acetonitrile (solvent A) and water to which was added formic acid 1% (solvent B) at a flow rate of 0.4 mL/min. A linear gradient was run, starting with 10% - 100% solvent A for 10 min, and stay at 100% for 1 min.

The mass spectrometer was operated in positive mode using dynamic multiple reaction monitoring (MRM). The nebulizer gas and desolvatation gas were, respectively, nitrogen heated at 300°C at 10 L/min and 400°C at 12 L/min.

Other common parameters used for the mass spectrometer were as follow: capillary voltage, 4.0 kV; pressure of nebulisation, 45 psi; nozzle voltage, 300 V.

The most abundant product ion after collision-induced fragmentation was used for quantitative purposes, and the second product ion for confirmation. Table 1 shows all the components, with their retention time, ionisation mode, precursor ion, cone voltage, product ions and collision energy. Sixteen mycotoxins (aflatoxins B_1, B_2, G_1, G_2, M_1, citrinin, diacetoxyscirpenol, gliotoxin, HT-2 toxin, nivalenol, patulin, mycophenolic acid, neosolaniol, ochratoxin A, T-2 toxin, verruculogen) were extracted and quantified from the quartz filter. The recoveries and quantification limits were presented in Table 2. Linearity was carried out by spiking increasing concentrations (triplicate) of the mycotoxin standards (0.1 to 50 µg/L). The quantification and detection limits (LQ and LD) were determined by spiked samples, based on a signal to noise ratio of 10:1 for quantification, and 3:1 for detection limit.

Table 2. Recoveries and quantification limits of mycotoxins in bioaerosols.

Mycotoxin	Recovery (%)	Quantification limit (ng/filter)
PAT	63	0.63
NIV	94	23.49
NEO	95	0.05
AFM1	81	0.06
AFG2	78	0.06
GLIO	73	1.36
AFG1	52	0.10
AFB2	70	0.07
DAS	97	0.05
AFB1	55	0.09
CIT	55	0.09
MPA	91	0.05
T2	56	0.09
OTA	88	0.06
VER	58	0.09
HT2	42	0.11

AFB1 – aflatoxin B_1, AFB2 – aflatoxin B_2, AFG1 – aflatoxin G_1, AFG2 – aflatoxin G_2, AFM1 – aflatoxin M_1, CIT – citrinin, DAS – diacetoxyscirpenol, GLIO – gliotoxin, HT2 - HT-2 toxin, MPA – mycophenolic acid, NEO – neosolaniol, NIV – nivalenol, OTA – ochratoxin A, PAT – patulin, T2 – T-2 toxin, VER – verruculogen.

Mutagenicity of bioaerosols using the Ames test. Quartz microfibre filters were suspended twice in 30 mL of methanol, acidified with acetic acid (0.5%). The solutions were kept in an ultrasonic bath for 3 min, and then shaken for 10 min in a multi-tube vortexer. The supernatant was transferred to an appropriate glass tube. All extracts were evaporated in a parallel evaporator (Syncore polyvap, Büchi Labotechnik G, Flawil, Switzerland), and then dried under a stream of nitrogen. The final residue was dissolved in 550 µL DMSO and then filtered through Millex HV 0.45 µm. Each filter extract was tested in triplicate.

The pre-incubation method of the *Salmonella typhimurium* assay was used, according to Mortelmans & Zeiger [28]. Strains were grown in Nutrient Broth No. 2 (Oxoid Ltd., Basingstoke, UK) with a 12 h shaking at 37°C. Ten µL of the bioaerosol extract were mixed with 100 µL of the bacterial

culture and 100 µL of 5% S9 mix when appropriate. The S9 fraction was obtained from Aroclor 1254-induced male Sprague-Dawley rats, supplied by Moltox (Boone, NC, USA). After 60 min agitation (185 rpm) at 37°C, 2 mL of molten agar with 10% histidine and biotin was added to the tubes and poured onto minimal glucose plates. After 48 h of incubation at 37°C, the number of revertants was counted by Noesis software (Saint Aubin, France). Because mutagens can damage DNA through various mechanisms, 3 strains were tested, enabling the detection of different types of mutations: TA98 (frameshift damages), TA100 (base-pair substitutions) and TA102 (base-pair substitution mainly consecutive to oxidative damage).

Statistical analysis. The Wilcoxon test was used to compare the levels of total and specific cfu between the 2 periods. Spearman correlations were performed at each season and between the 2 periods for mycotoxins produced by *Aspergillus flavus* group and *Aspergillus fumigatus* isolates.

SAS 9.2 software was used to analyse the data. $p<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Mycobiota of bioaerosols. Figure 1 presents the fungal diversity of bioaerosols collected in the cattle shed during the 2 periods (January-February and March-April). Among the 45 fungal species identified, *Stachybotrys chartarum* was observed for the first time in the air of dairy the shed. In comparison, only 28 species were identified in ambient air collected during preparation and distribution of corn silage and oilseed cakes [5].

During the first period, *Aspergillus fumigatus*, *Cladosporium cladosporioides*, *Penicillium chrysogenum*; *Stachybotrys chartarum*, *Ulocladium chartarum* were predominant.

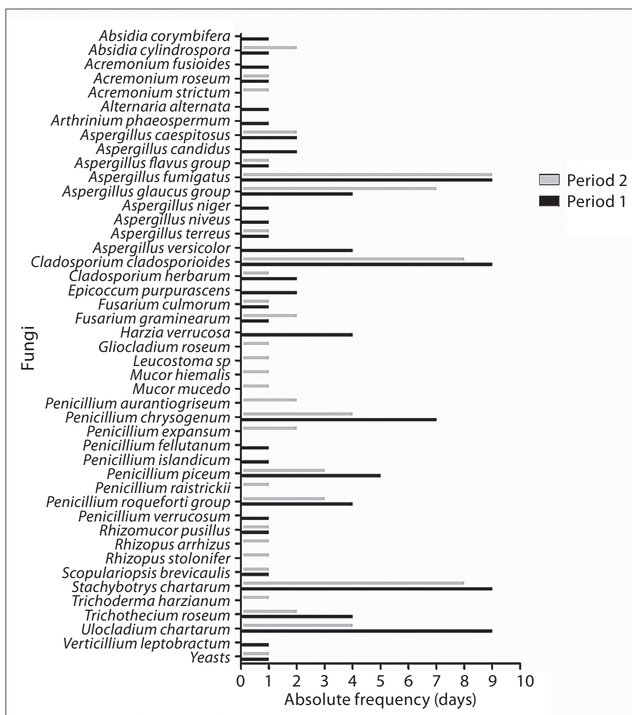

Figure 1. Frequency of fungal species collected during 2 periods in the cattle shed.

During the second season, the most common fungi were *Aspergillus glaucus* group, *Aspergillus fumigatus*, *Cladosporium cladosporioides* and *Stachybotrys chartarum*.

The contributions of recurrent strains (i.e observed during more than 6 days) are presented in Table 3. The percentages of *Aspergillus fumigatus*, *Aspergillus glaucus* group, *Cladosporium cladosporioides*, *Penicillium chrysogenum*, *Stachybotrys chartarum*, *Ulocladium chartarum* were, respectively, up to 14, 40, 17, 3, 41 and 19%.

Table 3. Overall percentage contributions of 6 major airborne fungal species to the total viable moulds measured in the ambient air of a cattle shed during 2 periods

Sampling period	Day	Fungal species*					
		A	B	C	D	E	F
1	1	4.6	-	-	1.7	0.7	16.8
	2	2.0	2.0	5.3	2.0	5.3	7.9
	3	4.4	2.2	2.2	2.2	1.1	11.1
	4	0.4	-	8.4	-	5.0	4.2
	5	-	-	5.8	1.3	4.5	2.7
	6	11.6	1.1	6.3	1.1	-	-
	7	1.2	-	3.2	0.4	2.4	5.9
	8	1.3	-	5.3	2.7	41.4	14.0
	9	3.3	-	4.5	-	3.0	4.8
	10	3.0	1.5	17.4	-	3.0	0.5
2	1	14.4		4.5	-	0.9	18.9
	2	3.4	12.9	11.2	-	-	1.7
	3	0.8	30.5	2.5	-	1.3	-
	4	5.5	39.7	2.7	-	4.1	-
	5	1.2	37.0	1.2	-	4.9	-
	6	-	-	7.5	1.5	2.0	0.5
	7	1.8	27.7	0.6	2.4	7.2	1.2
	8	0.4	-	-	-	-	-
	9	10.5	4.8	0.3	0.6	0.6	-
	10	4.2	5.4	-	3.1	0.4	-

- not detected

* The fungal species were: A – *Aspergillus fumigatus*; B – *Aspergillus glaucus* group; C – *Cladosporium cladosporioides*; D – *Penicillium chrysogenum*; E – *Stachybotrys chartarum*; F – *Ulocladium chartarum*.

Seasonal variations were observed in the cattle shed bioaerosols. The number of cfu was significantly different between the 2 studied periods for *Aspergillus fumigatus* ($p<0.001$), *Aspergillus glaucus* group ($p<0.01$), *Cladosporium cladosporioides* ($p<0.01$), *P. chrysogenum* ($p<0.05$), *Stachybotrys chartarum* ($p<0.005$) and *Ulocladium chartarum* ($p<0.05$).

In the study by Hanhela *et al.* [29], the most common fungi observed on Finnish dairy farms were the *Aspergillus glaucus* group and *Aspergillus fumigatus*. The most abundant allergen present in the atmospheric air in barns near Cooperstown (NY) and Rochester (MN) was *Aspergillus fumigatus* [30]. *Botrytis* and *Chrysosporium* were also detected in cow barns and in farm houses [31].

Stachybotrys chartarum has been found primarily in water-damaged buildings (median indoor concentration: 1.2×10^3 cfu/m^3) [32], and was found to be associated with idiopathic pulmonary haemorrhage in infants [33]. This strain is a saprophytic fungus frequently found on high cellulose materials (e.g. straw and hay), and can produce macrocyclic trichothecenes toxins, such as satratoxins [34].

According to Pieckova [35], the amount of viable moulds in indoor air is very variable. Moreover, a study in farm buildings located in southern Poland showed that the concentration levels of fungal aerosol in indoor environments were generally lower than outdoors [36].

In the presented study, the values for fungi per cubic meter of air (Tab. 4) varied from 2.5-25 cfu/m^3 (median: 6.48), and from 19-5.9 × 10^2 cfu/m^3 (median: 76.4), during the first and the second period, respectively. The number of total cfu significantly increased between the 2 studied periods (p<0.001). During each period, peaks of fungal contamination were observed during straw handling (Fig. 2). Animal behaviour may also influence the dust generated from bedding material. For example, young animals tend to be more active than older ones, and may disperse a large among of dust from the bedding [37].

Table 4. Concentrations of airborne fungal spores collected in ambient air

Sampling period	Day	Viable spores [a] (cfu/m^3)	Sampling period	Day	Viable spores [a] (cfu/m^3)
	1	8.8 ± 1.1		1	29.0 ± 4.4
	2	3.7 ± 0.4		2	2.9×10^2 ± 86.0
	3	2.5 ± 0.4		3	5.9×10^2 ± 1.4×10^2
	4	6.5 ± 1.1		4	170.0 ± 13.0
	5	6.5 ± 1.1		5	19.0 ± 2.6
1	6	2.6 ± 0.3	2	6	47.0 ± 5.3
	7	7.4 ± 1.3		7	4.2×10^2 ± 35.0
	8	25 ± 3.0		8	63.0 ± 4.8
	9	7.4 ± 0.5		9	86.0 ± 8.2
	10	6.3 ± 0.7		10	67.0 ± 11.0

[a] Mean ± S.D.

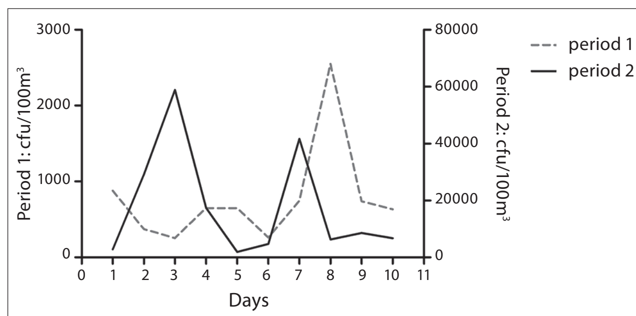

Figure 2. Monitoring of fungal spores during the 2 periods.

These levels of viable moulds were lower than these observed in others studies. Kullman *et al.* [38] showed concentrations of viable moulds in dairy barns from 1.7 × 10^3-1.6 × 10^6 cfu/m^3. In India, concentrations of viable moulds varied from 1.65 × 10^2-2.2 × 10^3 cfu/m^3 in aerosols collected from indoor cattle sheds [39]. The maximum values found in the presented study are lower than these described in sheds located in Egypt, Wisconsin (USA) and Finland, with a mean total fungal count of 1.5 × 10^3 cfu/m^3 [40], 8 × 10^3 cfu/m^3 [41] and 4.7 × 10^6 cfu/m^3 [29], respectively. However, in these studies, the sampling time was reduced to a few hours, and the observed levels could correspond to the fungal contamination peak.

Measurements of total fungi in European countries have shown that the highest emission rates of fungi were measured in broiler and poultry buildings, and the lowest in cattle buildings [37, 42]. The highest median concentration of culturable fungi has been found in Swiss poultry houses [43]. In horse stables, the median fungal concentration was approximately 1.78 × 10^2 cfu/m^3 [44].

In comparison to others agricultural activities, our study revealed lower levels of contamination. The exposure was found to be more severe during harvesting than in animal confinements [45]. In southern Georgia (USA), the total viable moulds have been estimated to be up to 10^7 cfu/m^3 during operations such as corn harvesting, corn loading or corn grinding [46]. Swan & Crook [47] found that workers handling grain, or working close to grain at farms located in England were exposed to 9.2 × 10^4 cfu/m^3. The mould levels observed in the air of sheds were lower than for other activities, such as corn silage and handling of oilseed cakes, with concentrations from 4.3 × 10^2 cfu/m^3-8.9 × 10^4 cfu/m^3 [5].

Mycotoxigenic ability of *Aspergillus flavus* and *Aspergillus fumigatus* isolates. Among the 25 isolates of *Aspergillus flavus*, no strain was able to produce aflatoxins *in vitro*. On the other hand, all the 19 isolates of *Aspergillus fumigatus* were found to produce 3 mycotoxins: gliotoxin, verruculogen and fumagillin (Fig. 3).

The mean concentrations in gliotoxin, verruculogen and fumagillin were, respectively, 96.7, 0.6 and 0.7 µg/g medium

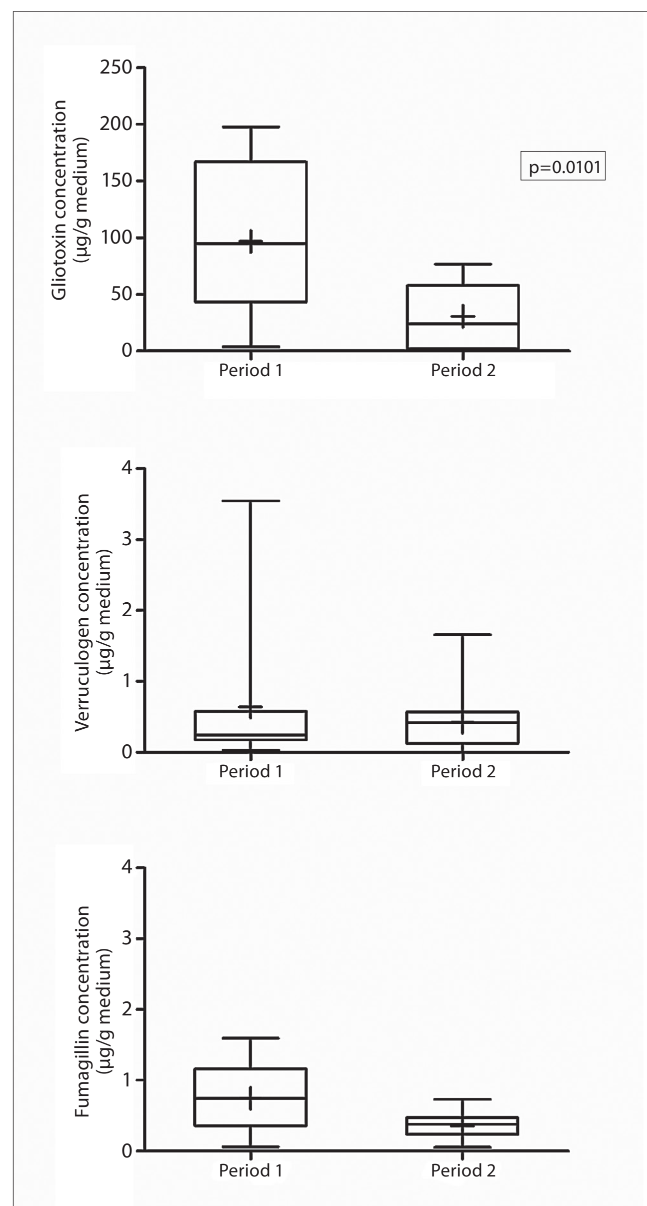

Figure 3. Production of gliotoxin, verruculogen and fumagillin by *A. fumigatus* isolates collected from bioaerosols during 2 periods in cattle shed.
The centre half of the data, from the first to the third quartile, is represented by a box with the median indicated by a bar. A line extends from the third quartile to the maximum value, and another from the first quartile. The mean is indicated by a plus.

during the first period, and 30.7, 0.4 and 0.4 μg/g medium during the second period. Fumagillin was correlated with the gliotoxin during the first period (r=0.85; p=0.0037) and with verruculogen during the second period (r=-0.66; p=0.038). The production of gliotoxin was significantly different between the 2 periods (p=0.0101).

Aspergillus fumigatus was previously detected in bioaerosols collected in the agricultural environment [35]. *Aspergillus fumigatus* spores are easily spread in the air and create a risk of exposure for both animals and humans. In addition, this mould is a well-known human and animal pathogen causing aspergillosis [48]. Gliotoxin has potent immunosuppressive effects. The presence of toxigenic isolates of *Aspergillus fumigatus* demonstrates that this mould and its related highly toxic metabolites, e.g. gliotoxin, verruculogen and fumagillin, should be monitored during agricultural activities, and could represent a potential health risk to exposed farmers and animals with affected immunity.

Multi-mycotoxin analysis from bioaerosols. Only aflatoxin B_1 and aflatoxin B_2 were detected in bioaerosols during 6 and 7 days, respectively. Nevertheless, no concentration can be provided because the data were under the quantification limits (aflatoxin B_1: 0.09 ng/filter ; aflatoxin B_2: 0.07 ng/filter). It was not possible to determine the possible exposure to *Stachybotrys chartarum* mycotoxins because no standard of satratoxins is commonly available. Future studies will consist in monitoring their production and purification from the *Stachybotrys chartarum* isolates collected in this study.

Mutagenicity of bioaerosols. Results were negative for the tests with strains TA 100 and TA 102 (data not shown). Table 5 presents the mutagenicity of air samples using the *Salmonella* assay consecutive to frameshift damages (TA98) in the absence and presence of metabolic activation (S9). For 3 samples of bioaerosols (period 2: days 8, 9 and 10), a significant response (ratio > 2) was detected with S9 mix fraction. These results provided the first data on the mutagenicity of bioaerosols originating from agricultural areas. Because bioaerosols are complex, moulds and mycotoxins cannot be considered as the only determinants of exposure in cattle sheds. The quantification and characterization of other constituents, such as volatile organic compounds, bacteria, insects and plant parts in the occupational environment, would be useful for the understanding of mutagenicity, and to evaluate the potential health risk of exposed farmers and animals. Others compounds like the PAHs emitted by agricultural machines could be also involved in mutagenicity.

Table 5. Mutagenicity of bioaerosols extracts in TA 98 strain

Sampling period	Day	TA 98 without S9		TA 98 with S9	
		Mean ± S.D.	Ratio	Mean ± S.D.	Ratio
1	1	25.3 ± 2.3	1.0	25.7 ± 3.8	1.0
	2	22.0 ± 3.0	0.9	28.3 ± 3.5	1.0
	3	26.7 ± 3.8	1.0	31.3 ± 2.9	1.2
	4	25.7 ± 7.6	1.0	28.0 ± 4.6	1.0
	5	20.7 ± 2.1	0.8	26.3 ± 2.5	1.0
	6	26.0 ± 6.6	1.0	27.7 ± 2.5	1.0
	7	24.0 ± 2.6	0.9	27.0 ± 1.0	1.0
	8	26.0 ± 5.6	1.0	28.7 ± 3.1	1.1
	9	28.0 ± 3.0	1.1	29.7 ± 3.5	1.1
	10	28.3 ± 5.1	1.1	27.0 ± 6.2	1.0
2	1	28.3 ± 2.3	1.1	28.3 ± 1.2	1.0
	2	22.3 ± 6.7	0.9	24.7 ± 4.6	0.9
	3	30.3 ± 7.1	1.2	26.3 ± 2.1	1.0
	4	23.0 ± 1.7	0.9	26.3 ± 3.8	1.0
	5	30.0 ± 3.6	1.2	25.7 ± 2.1	1.0
	6	28.3 ± 0.6	1.1	30.3 ± 4.0	1.1
	7	28.3 ± 4.6	1.1	32.7 ± 2.1	1.2
	8	42.8 ± 15.7	1.5	56.0 ± 6.6	**2.6**
	9	44.7 ± 6.7	1.6	50.0 ± 9.3	**2.3**
	10	27.5 ± 2.0	1.5	52.5 ± 13.3	**2.4**

Samples with mutagenic activity were presented in bold

CONCLUSIONS

These data showed for the first time that dairy farmers could be exposed to *Stachybotrys chartarum* during routine barn work. In further studies, mycotoxins of *Stachybotrys chartarum* could be searched for and monitored in cattle shed. Moreover, indicative mutagenicity was detected especially under conditions with the addition of an exogenous metabolization system (S9mix), reinforcing the need to continue the investigation of bioaerosol composition and mycotoxins in cattle sheds. To understand the accurate role of fungi in the mutagenicity of bioaerosols, fungal isolates belonging to *Aspergillus* and *Stachybotrys* genera will be also tested *in vitro* towards various strains of *Salmonella typhimurium* (Ames test), and for their ability to induced DNA adducts. Together, these data will highlight the genotoxic mechanisms involved, and will contribute to the evaluation of the potential health risks for dairy farmers.

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