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ABSTRACT

Hundreds of different cheeses are produced in France, where 23.9 kg of cheese were consumed per inhabitant in 2009, when it was ranked the second cheese-consuming nation. To meet this considerable demand, a large number of cheese factories exist where many workers, especially cheese-washers, may be exposed to fungal bioaerosols which can lead to adverse toxinic and allergic effects. Airborne bacteria, fragments or microbial by-products (endotoxins) are also found and contribute to total worker exposure. However, there is almost no published data concerning worker exposure or characteristics of bioaerosols emitted during these activities.

Here, we measured the parameters (concentrations, species present, and size distribution) of the culturable fungal bioaerosol emitted in a French natural-rind cheeses maturing cellar. Concentrations of airborne bacteria and endotoxins were also measured.

The main tasks were investigated using stationary or personal sampling over three consecutive days. Depending on the work area, high concentrations of culturable mesophilic microorganisms were measured (using closed-face cassettes): from 10^4 to 2×10^8 CFU.m^{-3} for fungi, and from 10^3 to 10^6 CFU.m^{-3} for bacteria. These concentrations are 10- to 100,000-fold higher than those measured at two reference points (indoor and outdoor) which are assumed not to be contaminated by the plant's activities. Endotoxin concentrations were between 10 and 300 EU.m^{-3} in the plant. Exposure was further assessed by identifying the predominant culturable fungi (allergenic *Mucor fuscus* and *Penicillium sp.*) and by measuring particle size distributions (cascade impactor). Airborne fungal entities (spores, mycelium strands and fragments, agglomerates, etc.) were found with aerodynamic diameters from 3 to over 20 µm.

A metrological approach was used to fully characterise the culturable fungal aerosols generated during cheese maturing in this plant. The results show that workers are exposed to concentrations of airborne culturable fungi, sometimes very high, throughout the manufacturing process. In addition to fungi, culturable bacteria and endotoxins are also present in the work atmosphere. All these microbial organisms thus contribute in a complex manner to total worker exposure. Despite the lack of both occupational exposure limit values and standardized measuring methods, our results suggest that an immunological risk may occur among workers, especially for cheese-brushers, cheese-washers and packagers who are the most exposed workers in the factory.

Keywords: Bioaerosols
Airborne microorganisms
Bacteria
Fungi
Endotoxin

INTRODUCTION

Historically and culturally, many cheese specialities are produced in different French regions. According to estimations, in France there are between 250 and 350 different varieties of cheese. Some of these cheese varieties are manufactured under “Appelation d’Origine Protégée” (Protected Designation of Origin) status (products produced, processed and prepared in a given geographical area using recognised know-how). In 2009, the average French inhabitant consumed 23.9 kg of cheese, produced by an estimated 350 different cheese factories, which together produced approximately 1.8 million tons of all types of cheese (fresh, soft, hard, blue, cow's milk, sheep's milk, goat's milk, etc.) (CNIEL, 2011).

The risk of respiratory disorders linked to airborne biological agents is particularly prevalent in the agricultural and agri-food sectors. Infectious risks are not the only risks associated with worker exposure; diseases of immunoallergic or toxinic origin have also been reported. The most frequent of these are: asthma, rhinitis, hypersensitivity pneumonitis, organic dust toxic syndrome and chronic bronchitis (ACGIH, 1999; Douwes et al., 2003; Eduard, 2009).

Workers in cheese-maturing cellars are exposed to fungi, mainly of the *Penicillium* genera. These are responsible for allergic and toxinic diseases (Campbell et al., 1983; Galland et al., 1991; Dahl et al., 1994; Guglielminetti et al., 2000). The atmosphere in the cheese-maturing cellars is generally thought to be an important factor in triggering a disease, often called "cheese-worker's disease" or "cheese-washer's disease", which is a form of occupational extrinsic allergic alveolitis (Martinet et al., 1999). Few studies describe occupational respiratory disorders and diseases among cheese workers, with most publications detailing isolated observations at a single site or on a restricted number of workers (Molina et al., 1975, 1977; Campbell et al., 1983; Dalphin et al., 1990; Galland et al., 1991; Dahl et al., 1994; Guglielminetti et al., 2000; Casper et al., 2008). Work in cold, humid and confined areas in itself is commonly linked to respiratory disorders, in particular broncho-pulmonary affections. Indeed, it is not even necessary to be a cheese handler oneself to suffer from these disorders. It is enough to work in the humid, contaminated atmosphere of such cheese cellars; workers who do packaging cheeses or cleaning the cellars can also present similar symptoms (Molina et al., 1977; Galland et al., 1991).

Although cheese production is based on ancient methods, the biological risk related to the presence of bioaerosols in the work atmosphere remains poorly known and largely under-documented. Few articles report bioaerosol concentrations measured in cheese-production areas or cheese-maturing cellars (Dahl et al., 1994; Hoekstra et al., 1998; Chaumont et al., 2001; Salustiano et al., 2003; Kure et al., 2008; De Santi et al., 2010). In these studies, the culturable fungi concentrations, determined by short-duration sampling with various high flow single-stage impactors, did not exceed 4.0×10^{4} CFU.m^{-3}.

This article presents an overview of the ambient concentrations and the level of worker exposure to airborne endotoxins, culturable fungi and bacteria in a plant specialising in cheese maturing. Worker exposure is more fully assessed by identifying the species and measuring the size distribution for airborne fungal particles.

MATERIALS AND METHODS

Activity in the cheese production plant

The plant specialises in maturing pressed, uncooked semi-hard cheeses with a natural, washed rind. Cheeses are collected, already formed, from a network of small producers. The products entering the plant, fresh cheeses before maturing, are known as "white products" and are an average of seven-days-old. Thus, the plant is not involved in the preliminary steps of cheese production (collection and maturation of the raw whole milk, transformation of the milk into cheese, salting, and forming). The cheeses are placed on wooden or stainless steel racks on top of mats made from plastic or natural straw. The temperature (T < 10 °C) and relative humidity (RH close to 100%) in the cellar are maintained close to constant.

The organoleptic properties of a cheese develop slowly and are mainly due to the natural transformations occurring through the action of bacteria introduced through the lactic ferments and the fungal flora developing on the surface. Cheeses, like other food products (e.g. dry sausages), may be inoculated with fungal species using a liquid culture of fungi (by spraying or soaking), or the flora may be allowed to develop spontaneously under the influence of the other cheeses and the atmosphere in the maturing cellar. The latter is used in this plant.

The cheeses are matured for several weeks during which workers perform three regular operations: (1) washing with brine, using a portable machine or by hand (wearing gloves); (2) dry brushing (or wiping), by hand (wearing gloves); (3) turning. These tasks ensure product conservation, allowing the cheeses to absorb salt and increase their water-content, aerate the pate and avoid softening, harden the rind, allow the rind to form evenly over the whole cheese, and favour the development of a fungal surface flora. The number, frequency and nature of these manipulations vary depending on the cheese's maturity. Dry brushing is used to remove part of the fungi flora which could develop in an uncontrolled manner on the surface of the product, and to limit the development of undesirable species, which can have a serious impact on the cheese's flavor and appearance.

At the end of maturation, cheese selectors choose the cheeses from all the products in the cellar to create homogeneous product lots meeting the requirements of different clients. These lots are transferred to a packaging workshop where several other people pack the cheese (waxed paper, cardboard, wooden crate, etc.) prior to storage and expedition. This packaging workshop consists of two communicating rooms. In the smallest room (2 to 3 workers) the cheeses from the cellar studied are packaged, while in the larger room (3 to 5 workers) cheese from the cellar studied and cheese from a different maturing cellar (not studied here) are packaged. In the packaging workshop, the temperature was between 14°C and 17°C and the RH was between 50 and 60%.

As the racks are gradually emptied by the cheese selectors, other workers transfer the material soiled by the cheese (empty racks, mats, aprons and gloves used during washing and brushing, etc.) to an area adjoining the cellar for manual or automatic washing. The temperature and the RH in this cleaning area were close to 25°C and 100%, respectively. The staff in this washing room is also responsible for replacing the cleaned material and cleaning the cellar floors.

About 30 workers, potentially exposed to airborne biological agents, are involved in the above described cheese production line in the factory.

Sampling methods

Airborne microbial particles were sampled using 37-mm closed-face cassettes (CFC). Culturable bacteria and fungi were sampled with CFC containing a sterile polycarbonate filter (Nuclepore®, Millipore®, 0.8 µm pore size) and a supporting absorbent pad (Millipore®). Airborne endotoxins were sampled with CFC containing two pyrogen-free glass fibre filters (Whatman®, GF/B filter, 0.68 mm thickness, 1.0 µm particle retention grade in liquid), a collection filter and a support filter. Sampling was performed at 2 L.min^{-1} (pump Gilian®, GilAir-3). The flow rate was measured before and after sampling using a bubble flow meter (Gilian®, Gilibrator). The cassettes were closed using a pneumatic press to ensure even, full sealing all around the circumference of the three pieces making up the cassette. The inlet orifice was maintained horizontal throughout sampling (in line with the recommendations of NF X43-257 - AFNOR, 2008). Further details can be found in Duquenne et al. (2012).

The size distribution of the culturable fungal particles emitted during cheese brushing was assessed using a Marple cascade impactor (model 298). This device consists of eight impaction stages (New Star Environmental Inc., 34-mm Mylar® circular discs with six radial slots) and an outlet filter (Pall-Corporation, 34-mm GLA-5000 PVC membrane, 5.0 µm pore size). Sampling was performed at 2 L.min^{-1} (Gilian®, GilAir-3). The cut-off aerodynamic particle diameters for each impaction stage were as follows (in µm): 0.52 – 0.93 – 1.55 – 3.5 – 6.0 – 9.8 – 14.8 – 21.3 (Rubow et al., 1987). The impactor inlet consisted of a cowl and visor. Due to the very high relative humidity (RH close to 100%), no grease was applied to the collection supports in the impactor to avoid problems recovering the biological particles and analysing the culturable fungi.

Sampling strategy

Measurements, based on stationary and personal sampling, were performed over three consecutive days, from the 20th to the 22nd of September 2011.

Stationary samples. All the ambient concentrations of culturable fungi and endotoxins were measured with closed-face cassettes for seven points (of which two reference points - Table 1a) by stationary sampling (at around 1.7 m from the ground). The ambient concentrations of culturable bacteria were measured only for the two reference points. These samples were taken over the three days (one sample per day and per location). The duration of sampling corresponded to a large proportion of the work time (minimum = 290 min, median = 389 min, maximum = 498 min).

Table 1. Name and description of the stationary (a) and personal sampling (b).

a - Stationary samples / Culturable fungi and endotoxins (3 days)	
Name	**Description of sampling site**
Outdoor Ref.	Outdoor reference sampling point, located in a car park 200 m from the building where cheeses are produced.
Indoor Ref.	Indoor reference sampling point, located in an office in a different building to that where cheeses are produced.
Cellar - Area 1	In the middle of several rows of racks where "white products" are stored. Activities include "brine washing / turning" of fresh cheeses (without external fungal flora).
Cellar - Area 2	In the middle of several rows of racks where maturing cheeses are stored. Activities include "brine washing / turning" of cheeses with fungal flora.
Brushing	Closest sampling point to (1 to 2 m from) the area where "dry brushing / turning" is performed by 2 to 3 workers. Areas of the cellar where almost fully mature cheeses are stored, presenting an often abundant fungal flora.
Cleaning area	In the middle of the washing room; close to an automatic machine used to clean dirty materials.
Packaging	At the centre of the activities surrounding cheese packaging.

b - Personal samples / Culturable fungi (3 days) and bacteria (2 or 3 days)	
Name	**Description of the activity monitored**
Cellar (INRS agent)	INRS agent responsible for sampling. Present in the cellar most of the time but not handling cheeses.
Cheese-washer 1	Worker responsible for "brine washing / turning" of cheeses (mainly "white products" without fungal flora).
Cheese-washer 2	Worker responsible for "brine washing / turning" of cheeses (mainly maturing cheeses with a surface fungal flora).
Cheese-brushers (2 different workers)	Workers responsible for "dry brushing / turning" of cheeses. Only handling maturing or fully mature cheeses with well-developed surface fungal flora.
Cleaning workers (2 different workers)	Workers in the cleaning room where soiled materials are cleaned. Other activities: washing the floor in the cellar, installing or dismantling racks, handling cheeses.
Cheeses selector	Worker responsible for choosing the cheeses in the cellar and creating homogeneous product lots. Other activities: "dry brushing / turning" of cheeses before select them or not.
Packaging (INRS agent)	INRS agent responsible for sampling. Present at the packaging station most of the time but not handling cheeses.
Packager 1	Handling and packaging cheeses.
Packager 2	Handling and packaging cheeses.

Personal samples. Exposures to culturable fungi and bacteria were measured by personal sampling for seven different tasks (Table 1b), using closed-face cassettes. Two INRS personnel were also equipped with closed-face cassettes. They were present alongside workers in the cellar or at the packaging station, but they did not participate in cheese production or packaging activities. Some workers were multitaskers, therefore the duration of sampling was adapted so that each sample could be used to assess a specific activity (minimum = 129 min, median = 248 min, maximum = 357 min). The personal concentrations of culturable fungi were all measured in triplicate over the three days. For the concentrations of culturable bacteria, some points were only duplicated.

Cascade impactors. Aerosol size distributions were measured for a 60-min sampling duration using a Marple impactor (at around 1.7 m from the ground). The impactor was placed at around 1 m from the activity of dry brushing of cheeses (the device was moved as the worker proceeded through the cellar). Three different measurements were performed.

Transport of samples. On each day of the study, samples were sent to the laboratory by post once sampling was completed. Filters were kept in their individual cassettes. The filters from the cascade impactor were carefully recovered from the device and individually placed in sterile 50-mL Greiner tubes. All samples were overnight shipped at room temperature and were analysed within 24 hours after being collected.

Samples analysis

Equipment and dilution water used were sterile and/or pyrogen-free when required. All analyses were performed in a laminar flow cabinet. Analysis methods are described briefly here, but full details of materials and reagent composition can be found in Simon et al. (2011) and Duquenne et al. (2012).

Culturable microorganisms count. Culturable microorganisms were harvested by introducing 10 mL extraction solution into each cassette followed by shaking for 20 min at 2000 rpm. The extract was serially diluted in a sterile tryptone salt solution. Culturable microorganisms were counted by the spread-plating technique. Briefly, a 100-µl aliquot of the diluted extract was spread over Petri dishes (2 plates per dilution, 3 dilutions) containing the appropriate culture medium. Mesophilic fungi were cultured on Malt Extract Agar (MEA), while bacteria were grown on Triptycase Soy Agar plus cycloheximide. All the inoculated media were incubated at 25 °C for 5 days. The limit of detection of the culturable method is 10 $CFU.mL^{-1}$, i.e. 100 CFU per cassette. The airborne culturable microorganism concentrations were expressed as Colony Forming Units (CFU) per cubic meter ($CFU.m^{-3}$).

Endotoxin analysis. The collection filter was carefully removed from the sampling cassette and transferred to a 50-mL tube containing 10 mL water. Extraction was performed at 2000 rpm for 60 min (room temperature). It was completed with a final centrifugation step at 2000 rpm for 10 min at 4 °C. The endotoxin concentration in the extract was assayed using the LAL kinetic chromogenic detection assay, using Kinetic-QCL® kits (Lonza Group Ltd.). The limit of detection of the endotoxin analysis is 0.005 $EU.mL^{-1}$, i.e. 0.05 EU per filter. The airborne endotoxin concentrations were expressed as Endotoxin Units (EU) per cubic meter ($EU.m^{-3}$).

Identification of fungi. Airborne culturable fungi were identified using macroscopic and microscopic morphological criteria and sequence based identification molecular methods. Dominant colonies were selected and picked up from the Petri dishes used for the enumeration of airborne cultural microorganisms. They were selected on macroscopic and microscopic criteria and cultured on the MEA medium for 7 days at 25 °C. The grown colonies were then sent to an external laboratory (IDmyk S.A., Limonest, France) for further macroscopic, microscopic and molecular identification. Molecular based identification of fungi was performed by sequencing of the D1-D2 region of 25S RNA gene and the HKGF2 gene coding for beta-tubulin.

Size distribution. Each of the filters from the cascade impactor was washed with 10 mL of extraction solution for 20 min with agitation at 2000 rpm to recover the biological particles. Extracts were then analysed using a similar protocol to that described above for culturable fungi.

Statistical analyses. As common with exposure data, the distribution of bioaerosol exposure concentrations fitted a log–normal, rather than a normal, distribution; therefore, data were log-transformed before subsequent analyses. ANOVA tests (95% confidence interval) and linear regression analyses were performed on log-transformed values using StatGraphics Centurion XV 15.2.00 software (StatPoint, Inc., USA).

RESULTS

Stationary sampling

Concentrations of culturable fungi. The ambient concentrations of culturable fungi for various locations in the factory are reported in Figure 1 and Table 2. At the reference points, the concentrations are all below 8.1×10^3 $CFU.m^{-3}$, whereas in production zones they generally exceed 10^5 $CFU.m^{-3}$. They can be up to 10^6 $CFU.m^{-3}$ at the packaging station and in zone 2 of the cellar. Near where cheeses were brushed, concentrations were around 10^7 $CFU.m^{-3}$ on the three days of sampling. The culturable fungal concentrations measured in the work areas were between 7 and around 15,000 times higher than those measured at the same time at the outside reference point (from 2 to around 10,500 times higher than the indoor reference).

Endotoxin concentrations. The ambient endotoxin concentrations are given in Figure 2 and Table 2. The concentrations measured at the reference points were all below 4.3 $EU.m^{-3}$. The concentrations in the maturing cellar (brushing, areas 1 and 2) were below 50 $EU.m^{-3}$. The highest endotoxin concentrations were measured at the packaging station (mean = 85.2 $EU.m^{-3}$; standard deviation = 36.4 $EU.m^{-3}$; n = 3) and in the cleaning room (mean = 275.7 $EU.m^{-3}$; standard deviation = 104.3 $EU.m^{-3}$; n=3). For these two areas, the concentrations were from 29 to 270 times higher than those measured at the same time outdoor (from 31 to 160 times higher than the indoor reference).

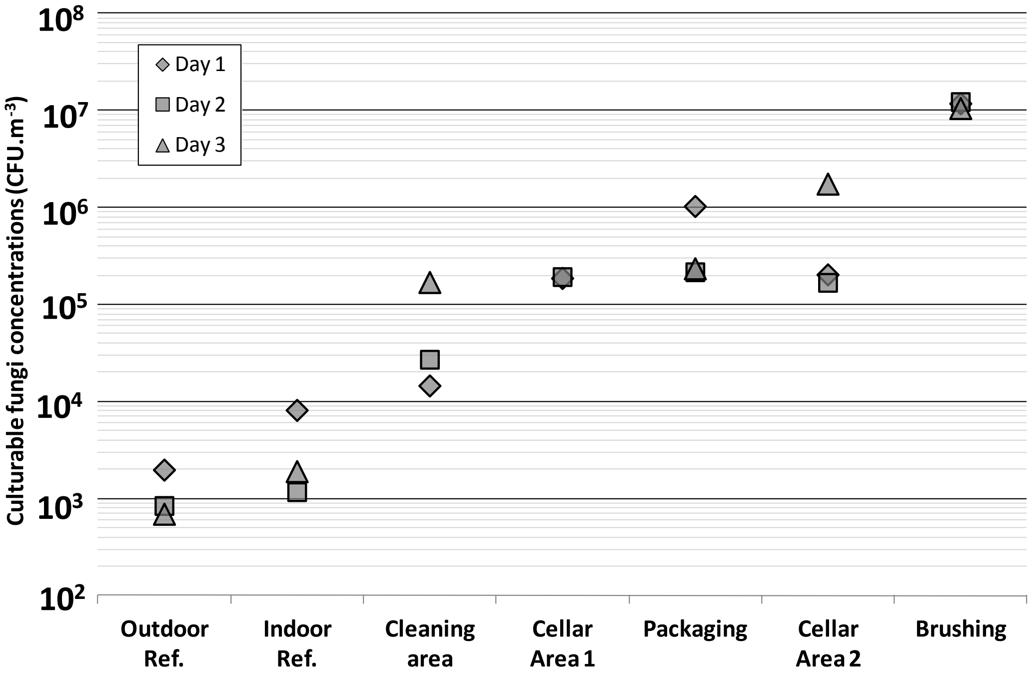

Fig. 1. Ambient concentrations of culturable fungi for different sampling locations (see Table T1 in online supplementary material for the full numerical data).

Table 2. Culturable fungi and endotoxin concentrations for the different stationary sampling points.

Stationary sampling points	Culturable fungi (CFU.m^{-3})			Endotoxin (EU.m^{-3})		
	AM	Min	Max	AM	Min	Max
Outdoor Ref.	1.2×10^3	6.9×10^2	2.0×10^3	2.4	1.2	4.3
Indoor Ref.	3.7×10^3	1.2×10^3	8.1×10^3	1.9	1.6	2.2
Cleaning area	7.0×10^4	1.4×10^4	1.7×10^5	275.7	158.2	357.3
Cellar Area 1	1.9×10^5	1.8×10^5	1.9×10^5	18.5	6.0	36.3
Packaging	4.9×10^5	2.1×10^5	1.0×10^6	85.2	61.2	127.1
Cellar Area 2	7.0×10^5	1.7×10^5	1.7×10^6	28.2	18.8	42.0
Brushing	1.1×10^7	1.0×10^7	1.2×10^7	16.1	11.7	19.6

AM: arithmetic mean of the 3 days; Min and Max: minimum and maximum values measured.

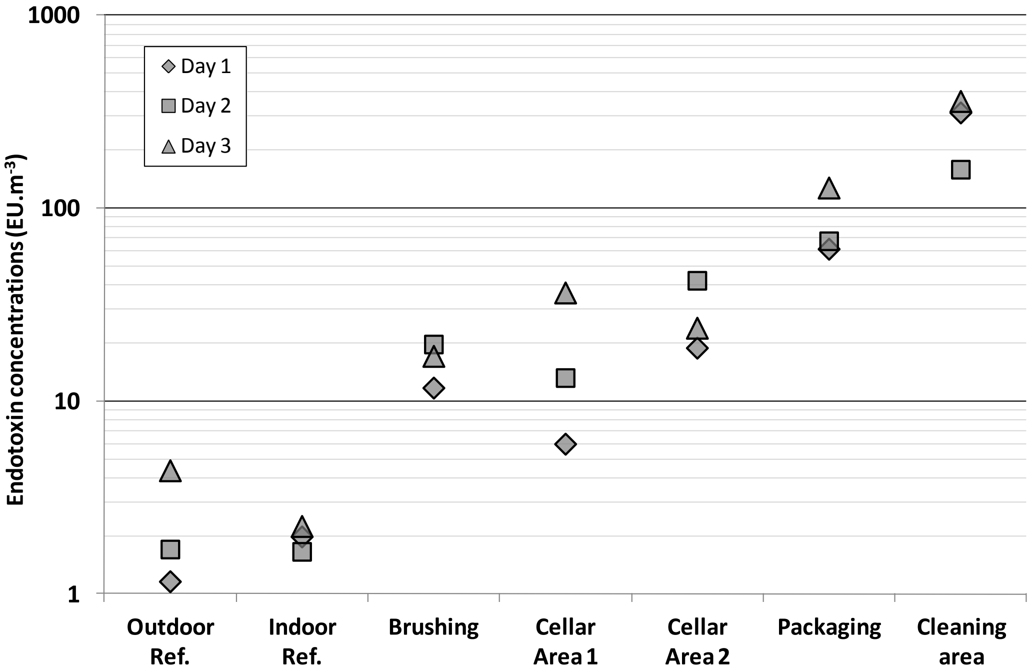

Fig. 2. Ambient endotoxin concentrations for different sampling locations (see Table T2 in online supplementary material for the full numerical data).

Personal sampling

Concentrations of culturable fungi. The concentrations of culturable fungi, based on personal samples for different workers, are reported in Figure 3 and Table 3. Of the 27 measurements made, 26 exceeded 10^5 CFU.m^{-3}. These personal concentrations are between 55- and around 180,000-fold higher than those measured for the reference samples of the outdoor atmosphere (from 25- to 130,000-fold higher than the indoor references). The task associated with highest exposure is dry brushing of cheeses (concentrations greater than 10^8 CFU.m^{-3}). Notably, exposure of the two INRS agents, who were present in the work area without actually handling the cheese, was similar to exposure of some of the workers.

Personal concentrations for cheese-brushers (Figure 3 - mean = 1.5×10^8 CFU.m^{-3}) are significantly higher ($p = 0.0001$) than the ambient concentrations measured closest to the activity of cheese brushing (Figure 2 - mean = 1.1×10^7 CFU.m^{-3}). Similarly, the personal concentrations for "Cheese-washer 2" (Figure 3 - mean = 1.6×10^7 CFU.m^{-3}), who always worked in “Cellar area 2”, are significantly higher ($p = 0.0101$) than the ambient concentrations measured in this area (Figure 2 - mean = 7.0×10^5 CFU.m^{-3}) at the same time.

Concentrations of culturable bacteria. The concentrations of culturable bacteria, based on personal samples, are also given in Figure 3 and Table 3. Of the 18 measurements made, 6 were between 10^4 and 10^5 CFU.m^{-3} and 7 exceeded 10^5 CFU.m^{-3}. In comparison, the concentrations at the two reference points are below 1.1×10^3 CFU.m^{-3} and 1.8×10^3 CFU.m^{-3} for the outdoor (mean = 5.4×10^2 CFU.m^{-3}; standard deviation = 4.5×10^2 CFU.m^{-3}; $n = 3$) and indoor (mean = 9.3×10^2 CFU.m^{-3}; standard deviation = 8.0×10^2 CFU.m^{-3}; $n = 3$), respectively (see also Table T3). So, the highest exposure levels were around 1000 and 5000 times higher than the concentrations measured at the reference points. A linear regression analysis revealed a significant and moderately strong correlation between the concentrations of culturable fungi and the concentrations of culturable bacteria ($n = 18$; $r = 0.685$; $p = 0.0017$). In most cases, the higher the culturable fungi concentration, the greater the culturable bacteria concentration.

Identification of airborne culturable fungi

About 20 different fungal species were identified in the collected bioaerosol samples. The three dominant airborne fungi belonged to *Mucor fuscus*, *Penicillium bialowiezense* (synonym: *P. bourgeianum*) and "*Penicillium* complex". The three dominant fungi accounted for 92% of the total culturable mycoflora found in the air samples taken in the plant. The "*Penicillium* complex" includes five *Penicillium* species (*P. camemberti*, *P. commune*, *P. biforme*, *P. caseifulvum, P. palitans*) that were not discriminated using the HKGF2 gene (equal homology to the reference sequences in databases).

The other (about fifteen) detected culturable fungal species which were not identified represented around 8% of the total culturable fungal flora in the air samples.

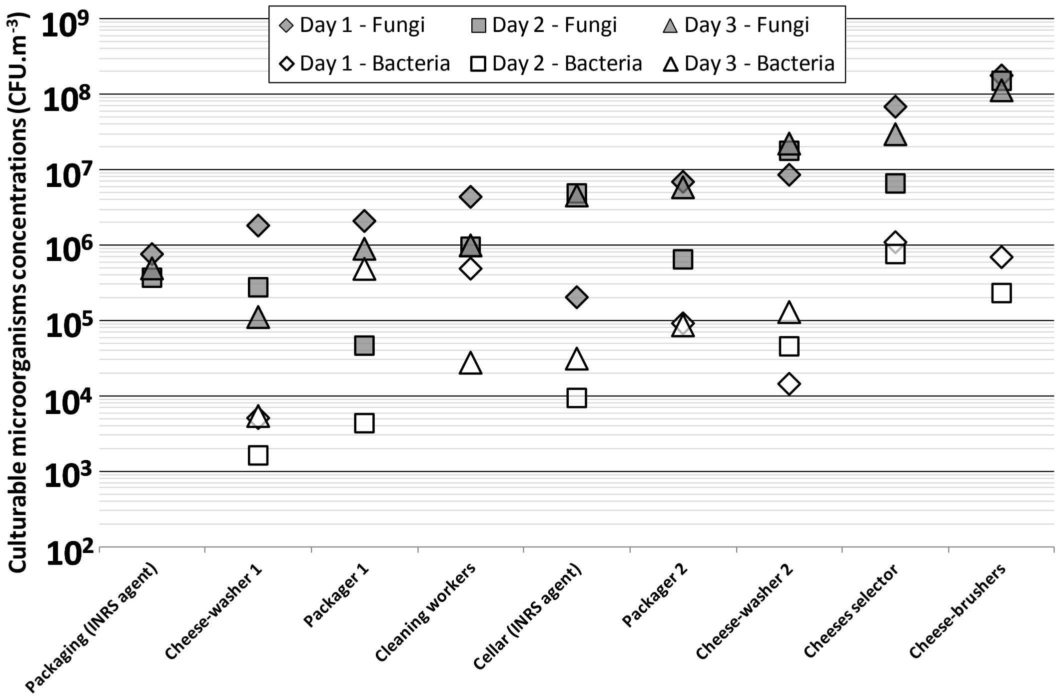

Fig. 3. Concentrations of culturable fungi and bacteria measured by personal sampling on workers (see Table T3 in online supplementary material for the full numerical data, including all the culturable bacterial concentrations for the two reference sampling points).

Table 3. Culturable fungi and culturable bacteria concentrations for the different personal sampling.

Personal sampling	**Culturable fungi (CFU.m^{-3})**			**Culturable bacteria (CFU.m^{-3})**		
	AM	**Min**	**Max**	**AM**	**Min**	**Max**
Packaging (INRS agent)	5.4×10^5	3.7×10^5	7.6×10^5	/	/	/
Cheese-washer 1	7.3×10^5	1.1×10^5	1.8×10^6	4.0×10^3	1.6×10^3	5.4×10^3
Packager 1	1.0×10^6	4.6×10^4	2.1×10^6	2.4×10^5	4.3×10^3	4.8×10^5
Cleaning workers	2.1×10^6	9.5×10^5	4.4×10^6	2.6×10^5	2.8×10^4	4.9×10^5
Cellar (INRS agent)	3.2×10^6	2.0×10^5	4.8×10^6	2.0×10^4	9.4×10^3	3.1×10^4
Packager 2	4.5×10^6	6.5×10^5	6.9×10^6	8.9×10^4	8.6×10^4	9.2×10^4
Cheese-washer 2	1.6×10^7	8.5×10^6	2.2×10^7	6.3×10^4	1.4×10^4	1.3×10^5
Cheese selector	3.5×10^7	6.6×10^6	6.8×10^7	9.2×10^5	7.6×10^5	1.1×10^6
Cheese-brushers	1.5×10^8	1.1×10^8	1.8×10^8	4.6×10^5	2.3×10^5	6.9×10^5

AM: arithmetic mean of the 2 or 3 days; Min and Max: minimum and maximum values measured;
/: not measured.

Size distribution

Figure 4 shows a representative measurement of the distribution of culturable fungal concentrations as a function of the aerodynamic diameter of the particles, close to the cheese brushing activity. Most culturable fungi emitted during brushing had an aerodynamic diameter between 3 and around 20 µm. These particles correspond to spores, agglomerates of spores, and mycelium released into the air as they were brushed off the surface of the cheeses. A lesser proportion of smaller (diameters below 3 µm - mycelium fragments) and larger particles (diameters greater than 20 µm - large agglomerates and large fungal structures) were also identified in the aerosol.

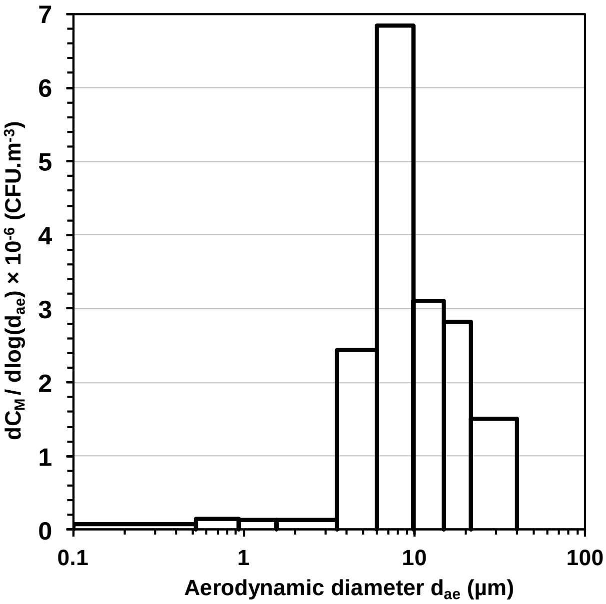

Fig. 4. Size distribution of the airborne culturable fungal concentration (dC_M) emitted close to the brushing activity (see also Figures F1 and F2 in online supplementary material for other measurements).

DISCUSSION

Concentrations measured at reference points

Whatever the location (rural, urban or peri-urban area), the season, or the country, the outdoor concentrations of culturable mesophilic microorganisms are generally below 10^4 CFU.m^{-3} and often close to 10^3 CFU.m^{-3} (Jones and Cookson, 1983; di Giorgio et al., 1996; Shelton et al., 2002; Medrela-Kuder, 2003; Adhikari et al., 2004; Fang et al., 2005; Lee et al., 2006; Lee and Jo, 2006; Kaarakainen et al., 2008; O'Gorman and Fuller, 2008). In public buildings, houses and other indoor environments without sources in various countries, the culturable microorganism concentrations are generally below 10^3 CFU.m^{-3}, and rarely exceed 10^4 CFU.m^{-3} (Reponen et al., 1992; Jaffal et al., 1997; Pastuszka et al., 2000; Pei-Chih et al., 2000; Shelton et al., 2002; Medrela-Kuder, 2003; Jo and Seo, 2005; Lee et al., 2006; Lee and Jo, 2006; Basilico et al., 2007; Kim and Kim, 2007; Tsai et al., 2007; Hussin et al., 2011). Several studies suggest that background endotoxin concentrations in outdoor and indoor environments in the absence of sources are below 10 EU.m^{-3} (Park et al., 2000; Carty et al., 2003; Mueller-Anneling et al., 2004; Zucker and Müller, 2004; Morgenstern et al., 2005; Madsen, 2006).

These bibliographic data agree well with the reference concentrations for culturable fungi (mean = 2.4×10^3 CFU.m^{-3}; maximum = 8.1×10^3 CFU.m^{-3}; n = 6), culturable bacteria (mean = 7.4×10^2 CFU.m^{-3}; maximum = 1.8×10^3 CFU.m^{-3}; n = 6) and endotoxins (mean = 2.2 EU.m^{-3}; maximum = 4.3 EU.m^{-3}; n = 6) that were measured in this study. The meteorological conditions (sunny with no wind) and the positioning of these reference points lead us to believe that they were neither affected by the activity performed in the plant nor by any other major source of bioaerosols.

Culturable fungi

Stationary samples. The ambient concentrations measured, mainly between 10^5 and 10^7 CFU.m^{-3} (Figure 1), underline the high fungal contamination of the air throughout the plant. These values are higher than those presented in previously published works. For example, in a Danish industrial dairy, concentrations of culturable fungi greater than 6.0x10^3 CFU.m^{-3} were measured in an area where cheeses were packaged (Dahl et al., 1994 – unknown air sampler). A study in cheese producing plants in the Netherlands reveals concentrations up to 8.0×10^3 CFU.m^{-3} (Hoekstra et al., 1998 – BioTest RCS Plus sampler). Chaumont et al. (2001) measured concentrations (SAS sampler) of over 2.5×10^4 CFU.m^{-3} during cheese brushing in cellars where Comté (pressed, cooked cheese) was matured, up to 2.0×10^3 CFU.m^{-3} in Emmental cellars (pressed, cooked cheese) and up to 4.0×10^4 CFU.m^{-3} in cellars where Mont d'Or was stored (soft cheese). Concentrations between 90 and 6.1×10^2 CFU.m^{-3} were measured in a cheese manufacturing facility in a Brazilian industrial dairy (Salustiano et al., 2003 – unknown impaction technique). Kure et al. (2008) measured the airborne culturable fungal concentrations in six plants producing pressed, uncooked cheeses; none of the values exceeded 2.7×10^2 CFU.m^{-3} (SAS / MAS-100 samplers and BIAP II slit-sampler). Finally, measurements in three maturing pits for pressed, uncooked "fossa cheeses" in Italy revealed concentrations between 5.3×10^2 and 7.5×10^2 CFU.m^{-3} (De Santi et al., 2010 – SAS sampler).

Such a comparison between published works (especially for results coming from field measurements) often remains delicate and complicated. Indeed, methods of bioaerosols sampling (filtration, impingment, duration, airflow, etc.) and analyses (measurement principle, culture medium, temperature of incubation, etc.) are major factors of variation of the measured concentrations in the air. Moreover, contamination of work atmospheres in cheese factories may also be highly variable depending on the type of cheese produced (fresh, soft, hard, pressed or not, cooked or uncooked pate, blue, with surface flora or not, cow's milk, sheep's milk, goat's milk, etc.), the working conditions, or the season for example.

Personal samples. All the occupational activities in the cheese-production line (brushing, washing, cleaning, selecting, packaging) in this plant expose workers to high concentrations of airborne culturable fungi (Figure 3). To our knowledge, these data are the first values from personal sampling to be published characterising exposure in a cheese factory / a cheese-maturing cellar.

The concentrations measured for the two INRS agents, who were present at the packaging station (mean = 5.4×10^5 CFU.m^{-3}; standard deviation = 2.0×10^5 CFU.m^{-3}; n = 3) and in the cellar (mean = 3.2×10^6 CFU.m^{-3}; standard deviation = 2.5×10^6 CFU.m^{-3}; n = 3), but who did not handle the cheese, are also high and of a similar order of magnitude to those of some workers. The high ambient concentrations therefore contribute ~~significantly~~ partially to worker exposure.

The concentrations measured based on personal samples are generally higher than the ambient concentrations measured by stationary sampling in the workplace. Thus, the occupational tasks and gestures involving manipulation of the cheeses by workers (brushing, washing, wiping, turning, etc.) seems to have direct consequences on the concentrations to which they are exposed.

Cheese-brushers and -selectors handle and brush large numbers of cheeses with an abundant surface flora throughout their working day. These workers are the most exposed to culturable mesophilic fungi. The concentrations measured for these two tasks (between 6.6×10^6 and 1.8×10^8 CFU.m^{-3}) are very high compared to the data available for other activity sectors such as composting, agri-food, agriculture, waste sorting, greenhouses, etc. (Goyer et al., 2001; Oppliger et al., 2005; Eduard, 2008; Duquenne et al., 2012; Hansen et al., 2012).

As yet, there is no occupational limit value for bioaerosols in France or elsewhere. The lack of such a value makes it more difficult to interpret results and to assess the risks linked to bioaerosols. Some guide values have, nevertheless, been suggested by some institutions, countries or in scientific articles; however, these are not strictly founded on exposure-response relationships. For example, for culturable fungi the Swiss guide value is 10^3 CFU.m^{-3} (SUVA, 2013), while the German value is 10^4 CFU.m^{-3} (Mandal and Brandl, 2011). Guide values of 10^4 spores.m^{-3} (Lavoie et al., 2007) or 10^5 spores.m^{-3} (Eduard, 2009) have also been suggested for total fungal spore concentrations (culturable or not, live or dead). A large majority of the personal concentrations measured in the cheese factory studied here exceeded these guide values.

Identified fungal species. *Mucor fuscus, Penicillium bialowiezense* and fungal strains belonging to a *"Penicillium complex"* were the dominant fungi identified in bioaerosol samples. These results corroborated previous findings suggesting that *Penicillium sp.* are prevalent in the microbial flora of cheeses (Lund et al., 1995). Culture conditions as well as selection criteria used to identify airborne fungi may have hampered the detection and identification of other fungal strains. Thus, the results reported in our study regarding fungal biodiversity of airborne mycoflora may be considered as indicative. This bias could be overcome by the use of technologies such as high-throughput sequencing performed directly on bioaerosols samples. Anyhow, both *Mucor sp.* and *Penicillium sp.* are potentially allergenic, and have been linked in the literature to symptoms or occupational diseases in workers. As the use of the beta-tubulin gene did not clearly allow the discrimination between some *Penicillum* species, it is not easy to appreciate the real allergenic potential of the isolated fungi. Anyhow, a few examples also exist for the specific case of cheese-makers (Schlueter, 1973; Campbell et al., 1983; Galland et al., 1991, Dahl et al., 1994, Guglielminetti et al., 2000).

Size distribution close to cheese brushing. Near where cheeses are brushed, the size distribution of the culturable fungal bioaerosol presents a median aerodynamic diameter close to 8 µm (Figure 4). The aerosol emitted is polydisperse and probably contains very heterogeneous fungal particles (spores, fragments, mycelium strands, agglomerates of different types, etc.). The size distribution clearly indicates that isolated spores, for which the aerodynamic diameter should be at most a few micrometers, are not a major component of the aerosol.

This type of granulometric data is essential if new preventive measures (capture, filtration, confinement devices, etc.) for a workstation are to be developed, for example. The size distribution of the fungal aerosol indicates that the concentrations measured at this sampling point correspond to the conventional inhalable fraction. It has been shown that the aspiration efficiencies for 37-mm diameter closed-face cassettes in calm air begin to underestimate the inhalable convention (CEN, 1993; ISO, 1995) for particles with aerodynamic diameters greater than around 40 µm (Görner et al., 2010). However, the majority of fungal elements emitted close to the brushing activity have aerodynamic diameters below this 40 µm threshold. Size distributions were not characterised for the other workstations, although it seems unlikely that they would be fundamentally different, or that they would contain many particles of greater than 40 µm diameter.

Culturable bacteria

All the workers equipped with personal samplers were exposed to higher airborne culturable bacterial concentrations than those measured at reference points with stationary sampling (Figure 3). To our knowledge, these are the first data concerning exposure to airborne bacteria to be published for this occupational sector. The high values for some concentrations (> 10^5 CFU.m^{-3}) are all the more notable given that filtration is known to be a stressful sampling method (mainly because of airflow desiccation), which can lead to underestimation of sensitive microorganisms, including bacteria (Macher and First, 1984; Jensen et al., 1992; Crook et al., 1997; Li et al., 1999; Wang et al., 2001). Moreover, Thorne et al. (1994) showed that culturable mesophilic bacteria concentrations may decrease after overnight mailing of the cassettes at room temperature (transport way that we used in this work). The culturable bacterial concentrations presented in this article should therefore be considered as minimum exposure levels for the workers during this measurement campaign. The same caveat do not apply, however, to the concentrations of culturable fungi, as fungi - and sporulating microorganisms generally - are not, or are only mildly sensitive to the stress induced by filtration, even with sampling durations of several hours (Lin and Li, 1998; Li et al., 1999; Nasman et al., 1999;

Wang et al., 2001; Durand et al., 2002). Likewise, culturable fungi concentrations remained steady after overnight shipping at room temperature of the sampling cassettes before the analysis (Thorne et al., 1994; Nasman et al., 1999).

Some culturable mesophilic bacterial concentrations measured in the cheese factory (> 10^5 CFU.m^{-3}) are in the higher concentration bracket when compared to similar measurements from various sectors including composting facilities, wastewater treatment plants, papermills, sawmills, household waste sorting plants, duck houses, etc. (Goyer et al., 2001; Oppliger et al., 2005; Martin et al., 2010; Duquenne et al., 2012).

Several countries (Germany, Denmark, the Netherlands, Switzerland) suggest a guide value of 10^4 CFU.m^{-3} for culturable bacteria concentrations (Mandal and Brandl, 2011; SUVA, 2013), although, once again, this value is not based on an exposure-response relationship. Several personal concentrations measured in the factory studied exceeded 10^4 CFU.m^{-3}.

Endotoxin concentrations

The ambient endotoxin concentrations in the plant were all greater than (at least 30-fold) the concentrations measured at the reference points. We could not find any previous publication characterising endotoxin concentrations in the cheese manufacturing sector, but our data can be compared with data coming from other occupations, where sampling had also been performed using 37-mm CFC. Thus, the endotoxin concentrations measured in the maturing cellar (between 6 and 42 EU.m-3) were close to the concentrations measured in wood industries, dental offices or metal machining workshops (Oppliger et al., 2005; Harper and Andrew, 2006; Gilbert et al., 2010; Singh et al., 2010; Gioffrè et al., 2012). The highest concentrations, measured at the packaging station and in the cleaning room (between 61.2 and 357.3 EU.m^{-3}), should be compared to those reported by Chang et al. (2001) for open-style swine houses or by Duchaine et al. (2001) for sawmills. The concentrations measured in the cheese factory are, however, lower than those measured in an indoor composting facility (between 500 and 5,400 EU.m^{-3} in Duquenne et al., 2012) or in conventional swine confinement buildings (between around 1,000 and 12,000 EU.m^{-3} in Duchaine et al. (2001) and 1,800 and 69,000 EU.m^{-3} in Bonlokke et al. (2009)).

Because of how difficult it is to establish a recognised dose-effect relationship, no regulatory value is currently available for endotoxin concentrations (Duquenne et al., 2013). However, various recommendations have been made to facilitate data interpretation. Among them, Rylander (1997) proposed a ‘no effect’ level (i.e. absence of respiratory tract inflammation) of approximately 100 EU.m^{-3}; the Dutch Expert Committee on Occupational Standards (DECOS, 2010) published a report proposing an occupational exposure limit of 90 EU.m^{-3}. The packaging station and the cleaning room are work areas in the plant where workers can be exposed to concentrations exceeding these recommended values.

CONCLUSIONS

The samples taken in the cheese-maturing cellar over three consecutive days made it possible to determine the bioaerosol concentrations to which workers are exposed in the various work areas. This study presents the first personal fungal and bacterial concentrations, as well as the first endotoxin concentrations, to be measured for this occupational sector.

The results show that high concentrations of culturable fungi are present in the work areas; the workers are particularly exposed to *Mucor fuscus* and to *Penicillium sp*. throughout the cheese-production line. This exposure is particularly high (between 10^6 and 1.8×10^8 CFU.m^{-3}) for cheese brushing, washing and selection activities. The concentrations measured suggest that an immunoallergic or even toxinic risk may occur for workers if an effective preventive method is not used at the most exposed work stations.

The work atmosphere in this plant is complex. The bioaerosols emitted by the activities contain a high proportion of fungi, but they also contain bacteria and endotoxins - sometimes in non-negligible concentrations - and probably also contain other microorganism compounds or by-products which were not measured. All these microorganisms, fragments and substances are found in the work atmospheres and contribute to overall worker exposure.

Our results indicate that the preventive measures already in place should be maintained in the plant (supply of masks, general ventilation, etc.). A respiratory protection program will also be necessary in order to manage the wearing of protective masks by the workers. Results also call for an increase in the information provided to workers, to help them understand the risks run when performing the different tasks and to involve them in defining any additional preventive approaches which should be applied. It is difficult to envisage reducing fungal concentrations in the cellar, as the presence of airborne fungi is essential to the cheese-maturing process. The specific atmosphere in this confined space allows fresh cheeses to be inoculated, and thus constitutes one of the most precious tools of the plant. The presence of airborne fungi is maintained over time specifically through repeated brushing of mature cheeses. In contrast, collective preventive measures can be implemented more easily in the cleaning room and at the packaging station as there are no particular constraints in these areas.

The observations, established for the plant studied at the time of the measurements, should not be generalised to all plants where cheeses are matured and packaged. The concentration levels and the microbial strains encountered are probably very variable from one plant to the next (e.g. depending on the workstation and how work is organised, the existing preventive measures, the type of cheese, factory throughput, geographical area, or season). This measurement campaign nevertheless shows that some cheese-maturing cellars can be a source of particularly high concentrations of fungal aerosols, and that this occupational sector should be more fully documented in the future.

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