



Granulometry, microbial composition and biological activity of dusts collected in French dairy farms

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ABSTRACT

Background: Dairy working increases the prevalence of lower airway respiratory diseases, especially COPD and asthma. Epidemiological studies have reported that chronic inhalation of organic dusts released during specific daily tasks could represent a major risk factor for development of these pathologies in dairy workers. Knowledge on size, nature and biological activity of such organic dusts remain however limited.

Objective: To compare size distribution, microbial composition and cellular effects of dusts liberated by the spreading of straw bedding in five French dairy farms located in Brittany.

Results: Mechanized distribution of straw bedding generated a cloud of inhalable dusts in the five dairy farms' barns. Thoracic particles having a 3–7.5 µm size constituted 58.9–68.3% of these dusts. Analyses of thoracic dusts by next generation sequencing showed that the microbial dust composition differed between the five French farms, although *Actinobacteria*, *Bacteroidetes*, *Firmicutes* and *Proteobacteria* represent more than 97.5% of the bacterial phyla detected in each sample. Several bacteria genera comprising of human pathogenic species, such as *Pseudomonas*, *Staphylococcus*, *Thermoactinomyces* or *Saccharopolyspora* were identified. *Cladosporium* and *Alternaria* fungal genera, which are potent environmental determinants of respiratory symptoms, were detected in dusts collected in the five farms and their levels reached 15.5–51.1% and 9–24.7% of assignable fungal sequences in each sample, respectively. Finally, all dust samples significantly and strongly increased the expression of the pro-inflammatory TNF-α, IL-1β, IL-6 and IL-8 cytokines at both mRNA and protein levels in human monocyte-derived macrophages. Their effects were dose-dependent and detectable from 1 µg/ml. The intensity of the macrophage responses however differed according to the samples.

Conclusions: Our results strengthen the hypothesis that organic dusts released during the distribution of straw bedding are mainly constituted of thoracic particles which are small enough to deposit on lower bronchial epithelium of dairy farmers and induce inflammation.

1. Introduction

The prevalence of respiratory symptoms related to chronic obstructive pulmonary disease (COPD), asthma or chronic bronchitis is higher in livestock farmers than in unexposed control subjects (Eduard et al., 2009; May et al., Guillien et al., 2016). Swine and dairy farmers more frequently display chronic cough, dyspnea, and accelerated decline in pulmonary function than non-farming individuals (Eduard et al., 2009; Guillien et al., 2016). The development of lower airway

respiratory diseases in dairy farmers is likely influenced by specific working tasks (Marescaux et al., 2016; Thaon et al., 2011). Particularly, a major risk factor contributing to the development of COPD or asthma may be the repeated inhalation of organic dusts released during grain handling, foddering and distribution of bedding materials (Gainet et al., 2007; Jouneau et al., 2012; Garcia et al., 2013; Basinas et al., 2014).

Dusts collected in dairy farms may contain not only different bacteria species, endotoxins and peptidoglycans from Gram-negative and Gram-positive bacteria respectively, but also several fungal species

Abbreviations: COPD, chronic obstructive pulmonary diseases; Toll-like receptors, OPC, optical particle counter; NGS, next generation sequencing; RA, relative abundance levels; PM, particle matter

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(Kullman et al., 1998; Lee et al., 2006). The various microbial membrane motifs of these organisms activate different pattern recognition signaling pathways, such as toll-like receptor (TLR) 2 and TLR4, and trigger chronic inflammation, as observed in murine models (May et al., 2012). *In vitro* experiments have shown that soluble extracts of farm-derived organic dusts activated bronchial epithelial cells and immune cells such as dendritic cells and macrophages (May et al., 2012).

However, according to their size, only a specific part of these organic dusts can really interact with the human bronchial tract where obstructive lung changes related to COPD are expected to occur. Indeed, among the particles inhaled through the nose and the mouth, only the thoracic fraction is able to penetrate beyond the larynx and spread into the thoracic region (CEN, 1993). The European standard EN481 defines the inhalable, thoracic and respirable fractions of an aerosol as decreasing functions of the particles forming this aerosol. The inhalable fraction corresponds to the part of an aerosol able to deposit anywhere in the respiratory tract, including nose and mouth, and is therefore constituted of extra-thoracic and thoracic particles. Penetration efficiency of inhalable particles down to the larynx (i.e., the extra-thoracic region) is 50% at 100 μm . For the thoracic fraction, the penetration efficiency of particles beyond the larynx is 99.7%, 50% and < 0.56% at 0.1 μm , 10 μm and at > 30 μm , respectively (CEN, 1993). The thoracic fraction of an aerosol also comprises of the respirable fraction defined as smaller particles that reach alveolar tissues (50% penetration efficiency at 4 μm). Regarding dairy farming, different studies have previously reported that American and European farmers are exposed inside the barns to inhalable particle matter (PM) during their daily working tasks and possibly to PM₁₀ and PM_{2.5} (Kullman et al., 1998; Cathomas et al., 2002; Garcia et al., 2013; Basinas et al., 2014). PM₁₀ and PM_{2.5} have a 50% cut-off aerodynamic diameter of 10 μm and 2.5 μm , respectively, and can diffuse in the tracheo-bronchial regions (CEN, 2014). Working tasks related to dairy farming thus likely release and expose farmers to thoracic dusts. Additional assessments of granulometry, microbial composition and biological activities of these thoracic dusts are however required to specify their potential role in lower respiratory diseases. First, it is needed to determine if thoracic dusts constitute the main fraction of the inhalable aerosols generated by dairy working tasks. Second, though it is well known that inhalable dusts contain endotoxins, different bacteria genera and additional microbial agents such as fungi can be present in thoracic dusts and mediate their biological effects. Third, the possibility that thoracic dusts activate immune cells, which contribute to COPD or asthma physiopathology, remains to be investigated.

In the present study, to gain insights onto dairy farm dusts, we compared, in five different French dairy farms located in Brittany, the size distribution, the global microbial composition and the pro-inflammatory effects on human macrophages of dusts specifically collected during the spreading of straw bedding, a working task known to markedly exposed dairy farmers to inhalable dusts (Basinas et al., 2014).

2. Materials and methods

2.1. Description of dairy farms

Dusts were collected from five typical dairy farms (Farms F1 to F5) located near Rennes, the capital of Brittany (France) where dairy farming is a main agricultural activity. These farms are family-run farms that develop an agricultural system combining crop and livestock farming. The farms have a mean area of $1405 \pm 365 \text{ m}^2$. They housed 60–130 milking cows in free stall barns, with (Farms F1 and F4) or without cubicles (Farm F2, F3 and F5), that were naturally ventilated through opening in walls and roofs (Table S1). In Brittany, dairy farmers classically use wheat straw for bedding and renew it daily. As generally observed in Breton dairy farms, straw was produced by the farmers and was mechanically distributed by a shredder in these five farms.

2.2. Dust size measurements

Dust sizes were determined using the optical particle counter (OPC) Grimm 1.108 (GRIMM Aerosol Technik Ainring GmbH & Co. KG, Germany). This optical laser light aerosol monitor measures the particle size distribution in 15 different channels ranging from 0.23 μm (its lower limit) to 20 μm and in a channel for particles with a size greater than 20 μm . The OPC Grimm was set to register a measure every 6 s. To simplify the readability of the results, we have aggregated the data recorded in some channels and created 8 bins: 0.23–1 μm , 1–3 μm , 3–5 μm , 5–7.5 μm , 7.5–10 μm , 10–15 μm , 15–20 μm and > 20 μm . Results were expressed as mean mass concentrations for size bins and for inhalable, thoracic and respirable fractions. The extra-thoracic and tracheobronchial fractions were also calculated as follow: extra-thoracic fraction = inhalable fraction - thoracic fraction and tracheobronchial fraction = thoracic fraction - respirable fraction. During distribution, dust sizes were measured by placing the OPC on a tripod in a vacant zone of the stall, located at 4–5 m from the stall corridor from which straw bedding is dispersed (Fig. S1).

2.3. Thoracic dust collection

In each farm, during the measurement of dust size by the OPC Grimm, dusts were simultaneously collected on three different Teflon filters (37 mm diameter, 0.7 μm porosity, Millipore, Billerica, MA, USA) placed in cassettes of three CATHIA sampling heads (Tecora, Paris, France) selecting the thoracic fraction of airborne particles. Each CATHIA head was connected to a sampling pump (model Bravo R/RP Tecora, France) operating at 7 L/min, as recommended by the manufacturer and in agreement with the thoracic convention EN 481. These devices were then placed on a tripod, 1.5 m high, to form a one-meter equilateral triangle around the OPC Grimm 1.108. Sampling was begun when the farmers started to project straw in the stable and it was stopped approximatively 16 min after the end of the task in order to sample dusts remaining in the air. Dusts were collected in the five farms between December 2015 and February 2016. The mass of dusts collected on filter was determined by differential weighing. Prior to each weighing, Teflon filters were conditioned one night in a controlled environment (hygrometry 35–50% and temperature 20–22 °C). Then, filters were weighed on an electronic micro-balance (Precisa 2000, minimum resolution of 0.01 mg, Mettler Toledo, Colombus, Ohio, USA) before and after dust sampling. The limit of quantification of the method is 0.08 mg per filter. The sum of the three net differences between pre- and post-sampling filter weighing was used to calculate the total dust mass sampled during the task. Just after weighing, the three Teflon filters were successively washed with the same sterile phosphate buffer saline solution, under a laminar flow hood, to re-suspend thoracic dusts into a unique stock suspension for each farm. The suspension was next aliquoted and frozen at –20 °C. A laboratory filter blank extract was also prepared by washing filters that were not exposed to organic dusts.

To determine the size distribution of dusts collected on Teflon filters, the masses of inhalable dusts measured by Grimm were multiplied by the “thoracic relative to inhalable” penetration efficiency derived from the European EN 481 convention for the following median bin sizes: 0.615 μm (0–1 μm), 2 μm (1–3 μm), 4 μm (3–5 μm), 6.25 μm (5–7.5 μm), 8.75 μm (7.5–10 μm), 12.5 μm (10–15 μm), 17.5 μm (15–20 μm) and 25 μm (> 20 μm). The corresponding penetration efficiencies for these 8 median bin sizes are: 100% (0.615 μm), 100% (2 μm), 99.58% (4 μm), 93.74% (6.25 μm), 75.92% (8.75 μm), 43.02% (12.5 μm), 15.73% (17.5 μm) and 2.97% (25 μm). To be expressed in percentage, the masses of thoracic dusts measured in each size bin were then divided by the total mass concentration of thoracic dusts measured by Grimm for the 8 size bins.

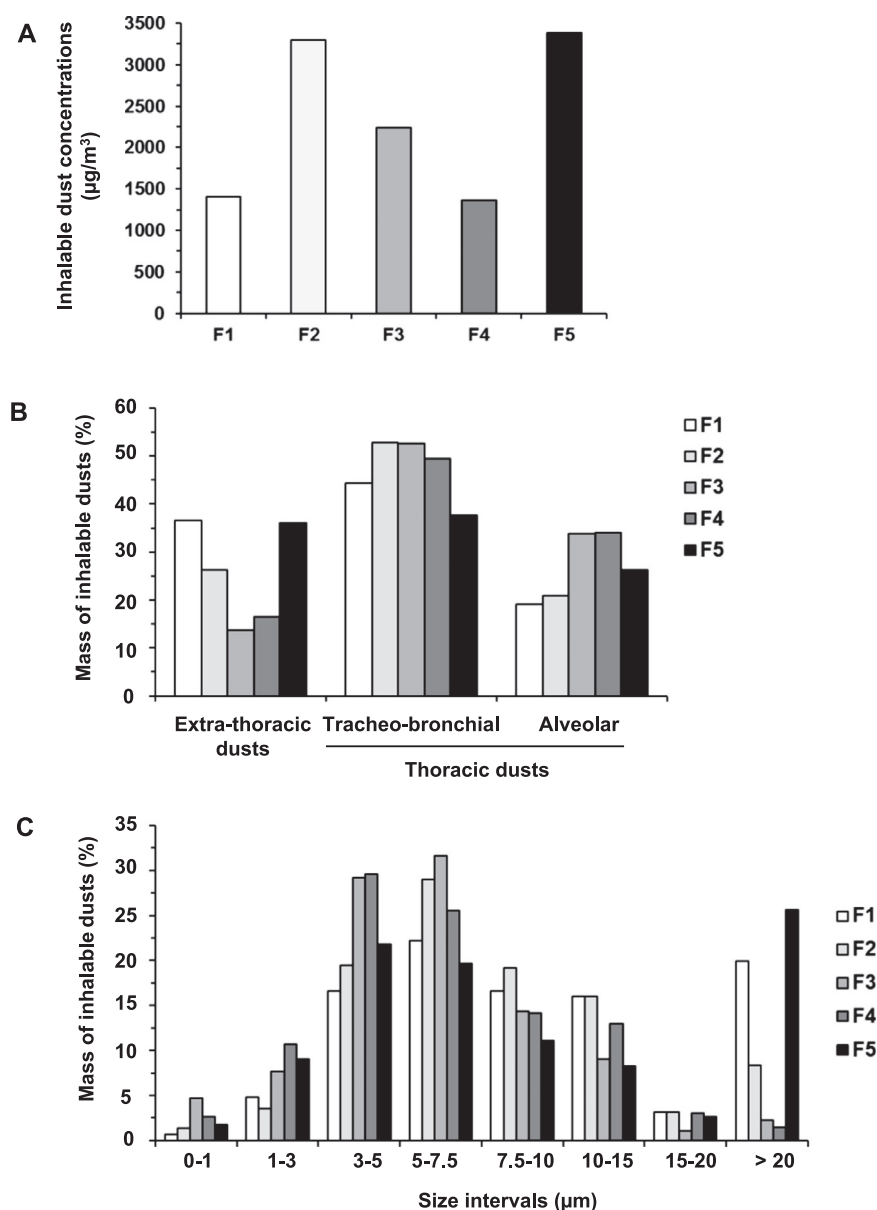


Fig. 1. Concentrations and size of inhalable dusts released during the distribution of straw bedding. Ambient dusts were analyzed in the stable of five different dairy farms (F1 to F5) during straw distribution, using a Grimm 1.108 OPC placed on a tripod in a vacant zone. The results are expressed as (A) inhalable dust concentrations, (B) mass percentages of inhalable dusts for extra-thoracic, tracheobronchial and alveolar fractions and (C) mass percentages of inhalable dusts by particle size intervals.

2.4. NGS

Total DNA was extracted from thoracic dust suspensions using the method developed by Godon et al. (1997). A portion of the 16S rRNA was amplified using the barcoded, universal primer set (515WF/918WR) (Wang et al., 2009). A portion of the 18S rRNA was amplified using universal 18S primer set (574WF/952WR) (Hadziavdic et al., 2014). PCR reactions were performed using AccuStart II PCR ToughMix kit, cleaned (HighPrep PCR beads, Mokascience) and then submitted for sequencing on Illumina HiSeq. 2000 instrument at GeT-PlaGe (Auzeville, France). Negative controls for DNA extraction kits and PCR reagents were also performed to ensure that materials were not contaminated. Sequences were processed using Mothur (version 1.33.1) according to MiSeq SOP pipeline (Schloss et al., 2009). Barcodes, primers, and sequences showing homopolymers of more than 8 bp have been discarded. Sequences showing 100% homology were grouped in unique sequences, then in operational taxonomic unit (OTUs, based on 97% homology). Sequences were next assigned to match to a sequence in SILVA (version 123) and PR2 databases for prokaryotes and eukaryotes, respectively, to identify the genus level. We considered assigned sequences with relative abundance (RA) higher than 0.002% and we further analyzed those with a RA > 1%.

2.5. Cell cultures and treatments

Peripheral blood mononuclear cells were first isolated from bloody buffy coats of healthy donors through Ficoll gradient centrifugation. Human monocytes were then prepared by a 2-h adhesion step in 6-well microplates, which routinely obtained > 90% of adherent CD14-positive cells as assessed by immunostaining (Bourdonnay et al., 2009). These monocytic cells were next cultured for 6 days in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 20 UI/ml penicillin and 20 µg/ml streptomycin, in the presence of 800 UI/ml GM-CSF to get macrophages as previously reported (Bourdonnay et al., 2009). After 6 days, according to cell cultures, approximately 0.5 million of adherent macrophages were present in each well of the 6-well microplates. Cells were then washed and treated, in 2 ml of complete RPMI 1640 culture medium, with the indicated concentrations of each dust suspension, for 4 h and 24 h to quantify cytokine mRNA and protein levels, respectively. After treatments, cell viability was always > 90%. To verify the direct impact of Teflon filters on gene expression, cells were also exposed to the filter blank extract for 4 h.

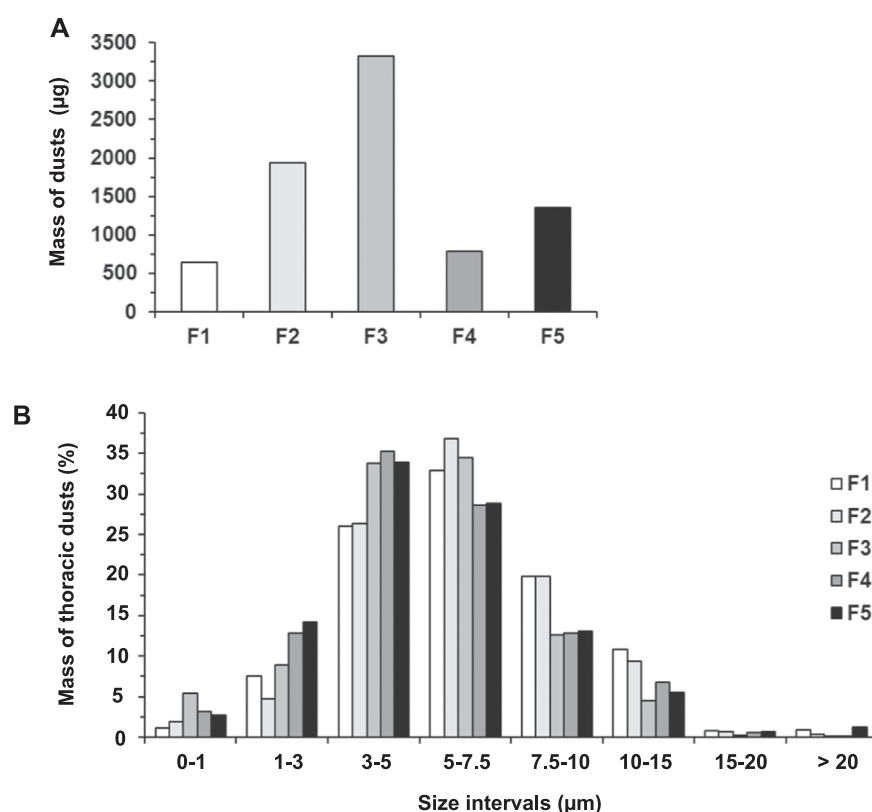


Fig. 2. Granulometric analysis of thoracic dusts collected by CATHIA samplers. (A) Total masses of thoracic dusts collected on filters of three CATHIA sampling heads that selected the thoracic fraction of airborne particles. These devices were positioned on a tripod to form a one-meter equilateral triangle around the Grimm 1.108 OPC during distribution of straw bedding in the five farms (F1 to F5). (B) Estimation of the percentages of dust masses present in each size interval of the thoracic fraction. For each farm, calculations were performed using both the size distribution by size interval of inhalable dusts determined by the OPC measurements (Fig. 1C) and the theoretical sampling efficiency of thoracic fraction defined by the thoracic convention EN 481 for midpoint of each size intervals.

2.6. RNA isolation and Reverse Transcriptase-Polymerase chain reaction (RT-PCR) assays

At the end of the 4 h treatment with thoracic dusts, total RNAs were extracted by the TRIzol method and then analyzed by RT-PCR, in duplicate, using the fluorescent dye SYBR Green methodology and a CFX384 real-time PCR system (Bio-Rad). Human “QuantiTect Primer assay” sets from Qiagen were used to quantify TNF- α , IL-1 β , IL-6 and IL-8 mRNA transcripts. Specificity of gene amplification was checked at the end of PCR through analysis of dissociation curves of the PCR products. Amplification curves were analyzed by the comparative cycle threshold method, using the CFX Manager™ Software. For each well, this software automatically sets the fluorescence baseline individually and then computes the optimal quantification cycle (Cq) on a per fluorophore basis by applying a multivariable, nonlinear regression model. Then, mean Cq values were used to normalize expression of the steady-state target mRNA levels to those of the 18 S ribosomal protein, using the $2^{(-\Delta\Delta Cq)}$ method.

2.7. Quantification of cytokine levels

Levels of TNF- α , IL-1 β , IL-6 and IL-8 secreted in culture medium were quantified by ELISA, using specific Duoset ELISA development system kits (R & D Systems) following the manufacturer's instructions.

2.8. Statistical analysis

For studies on human cells, data are expressed as mean $\pm$ SD of four independent experiments performed on four different primary cultures of macrophages. Differences between mRNA and cytokines levels were determined using the ordinary non-parametric Kruskal Wallis test. When variations among data column medians were significantly greater than expected by chance ($p < 0.05$), this analysis was followed by the Dunn's Multiple Comparison Test.

3. Results

3.1. Granulometric characterization of inhalable dusts produced during straw distribution

The present study was designed to characterize organic dusts produced during mechanized distribution of straw bedding in five different French dairy farms (F1 to F5). To this goal, we first analyzed size distribution of inhalable dusts using a Grimm OPC. The mean background dust concentration in the 5 farms was $63.1 \pm 40.7 \mu\text{g}/\text{m}^3$ (Table S2). The mean concentration of inhalable dusts released during straw dispersion ranged from 1354 (F4) to 3380 (F5) $\mu\text{g}/\text{m}^3$ (Fig. 1A). The peak values were measured between 4797 to 32,250 $\mu\text{g}/\text{m}^3$ (Table S2). After this working task, which lasted approximately 20 min, dust concentrations rapidly decreased to their background levels in the five farms (data not shown). Among these inhalable dusts, the thoracic fraction was always predominant (64–86.4%) in samples collected from the five farms and it is mainly comprised of dusts that could deposit on the tracheobronchial area (Fig. 1B). However, it can be noted that the mass of dusts that could not penetrate below the larynx, (i.e the extra-thoracic fraction), was variable according to the farms (Fig. 1B). It accounted for 13.6% in the farm F3 to 36% in the farms F1 and F5. In addition, Fig. 1C demonstrates that, among inhalable dusts, the highest percentages of dust masses were measured in the 3–5 μm and 5–7.5 μm size intervals. These dusts ranging from 3 μm to 7.5 μm represented 39% (F1) to 60% (F3) of the total dust masses.

3.2. Size distribution of thoracic dusts collected by CATHIA devices

In each farm, simultaneously to Grimm measurements, thoracic dusts were also collected on filters by a CATHIA device during straw distribution. The highest amount of thoracic dusts collected on filters was measured in the F3 sample (Fig. 2A). This result is concordant with our previous observation showing that the extra-thoracic fraction represented only 10% of the inhalable dusts collected in the F3 sample

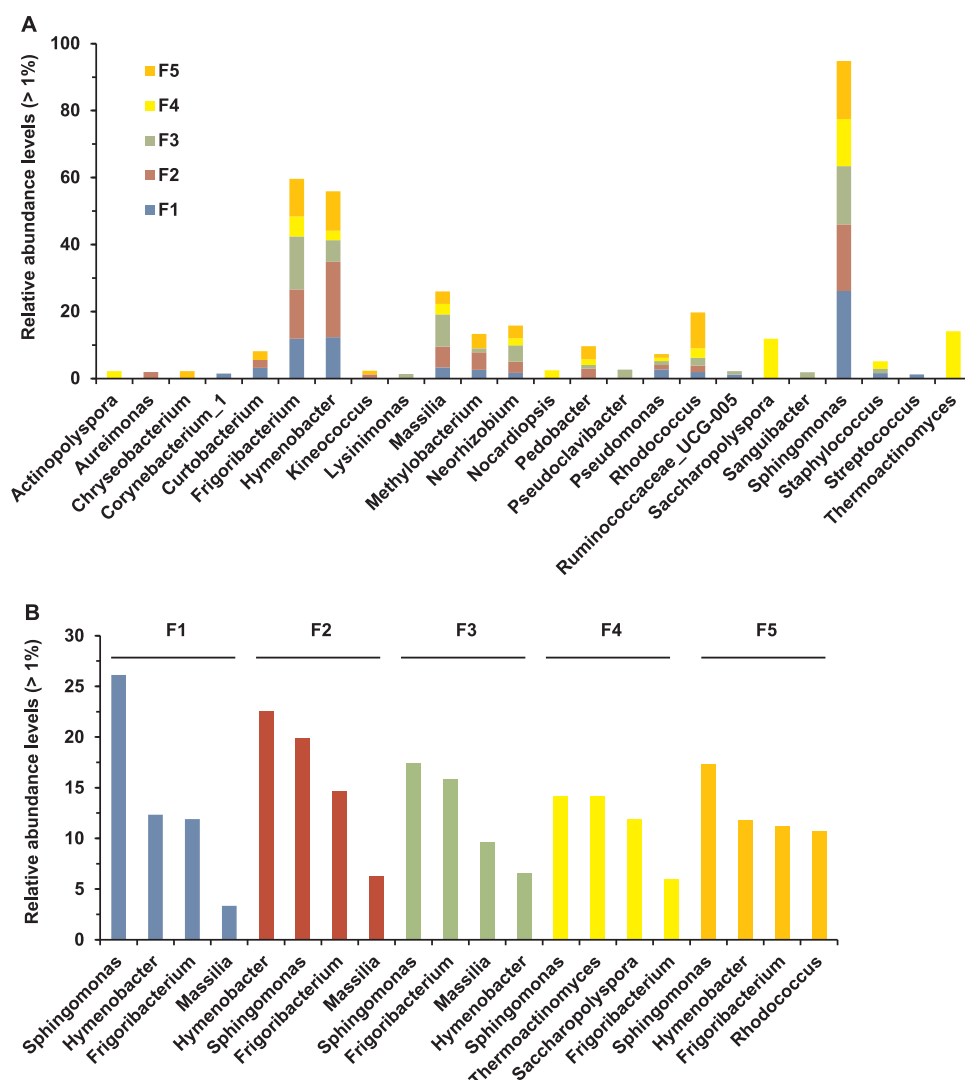


Fig. 3. Bacterial genera with relative abundance (RA) levels > 1% of in dust samples. RA values are expressed as percentages of the total number of genera sequences identified in dairy dusts by NGS. (A) All genera with RA > 1% present in dust samples. (B) Top four of the genera sequences in each dust sample.

(Fig. 1B). Taking into account the efficiency of dust penetration into the thoracic region defined by the European standard EN481, we then estimated the size distribution of thoracic dusts collected on filters using the Grimm measurements previously recorded for inhalable dusts. Fig. 2B shows that dusts with a 3–7.5 μm size formed the majority of thoracic dusts collected in the five farms. Indeed, these dusts represented 58.9% (F1) to 68.2% (F3) of thoracic dusts collected on filters. In contrast, and as expected, dusts having a size > 15 μm accounted for less than 2% in all dust samples.

3.3. Analysis of the microbial composition of thoracic dusts by NGS

Using the NGS technology, we next analyzed microbial and fungal composition of dusts collected in the five dairy farms during distribution of straw bedding. A total of 201,617 prokaryote sequences have been analyzed for the five samples, with 37,871 to 48,691 sequences per sample. Fig. S2 shows that, within the domain “Bacteria”, the Gram-negative *Proteobacteria* and *Bacteroidetes*, and the Gram-positive *Actinobacteria* and *Firmicutes* are the four main phyla whose sequences represent more than 97% of the total bacterial DNA sequences. However, the RA levels of these phyla markedly differ within dust samples. Indeed, *Proteobacteria* is the most abundant phylum in samples F1 to F3 (41–43%) whereas *Actinobacteria* comprises of a majority in the samples F4 (35.4%) and F5 (38.4%). *Firmicutes* is less abundant in all samples, except in sample F4 (24.7%), and sequences assigned to *Bacteroidetes* represent 9.7% (F4) to 27.8% (F2) of the total sequences related to

these four phyla. Consequently, the samples F1, F2, F3 and F5 are mainly constituted of Gram-negative organisms (55% to 69%) whereas 60% of Gram-positive organisms are present in the F4 sample (Fig. S2).

On a genus point of view, all assignable sequences with RA > 0.002% are listed in the Table S3. The most abundant sequences in each dust sample, i.e. sequences with RA > 1%, account for 74.2%, 86%, 71%, 70.8% and 76% of all sequences detected in dust sample F1, F2, F3, F4 and F5 respectively. Fig. 3A shows that sequences with RA > 1% are assigned to 24 different genera of which seven are systematically detected in the five dust samples: *Frigoribacterium*, *Hymenobacter*, *Massilia*, *Neorhizobium*, *Pseudomonas*, *Rhodococcus* and *Sphingomonas*. The top four genera in each dust sample are shown in the Fig. 3B. Among these most abundant genera, *Sphingomonas* (17.3% to 26.1%) and *Frigoribacterium* (6% to 15.8%) are the two genera detected in the five dust samples. *Sphingomonas* is an environmental bacteria isolated from many different land and water habitats, as well as from plant root systems. *Frigoribacterium*, an *Actinobacteria*, was first isolated in cattle barn (Kämpfer et al., 2000). *Massilia* (3.3% to 9.6%), a root colonizing *Proteobacterium*, and *Hymenobacter* (2.8% to 22.5%), an environmental bacteria from the *Bacteroidetes* phylum, are also detected in the top four genera in three (F1 to F3) and four (F1-F3, F5) dust samples, respectively. *Thermoactinomyces* (*Firmicutes*), a bacteria commonly found in natural high-temperature habitats, and *Saccharopolyspora*, an *Actinobacteria* found in soil, are the two Gram-positive genera specifically present in the top four sequences of the F4 sample. As it was not possible to identify the bacteria at the Species level, pathogenic strains

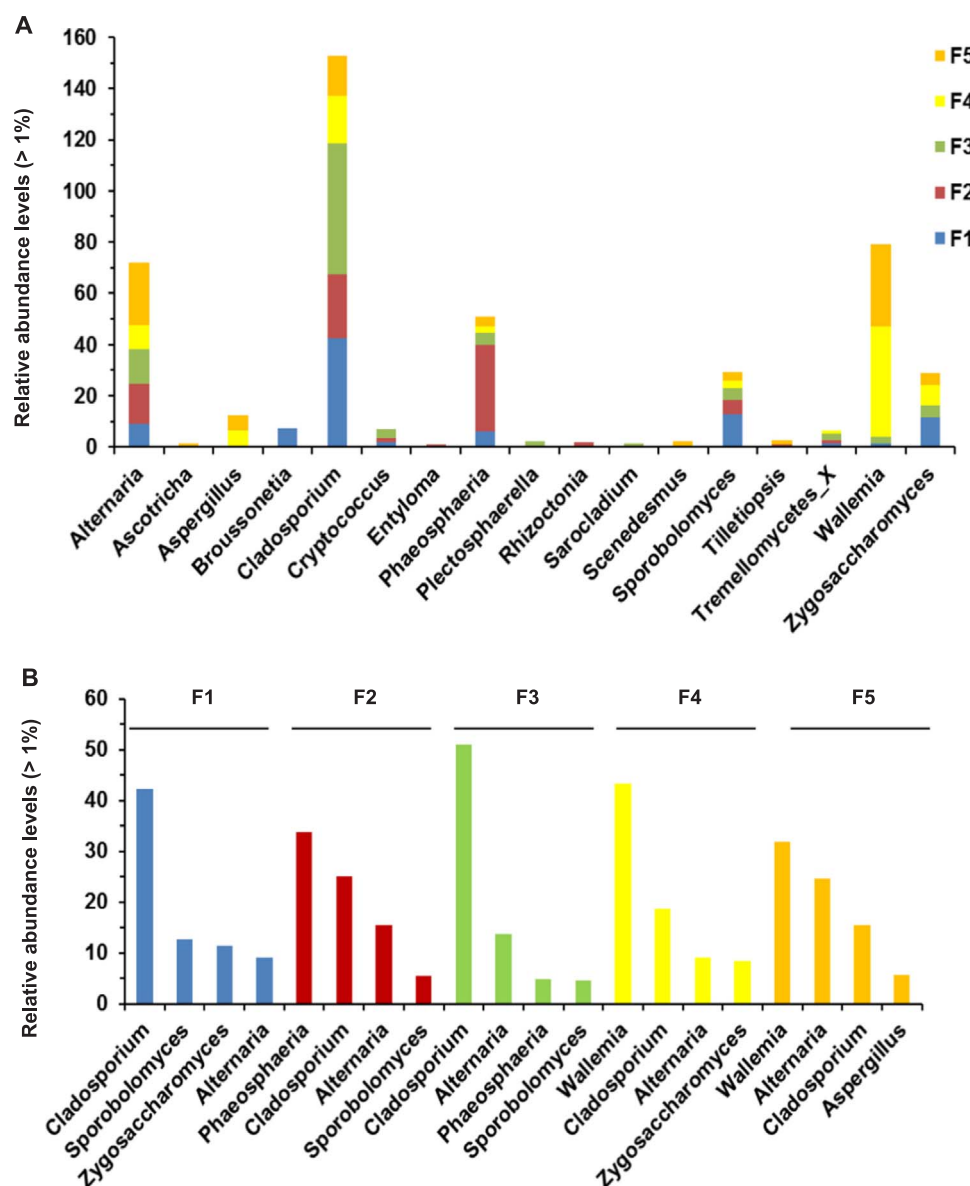


Fig. 4. Fungal genera with relative abundance (RA) levels > 1% of in dust samples. RA values are expressed as percentages of the total number of genera sequences identified in dairy dusts by NGS. (A) All genera with RA > 1% present in dust samples. (B) Top four of the genera sequences in each dust sample.

for humans could not be specified. However, among the genera with RA > 1% and displaying classical pathogenic species, *Staphylococcus* and *Pseudomonas* were identified in three (F1, F2, F4) and five dust samples, respectively (Fig. 3A). In addition, *Rhodococcus*, *Stenotrophomonas*, *Acinetobacter* and *Corynebacterium* were also detected with a lower frequency in different samples. Sequences with RA < 1% are comprised of 49%, 54%, 50%, 57% and 54% Gram-negative bacteria in the dust sample F1, F2, F3, F4 and F5 respectively (Table S3). It is noteworthy that sequences assigned to the *Legionella* genus are detected at RA < 1% in the sample F2 but not in any other samples.

3.4. Analysis of the fungal composition of thoracic dusts by NGS

A total of 261,904 eukaryote sequences have been analyzed for the five samples, with 40,852 to 53,235 eukaryote sequences per dust sample. All assignable sequences with RA > 0.002% are listed in the Table S4. The sequences with RA > 1% account for 93.7%, 91.6%, 93.8%, 94.1% and 94.9% of all sequences detected in dust sample F1, F2, F3, F4 and F5 respectively. Fig. 4A shows that sequences with RA > 1% are assigned to 17 different genera of which four are systematically detected in all dust samples: *Alternaria*, *Cladosporium*, *Phaeosphaeria* and *Sporobolomyces*. Among the top four genera,

Cladosporium (15.5% to 51.1%) and *Alternaria* (8.96% to 24.7%) are detected in the five dust samples (Fig. 4B). They constitute the most common outdoor genera detected in air samples. Other predominant genera include *Sporobolomyces* (a soil yeast), *Zygosaccharomyces* (a yeast contaminating food items), *Phaeosphaeria* (a wheat pathogen ascomycete) and *Wallemia* (a xerophilic fungus frequently found in agricultural dusts) present in the top four genera of different samples. In addition, *Cryptococcus* and *Aspergillus*, which contain well-known pathogenic species for humans, are also detected with RA > 1% in samples F1 (1.74%), F2 (1.9%), F3 (3.4%), and in samples F4 (6.6%), F5 (5.7%), respectively.

3.5. Pro-inflammatory responses induced by thoracic dusts in human macrophages

We finally determined the ability of thoracic dusts to trigger pro-inflammatory responses in human monocyte-derived macrophages. We exposed macrophages to 0.1–10 µg/ml (or approximately 0.026–2.6 µg/cm²) thoracic dusts, re-suspended in RPMI 1640 culture medium, for 4 h and 24 h to quantify expression of TNF-α, IL-1β, IL-6 and IL-8 at mRNA and protein levels, respectively. Each dust sample was incubated with four different primary cultures of human

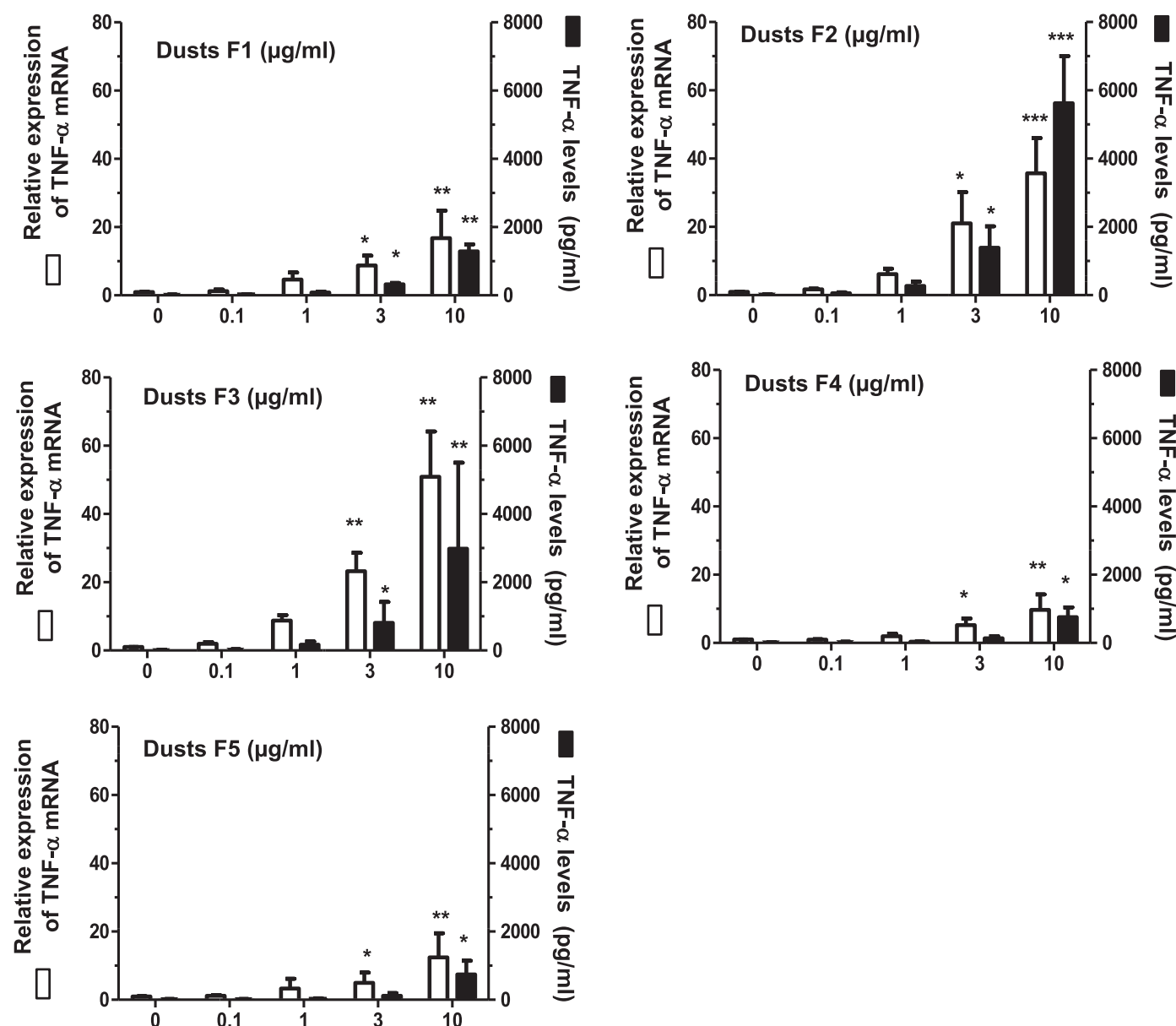


Fig. 5. Effects of dairy dusts on TNF-α expression in human macrophages. Human monocyte-derived macrophages were exposed for 4 h (mRNA levels) or 24 h (protein secretion) to increasing concentrations of dusts collected in dairy farms F1 to F5. mRNA levels of target genes were determined by quantitative RT-PCR and normalized to endogenous ribosomal 18S RNA levels. Data are expressed relatively to mRNA levels found in “0” control cells not exposed to dusts, arbitrarily set at the value of 1. The levels of TNF-α secreted in culture medium were quantified by ELISA. All results are expressed as means $\pm$ SD of four independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus “0”.

macrophages prepared from four different human donors. First, control experiments showed that the filter blank extract had no effect on gene expression in human macrophages (data not shown). In contrast, thoracic dusts collected in the five farms dose-dependently increased the levels of both TNF-α transcripts and TNF-α protein secretion (Fig. 5). Their effects were always maximal at 10 µg/ml, but the levels of gene stimulation and protein secretion were different according to the samples. The dusts from the F2 sample induced the highest release of TNF-α, at a level that was approximately 5- to 6-fold higher than those measured for the F1, F4 and F5 samples. Similar results were obtained for the three other cytokines. All thoracic dust samples markedly increased the levels of transcripts coding for IL-1β, IL-6 and IL-8 proteins (Figs. 6–8). IL-1β secretion was around 25–50 pg/ml for macrophages exposed to F1, F3, F4 and F5 samples but approximately 4-fold higher for cells treated with the F2 dust sample (Fig. 6). The secretions of IL-6 (Fig. 7) and IL-8 (Fig. 8) were also maximal for macrophages treated with the F2 dust sample.

4. Discussion

The present study demonstrates that mechanized distribution of straw bedding in these five French dairy farms generates high concentrations of thoracic dusts that contain several different bacterial and fungal DNA sequences, and induce pro-inflammatory responses in macrophages.

First, measurements of inhalable dust concentrations by static samplings show that mechanical distribution of straw bedding produced a dense dust cloud that lasted approximately 15–20 min in each farm. Dust concentrations (from 21 to 3380 µg/m³) are in the range of those measured by other static samplings in Californian (74–1690 µg/m³) and Swiss (154–4747 µg/m³) dairy farms during a working day inside the cattle building (Cathomas et al., 2002; Garcia et al., 2013a). Using personal samplers placed at breathing zone, Garcia et al. (2013b) compared dust exposure of Californian dairy workers engaged in seven different tasks and concluded that farmers performing re-bedding had

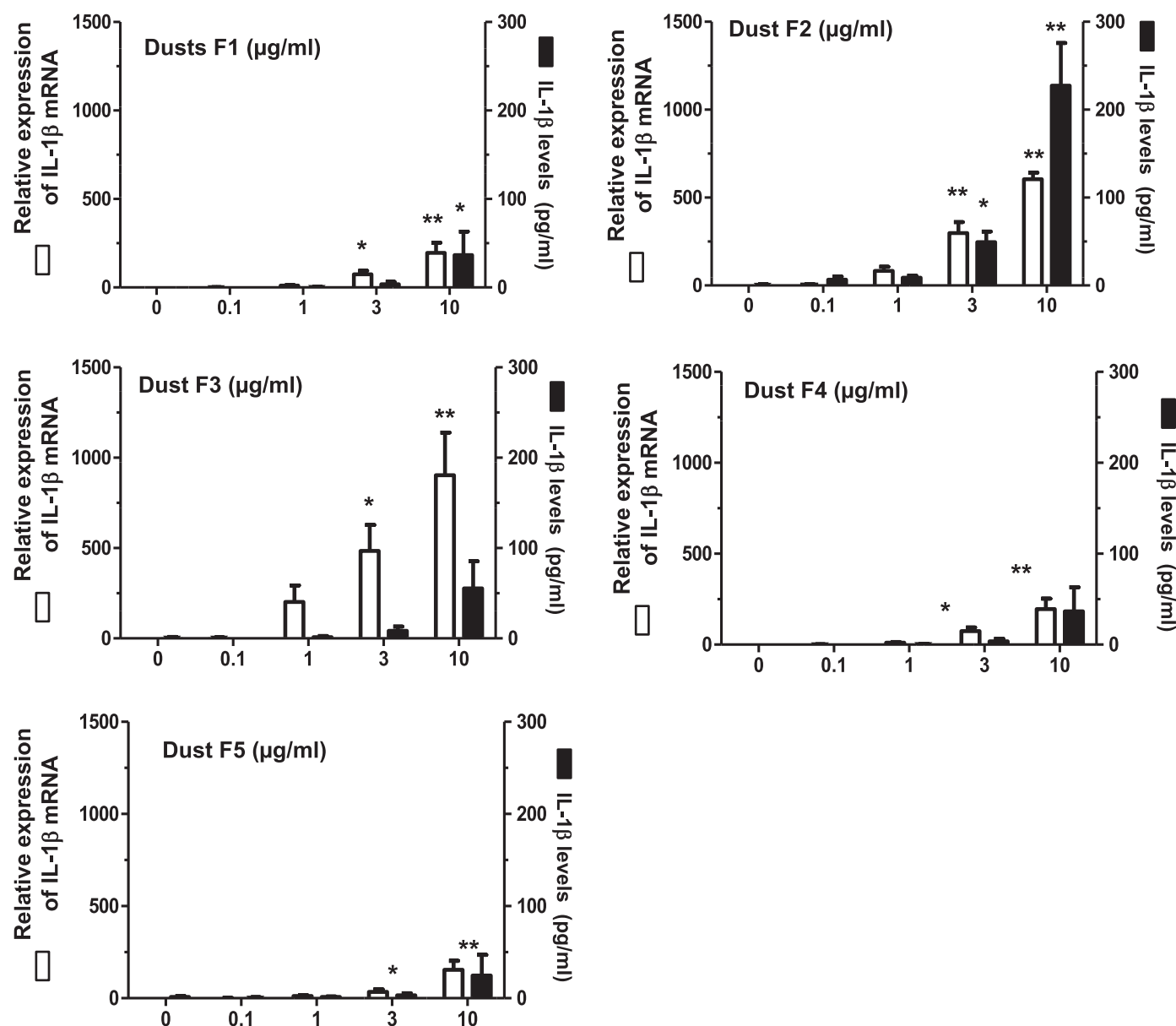


Fig. 6. Effects of dairy dusts on IL-1 β expression in human macrophages. Human monocyte-derived macrophages were exposed for 4 h (mRNA levels) or 24 h (protein secretion) to increasing concentrations of dusts collected in dairy farms F1 to F5. mRNA levels of target genes were determined by quantitative RT-PCR and normalized to endogenous ribosomal 18S RNA levels. Data are expressed relatively to mRNA levels found in “0” control cells not exposed to dusts, arbitrarily set at the value of 1. The levels of IL-1 β secreted in culture medium were quantified by ELISA. All results are expressed as means $\pm$ SD of four independent experiments. * $p < 0.05$, ** $p < 0.01$ versus “0”.

the highest sample mean concentration for inhalable dusts. Similarly, in the Basinas's study, multivariate models indicated that task with the strongest influence on both inhalable dust and endotoxin exposure in Danish cattle barns was the preparation and handling of bedding (Basinas et al., 2014). Altogether these results strengthen the hypothesis that distribution of bedding materials, including straw spreading, is one of the main tasks exposing dairy farmers to inhalable dusts. In addition, we show in the present study i) that thoracic particles steadily constitute the main fraction of inhalable dusts liberated during straw bedding distribution and ii) that dusts are small enough to efficiently penetrate into the tracheobronchial regions, as their masses are mostly grouped in the 3–7.5 μm size intervals. Cathomas et al. (2002) previously reported that the PM₁₀ fraction constituted approximately 63% of the total suspended particles collected during a working day in Swiss cattle barns. However, in this report, the task(s) that specifically produced PM₁₀ could not be determined since dusts were collected on the same filters during the whole activities including milking, feeding, barn

cleaning, manure removal or bedding distribution. Moreover, the specific size distribution of the collected dusts was not assessed. It can be noted that the mass percentages of extra-thoracic particles measured in the five Breton dairy farms are highly variable, although they never exceed those of thoracic dusts. Differences in concentrations of these largest particles may result from the type of shredders used for straw distribution, from adjustments of these machines and/or storage conditions of straw before measurements. Collectively, our results thus demonstrate that the dispersion of straw bedding in the five Breton dairy farms constitutes a critical task that mainly releases small thoracic dusts.

Secondly, using NGS analysis of the bacterial 16S RNAs, we show that thoracic dusts collected during straw spreading are mainly composed of Gram-negative bacteria, especially *Proteobacteria*, in 4 out of 5 samples. The *Proteobacteria* *Sphingomonas* was one of the two most abundant bacteria genera detected in the five dust samples. *Sphingomonas* species are frequently identified in the environment,

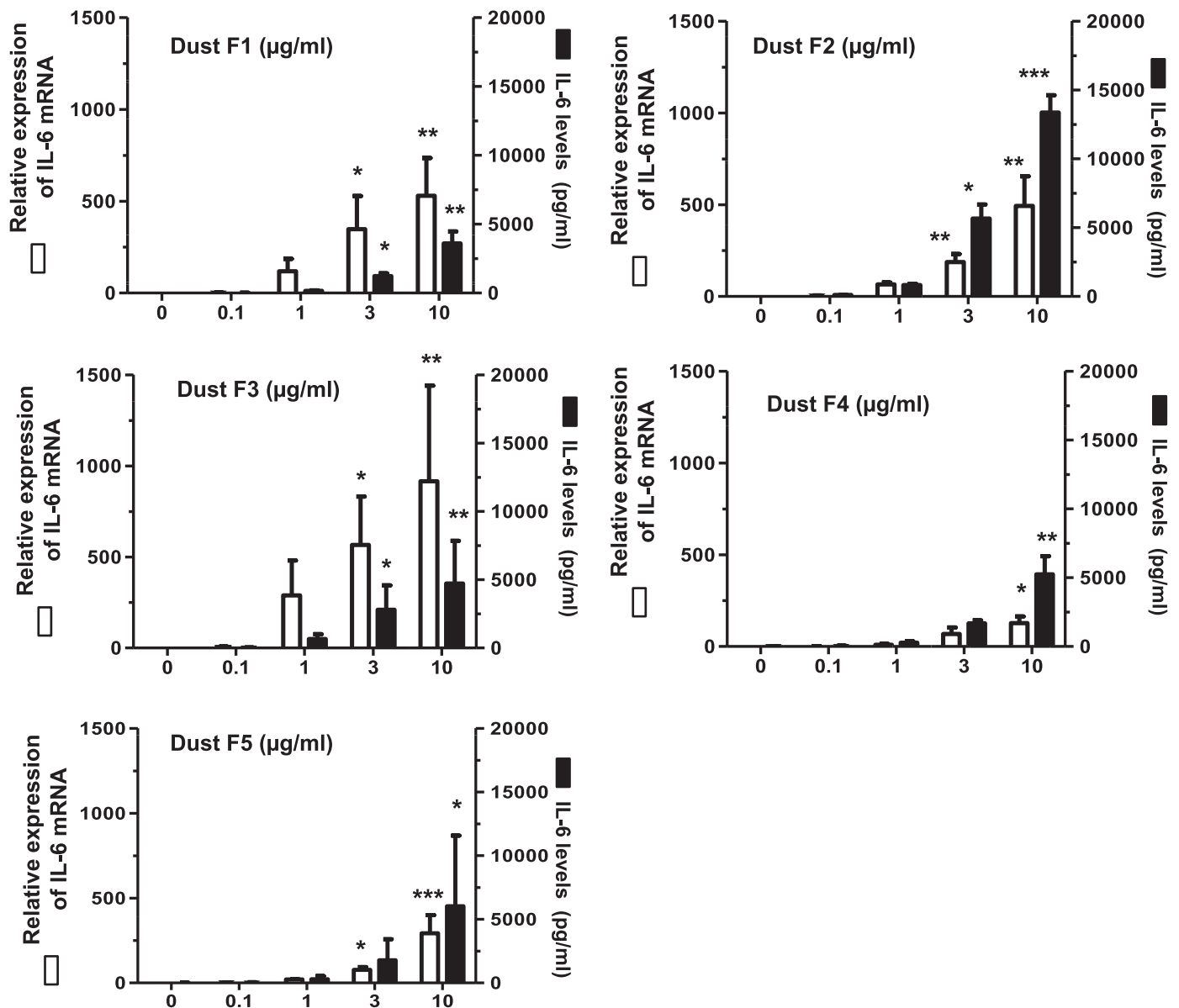


Fig. 7. Effects of dairy dusts on IL-6 expression in human macrophages. Human monocyte-derived macrophages were exposed for 4 h (mRNA levels) or 24 h (protein secretion) to increasing concentrations of dusts collected in dairy farms F1 to F5. mRNA levels of target genes were determined by quantitative RT-PCR and normalized to endogenous ribosomal 18S RNA levels. Data are expressed relatively to mRNA levels found in “0” control cells not exposed to dusts, arbitrarily set at the value of 1. The levels of IL-6 secreted in culture medium were quantified by ELISA. All results are expressed as means $\pm$ SD of four independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus “0”.

especially in soil and water. They are not considered as major infectious agents for humans, although *Sphingomonas paucimobilis* could be an opportunistic pathogen in immunocompromised individuals (Ryan and Adley, 2010). Besides *Proteobacteria*, Gram-positive bacteria still represent 30–40% of all genera identified in inhalable dusts and they largely predominate in the F4 dust sample (60%). *Thermoactinomyces* (Firmicutes) and *Saccharopolyspora* (Actinobacteria) were specifically detected in the top four sequences with RA > 1% in this last sample. The species *Thermoactinomyces vulgaris*, *Thermoactinomyces candidus* and *Saccharopolyspora rectivirgula* (*Micropolyspora faeni*) are frequently detected in dairy farms, notably in those performing straw spreading (Kotimaa et al., 1984, 1991). These bacterial species are known as classical agents causing the hypersensitivity pneumonitis called the Farmer's lung disease, a pathology observed in dairy farming regions (Kotimaa et al., 1984; Cormier et al., 1985; Roussel et al., 2005). Moreover, it was not surprising to identify *Frigoribacterium* as the most abundant Gram-positive genera in all dusts sample since this *Actinobacteria* was first isolated from airborne dusts during the distribution

and handling of feed and bedding in Finnish cattle barns (Kämpfer et al., 2000). As expected, the bacterial patterns found in our dust samples markedly differ from those described in American swine farms which do not use straw bedding. These patterns are dominated by the Gram-positive genera *Actinobacteria* and *Firmicutes* (Boissy et al., 2014). Notably, *Clostridium* and *Lactobacillus* (Firmicutes), the two most abundant genera found in swine dust samples (Boissy et al., 2014), were only detected at very low levels in the dairy dust samples (see Table S3). Although pathogenic species cannot be identified by the NGS methodology, classic pathogenic genera with RA > 1% have been found, such as *Staphylococcus* and *Streptococcus* which are potentially associated with the presence of animals in farms. *Pseudomonas* was also detected in the five dust samples. Acquisition of *Pseudomonas aeruginosa* is known to increase the risk of exacerbation in patients with COPD (Murphy et al., 2008). In addition, *Rhodococcus*, *Stenotrophomonas*, *Acinetobacter* and *Corynebacterium*, measured in low proportions, have been associated with nosocomial respiratory infections and can represent health risk to immunocompromised individuals (Savini et al.,

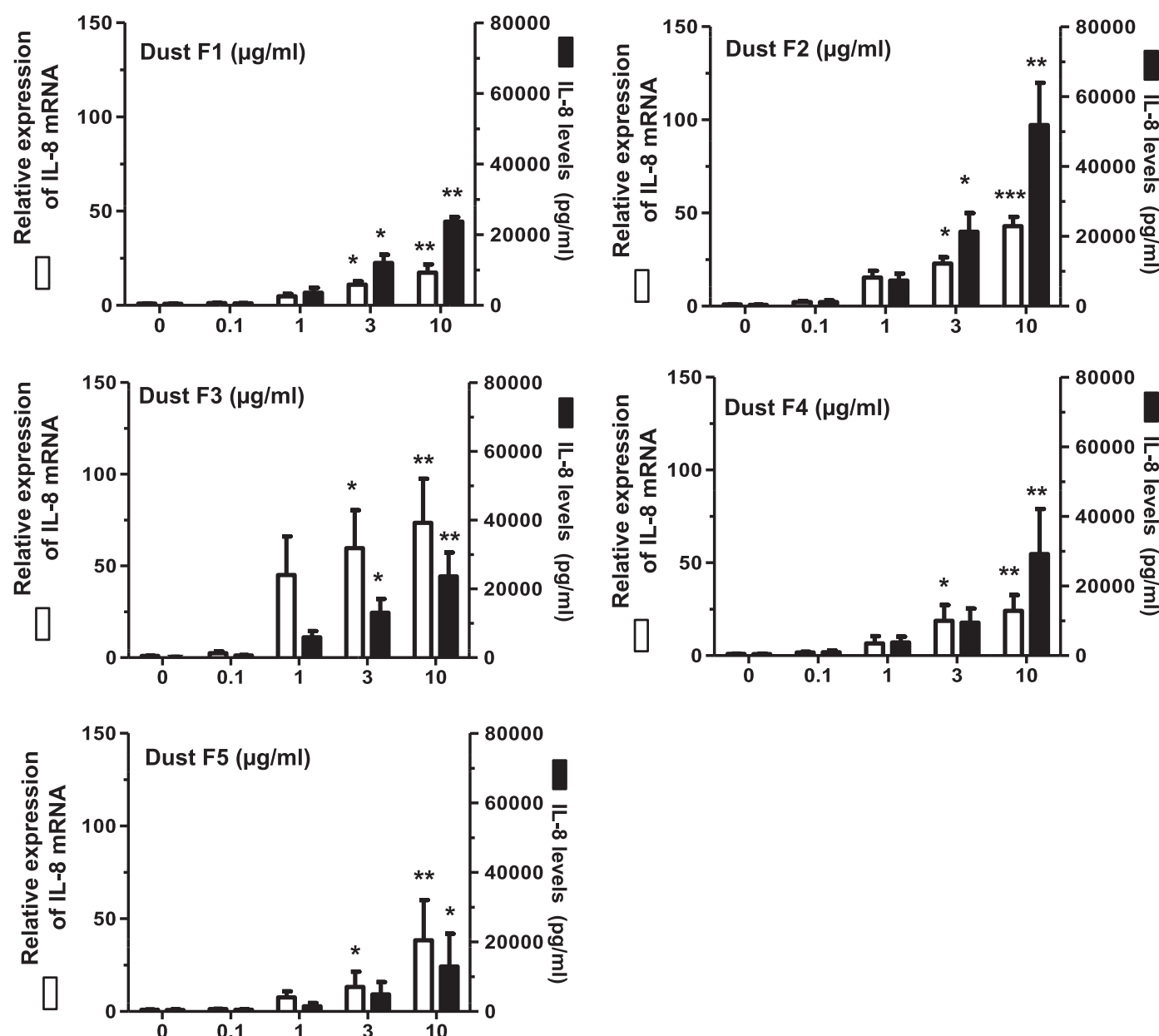


Fig. 8. Effects of dairy dusts on IL-8 expression in human macrophages. Human monocyte-derived macrophages were exposed for 4 h (mRNA levels) or 24 h (protein secretion) to increasing concentrations of dusts collected in dairy farms F1 to F5. mRNA levels of target genes were determined by quantitative RT-PCR and normalized to endogenous ribosomal 18S RNA levels. Data are expressed relatively to mRNA levels found in “0” control cells not exposed to dusts, arbitrarily set at the value of 1. The levels of IL-8 secreted in culture medium were quantified by ELISA. All results are expressed as means $\pm$ SD of four independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus “0”.

2012; Baranzelli et al., 2013).

NGS analysis of 18S RNA shows that the fungal genera *Alternaria* and *Cladosporium* predominate in all dust samples. These species have been associated with an increased risk of airway allergic diseases (Sharpe et al., 2015). Especially, sensitization to *Alternaria alternata* spores, a very common outdoor aeroallergen, constitutes a major risk factor for asthma (Gabriel et al., 2016). *Cladosporium*, but not *Alternaria*, species have also been commonly detected in bedding materials in Finnish dairy farms (Kotimaa et al., 1991; Hanhela et al., 1995).

Other potentially pathogenic genera have been found in some samples. *Wallemia* is notably the most frequently mold detected in samples F4 and F5. In addition to bacterial species, *Wallemia sebi* is a well-known fungal agent classically reported to cause Farmer's lung disease (Reboux et al., 2001). In eastern France, *Eurotium amstelodami*, the teleomorph of *Aspergillus vitis*, and *Absidia corymbifera* are two other molds mainly involved in this hypersensitivity pneumonitis (Reboux

et al., 2001). *Aspergillus* sequences have been detected in the five samples and predominantly in F4 and F5 samples (Table S2). However, sequences related to *Absidia* were not found, even at low levels, in the five dust samples collected in the Breton dairy farms (western France). It is noteworthy that the F4 dust sample is particularly enriched with sequences of *Thermoactinomyces*, *Saccharopolyspora* and *Wallemia*, three microbial agents promoting the Farmer's lung disease.

Cryptococcus, which has been detected in different samples at RA > 1%, has been associated with pneumonia (Brizendine et al., 2011). *Sporobolomyces* species, present at high levels in 3 out of 5 five dust samples, have been exceptionally associated with infection in immunocompetent or immunocompromised patients (McNicholas et al., 2012).

Collectively, the results of NGS analysis show that global bacterial and fungal composition is different for the five dust samples and thus suggest that a specific microbial pattern of dusts released during the

distribution of straw bedding cannot be generalized for Breton dairy farms. Further studies are required to characterize the specific species present in these samples.

In the last part of this study, we investigated the pro-inflammatory responses of human macrophages exposed to the five dust samples. Our results demonstrate that dusts released during straw spreading dose-dependently induced TNF- α , IL-6 and IL-8 expressions at both mRNA and protein levels in primary cultures of human monocyte-derived macrophages. The levels of gene inductions were nevertheless variable probably due to the specific microbial composition of each sample. Particularly, gene expressions always achieved the highest levels in the F2 dust sample in which sequences related to *Hymenobacter* and *Phaeosphaeria* are the most abundant. Although these two genera have never been reported to promote inflammation, their association in the F2 sample, in addition to other microbial agents, may have triggered these marked responses of human macrophages. Previous studies have reported that dusts collected in American dairy farms, or their soluble extracts, also stimulated expression of these three pro-inflammatory genes in human U937 macrophages and monocytes (Poole et al., 2010; Vogel et al., 2012). These dust effects appear to be controlled in part by the transcription factor NF- κ B through endotoxin-dependent activation of TLR4 since endotoxin depletion or TLR4 blocking significantly reduced cytokine expressions (Poole et al., 2010; Vogel et al., 2012). In addition, bacterial peptidoglycan, a major component of Gram-positive bacteria detected in settled dust samples from swine and dairy farms, can contribute to pro-inflammatory gene expression by stimulating TLR2 and nucleotide-binding oligomerization domain 2 (NOD2) signaling pathways (Poole and Romberger, 2012).

Our results show that the five dust samples also induced IL-1 β secretion. Activation of the NALP3 inflammasome allows the caspase-1-dependent processing of pro-IL-1 β and the release of active IL-1 β from macrophages (Martinon et al., 2004). In contrast to the lipopolysaccharide endotoxin, peptidoglycans and its derivative muramyl dipeptide can trigger the inflammasome-dependent secretion of IL-1 β , which secondary promotes induction of TNF- α , IL-6 and IL-8 gene expression in macrophages (Martinon et al., 2004; Hedl and Abraham, 2011). These results thus suggest that both endotoxins and peptidoglycans can mediate pro-inflammatory responses of human macrophages exposed to the thoracic dusts collected in Breton dairy farms.

It should be noted that fungal spores, notably those from *Aspergillus* and *Cladosporium*, *Aspergillus fumigatus* antigens and alternariol, a metabolite produced by *Alternaria*, can induce pro-inflammatory gene expression in monocytes/macrophages (Shahan et al., 1998; Jouneau et al., 2011; Solhaug et al., 2015). Moreover, Bellanger et al. (2011) showed that β -glucans from fungal hyphae of *Eurotium amstelodami* are able to trigger a marked inflammatory response in airway human cells. Consequently, fungal species contained in thoracic dusts may also contribute to pro-inflammatory responses of dust-exposed macrophages.

Finally, as shown in Figs. 5–8, dust effects were detectable in cells treated from 1 μ g/ml or 0.26 μ g/cm². This level of settled dusts was previously reached *in vitro*, using an aerosol-to-cell exposure system, when the mass concentration of dairy parlor PM₁₀ inside the gravity settling chamber was 2.6–3 mg/m³ (Hawley et al., 2015). These aerosol concentrations coincide with those measured in French dairy farms during straw spreading. These observations thus suggest that thoracic dust concentrations used in the present study may be associated with environmentally-relevant atmospheric dust levels.

5. Conclusions

In summary, our results demonstrate that mechanized distribution of straw bedding releases a cloud of organic thoracic dusts that are small enough to reach the bronchial tract where inflammation and chronic obstruction related to COPD or asthma are expected to occur. These dusts can trigger at least *in vitro*, marked pro-inflammatory

responses of macrophages, probably due to their complex organic composition. Distribution of straw bedding can thus constitute a sensitive working task that exposes dairy farmers to biologically active particles. Further investigations are nevertheless required to determine if chronic exposure to these organic particles can stably impair lung functions.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.envres.2017.07.025>.

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Supplementary data

Supplementary data contain two additional Figures and legends (Figures S1 and S2), and four Tables (Table S1 to S4).

Granulometry, microbial composition and biological activity of dusts collected in French dairy farms

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Figure S1. Schematic configuration of sampling in stalls.

Figure S2. Taxonomic classification at Phylum level of the bacterial sequences identified in dust samples F1 to F5. Each pie chart represents the relative abundance of the four main bacterial phyla expressed as percentages of the total number of genera sequences identified in each sample by NGS.

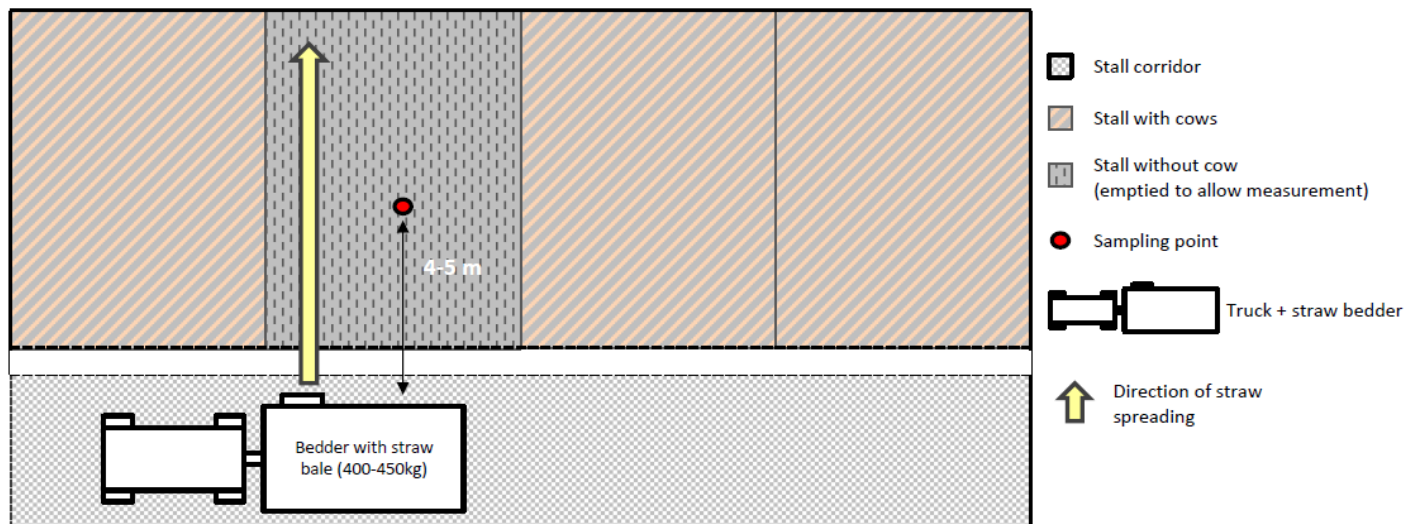


Figure S1

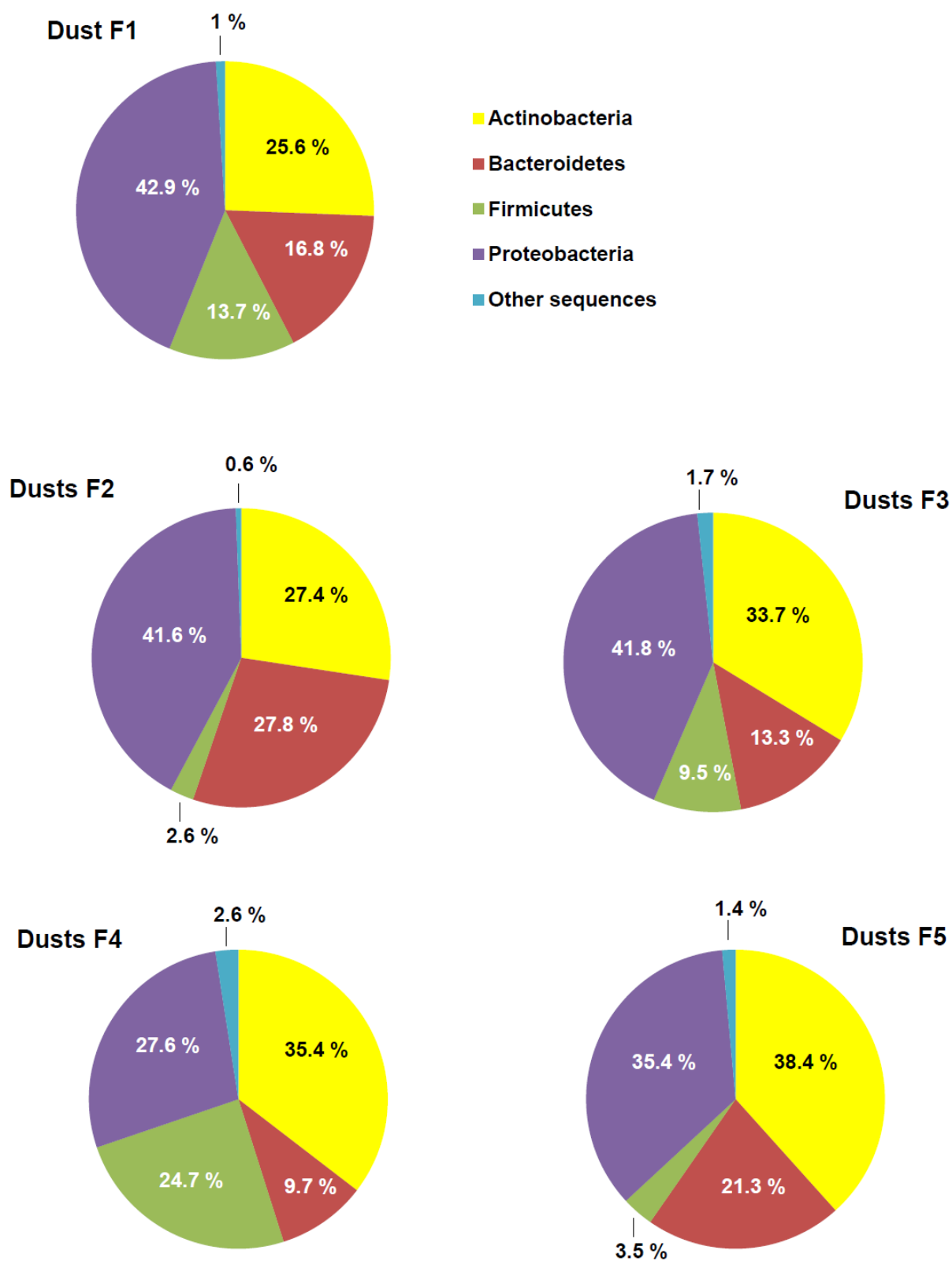


Figure S2