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Factors determining the exposure of dairy farmers to thoracic organic dust

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Abbreviations: MSA: Mutualité Sociale Agricole, GM: geometric mean, TWA : time-weighted average, PPI: parallel particle impactor, CFU: colony-forming unit, LOQ: limit of quantification

Abstract

Bronchial respiratory diseases are more common in dairy farmers than in the general population, perhaps because the repeated inhalation of organic dust contributes to the development of these disorders. However, the factors determining the exposure of farmers to particles that can enter the lower bronchial tract and interact with it, i.e. the thoracic fraction of the inhalable dust, remain to be identified.

We therefore measured the exposure of dairy farmers to thoracic organic dust and identified the farm features and tasks that increased exposure. We measured thoracic particles (n = 110) and farm characteristics and occupational tasks in 29 Brittany dairy farms. The mean (GM) (geometric standard deviation, GSD) concentration of thoracic dust in air inhaled by farmers was 0.24 mg/m^3 (2.8) and the concentrations of endotoxins, Gram-positive bacteria and fungi in the thoracic fraction were 128 EU/m^3 (4.0), 960 CFU/m^3 (6.3) and 690 CFU/m^3 (5.4), respectively. Model-based estimates of the association between exposure, farm features and tasks indicated that manual grain and feed handling and mechanical bedding spreading significantly increased exposure to thoracic dust, endotoxins, bacteria and fungi. Exposure to bacteria and fungi was reduced by cowsheds divided into cubicles, whereas using automatic muck scrappers in alleyway and automatic milking tended to increase exposure to bacteria and endotoxins. Finally, exposure to endotoxin and fungi were reduced by warmer farm buildings and well-ventilated buildings having walls with large openings.

In conclusions, major occupational tasks and specific farm features determine the exposure of Breton dairy farmers to thoracic organic dust.

Keywords: respiratory disease, organic dust, dairy farms, thoracic particles, determinants

1. Introduction

Several studies have shown that dairy farmers are more likely to suffer from respiratory disorders like chronic bronchitis, non-atopic asthma and chronic obstructive pulmonary disease (COPD) than is the general population (Dalphin et al., 1998; Gainet et al., 2007; Omland et al., 2011; Reynolds et al., 2013, Guillien et al., 2016). This increased risk of pulmonary disease may result from repeatedly inhaling organic dust (Eduard et al., 2009; Thaon et al., 2011; Jouneau et al., 2012; Marescaux et al., 2016).

Organic dust contains particles of plant, animal and/or microbial origin (Douwes et al., 2003). The most widely investigated microbial agent in organic dust from dairy farms, is endotoxin, a major component of the outer membrane of Gram-negative bacteria. Lipopolysaccharides, that contain endotoxins and lipoglycans, induce severe inflammation in murine models and can trigger lower respiratory tract symptoms in farmers (Donham et al., 1995; Vogelzang et al., 1998; May et al., 2012). However, components of Gram-positive bacteria (peptidoglycans) and fungi (glucans) are also present in organic dust and probably help trigger chronic inflammation (Larsson et al., 1999; May et al., 2012; Poole and Romberger, 2012).

It is essential to know how much of each type of particle reaches those bronchial areas where COPD, asthma and chronic bronchitis are thought to develop in order to investigate the impacts of exposure on disease development. Particle size is the main factor determining how organic dust interacts with the respiratory tract and only a fraction of inhaled particles is thought to reach the bronchial tract. The European EN481 standard (CEN, 1993) has defined three aerosol fractions linking particle size to the distance particles can penetrate into the respiratory tract. One is the inhalable fraction: particles that can penetrate throughout the respiratory tract, including extra-thoracic particles that are deposited up to the larynx. The second is the thoracic fraction: particles that reach the bronchial region and below. And the third is the respirable fraction: the fraction that can enter the alveolae. The thoracic fraction

thus appears to be the most suitable for describing the exposure of dairy farmers to organic dust.

Most studies on the factors determining the exposure of dairy farmers to bioaerosols have collected inhalable fractions of organic dust while the farmer has been working (Spaan et al., 2006; Samadi et al., 2012; Garcia et al., 2013; Basinas et al., 2014). They have clearly demonstrated that several operations expose dairy farmers to inhalable particles but did not identify the specific tasks that produced the thoracic dust. We have recently shown that 3-10 µm diameter thoracic dust is the main product of mechanical straw spreading (Pfister et al., 2017), a task that greatly increases exposure to inhalable dust (Garcia et al., 2013; Basinas et al., 2014). This suggests that workers on dairy farms are indeed exposed to thoracic dust although the factors determining such exposure remain to be identified.

We therefore measure the exposure of dairy farm workers to thoracic particles, determined the concentrations of endotoxin, culturable bacteria and fungi in these particles, and identified the farm features and tasks that influence exposure to them. We repeatedly measured the quantities and components of thoracic dust around workers on 29 French dairy farms as they carried out specific tasks, the features of these farms and analysed our data using linear and logistic mixed-effect statistical models.

2. Materials and methods

2.1 Sampling

We studied 42 dairy farmers on 29 dairy farms randomly selected from the Breton "Mutualité sociale agricole" (MSA), the French agricultural social security system. All the farms were in the department of Ille-et-Vilaine region of Brittany. Each participating farm was visited three times during the year to cover the various activities and climate changes. Two or three farmers from farms that were not run by a single farmer took part in the study. Measurements were done as defined by the annual calendar of Brittany dairy activities published by the MSA. Winter (November 1 to February 28), during which cows are kept in cowsheds on most farms and involving many activities in barns. Spring (March 15 to May 31), when cows are let out to pasture and farmers prepare for arable crops (seeding, ploughing, fertilising, etc). Summer (July 1 to October 31) when the cows are mostly outdoors and less work is done in barns. Measurements at each period were planned independently of their location or activity and were limited only by the farmer. The study was approved by the local ethics committee (registration number 14.72).

2.2 Exposure monitoring

We performed 3 to 7 measurements on each farm, always in the morning as the activities during the morning and afternoon were generally similar. The samples covered all tasks performed by dairy farmers in the cowshed and outdoors. A total of 37-38 measurements were recorded at each season for a total of 110-112 measurements of thoracic dust, endotoxins, cultivable Gram-positive bacteria and fungi. Each sampling session took 227 min (SD = 71 min) in winter, 216 min (SD = 68 min) in spring and 247 min in summer (SD = 94 min). Thoracic dust, endotoxins, cultivatable bacteria and fungi were collected on 37-mm glass fibre filters (pore size: 0.8 µm; Millipore, Billerica, MA, USA) using a thoracic parallel particle impactor (PPI)-T (Tecora, Paris, France) connected to a sampling pump (Aircheck 2000,

Tecora, Paris, France) operating at 2 L/min (Görner et al., 2017). Airflow was checked before and after sampling. The air inlet of the PPI was attached at shoulder level in the personal breathing zone. The sample-bearing filters were transferred from the PPI to a 37 mm diameter cassette and kept at 4°C for transport to the laboratory. One field negative control and one laboratory negative control were processed with each week's samples.

2.3 Gravimetric analysis and extraction of thoracic dust

The glass fibre filters were weighed before and after sampling. Filters were placed in a controlled environment (35-50% humidity, 18-22°C) overnight prior to each weighing, They were then weighed on an electronic micro-balance (model Precisa 2000, Mettler Toledo, Colombus, Ohio, USA) just before and after dust sampling. The limit of quantification (LOQ) of the method was 0.08 mg per filter. Weights below the LOQ (n=15) were assigned an imputed value following the EN 689 standard (CEN 1996). Microorganisms were extracted from the filters by placing them in 10 mL pyrogen-free water (Lonza, Walkersville, USA) containing 5% Tween-20 **(**Sigma-Aldrich, Saint-Quentin Fallavier, France) immediately after weighing. The filters and extractants were shaken (2000/min) for 1 hr at room temperature and centrifuged at 2000 g for 10 min at 4°C. The resulting pellets were suspended for microbial analyses.

2.4 Endotoxin measurements

Endotoxins were quantified using the kinetic Limulus Amebocyte Lysate test (Lonza, Walkersville, USA). Suitably diluted samples (from 1:1 to 1:1000) were tested in duplicate. According to manufacturer, the LOQ of the method was 0.005 EU/ml. As each filter was extract with 10 ml pyrogen-free water, the final LOQ was 0.05 EU per filter.

2.5 Cultivable bacteria and moulds

Cultivatable bacteria were grown on Trypton soy agar (Biokar, Beauvais, France). Suspensions (100 µl) were tested in duplicate by plating out on agar petri dishes at 1:10, 1:100,

and 1:1000 dilutions and incubating them for 48 hr at 36°C. Gram-positive were discriminated from Gram-negative bacteria by staining with gentian violet, Lugol, 96% alcohol (V/V) and carbol fuchsin reagents (Millipore, Radnor, Pennsylvania, USA). Cultivable fungi and spores were grown on dichloran-glycerol agar (Biokar, Beauvais, France). Duplicate (100 µl) suspensions were plated out on agar (1:10, 1:100, 1:1000 dilutions) and incubated for 3-7 days at 24°C. Colonies were counted on days 3, 5 and 7 after inoculation. As each filter was extracted with 10 ml pyrogen-free water, the LOQ was 100 CFUs per filter for both bacteria and fungi. Values below the LOQ (n = 29 for bacteria and n = 47 for moulds) were assigned an imputed value following the EN 689 standard (CEN, 1996).

2.6 Farms

The selected dairy farms were typical family-run Breton farms; nine were run by a single farmer and the others by 2 to 5 farmers. Our measurements were done on a maximum of 3 farmers per farm. A typical farm had free stalls, a main cowshed for the dairy cows and one to five other compartments for calves or feed storage. The mean number of dairy cows was 78 (SD = 31). The main cowsheds were generally ventilated by opening in the walls. We used two indicators of ventilation: the area of wall opening and the area of wall opening normalised to cowshed floor area.

2.7 Collection of data on determinants

All data were recorded on formatted sheets, prepared according to the specific Breton dairy practises and to the results of previous studies describing the exposure of farmers (Jouneau et al., 2012; Basinas et al., 2014; Samadi et al., 2012; Garcia et al., 2013). The time each farmer took to perform each task during sampling was recorded by the technician (Institut Technique des Gaz et de l'Air, Saint-Grégoire, France) conducting the measurements. Particular attention was paid to the tasks that could be done manually or mechanically using a

tractor. The characteristics of feeding systems, cowsheds, slurry and manure management, and milking methods were also recorded.

2.8 Data analysis

The time taken to complete the morning shift was 210 min, but it varied greatly from one farm to another and between farmers. We therefore expressed the exposure of farmers using the 210-min time-weighted average. Natural logarithms of the time-weighted average (TWA) values were also calculated to normalize the distribution and perform statistical analyses. The geometric mean (GM) and geometric standard deviation (GSD) were used to describe exposure.

2.9 Effects of tasks and farm characteristics on exposure to contaminants

All tasks and farm features with less than 5 observations were excluded from the analyses. The effect of tasks and farm features on exposure to thoracic dust, endotoxins and cultivable bacteria were investigated with mixed-effect linear models. As the number of potential exposure determinants was large, we used a 2-step procedure: (1) For each contaminant, a univariate analysis was performed with each determinant. Farms and their workers were considered to be nested random effects. We used a likelihood ratio test to compare a naïve mixed-effect model (including only random effects) with another model including each determinant as a fixed effect. Determinants with a *P*-value below 0.25 were selected as candidates for multivariate analysis. (2) multivariate linear mixed-effect models were then implemented. The candidate variables selected from the univariate analysis were introduced iteratively following a forward process based on the minimization of the Akaike Information Criterion (AIC). The tasks were treated as quantitative variables, using their duration. Most farms characteristics were treated as dichotomous variables (presence = 1, absence = 0). However, humidity, temperature, number of cows, and area of the wall opening were all treated as quantitative variables using their respective units. The wall opening area,

normalized to cowshed area was used as a quantitative variable or a dichotomous variable (using the 75th percentile value of 0.36m²/m² as cut-off). The variability produced by the multivariate models was calculated to evaluate their quality.

A logistic mixed-effect model was used instead of a linear mixed-effect model because many of the fungus measurements were below the LOQ (n = 47, 41%). Exposure to fungi was recoded as a dichotomous variable using 1000 CFU/m^3 value as cut-off to define exposed (= 1) and unexposed (= 0) farmers. This cut-off was derived from the Swiss guide value for occupational exposure to moulds (SUVA, 2013). The previous procedure was then applied (univariate selection followed by the iterative forward procedure) to investigate the potential impact of determinants on exposure to fungi. The area under the ROC curve was used to assess the discriminating ability of the multivariate logistic model. All statistical analyses were performed using the R package lme4 (Bates et al., 2014) and ROCR (Sing et al., 2005).

Possible correlations between tasks and farm features were determined prior to modelling by calculating the Spearman correlation coefficients.

3. Results

3.1 Farm features and tasks performed by workers

The quantitative features of the 29 dairy farms that took part in the study are summarized in Table 1a and the qualitative farm features and tasks, together with their relative frequencies are listed in Tables 1b and 2. The corn (maize) silage or the corn silage/haylage mix used as fodder on most dairy farms was distributed in the cowshed. Two farms used a fodder system composed of only raw grassland or haylage. Fodder was usually distributed mechanically using a tractor. Grain or dry compounds, mixed manually or mechanically with silage in a feed mixer, were also frequently added to feed. Most farmers kept their cows feeding out in meadows as long as the weather was favourable, but cows were permanently housed in cowsheds on farms equipped with an automatic milking system. Cows were milked manually in herringbone parlours on 25 farms. The other farms had automatic milking systems. The main bedding was straw on 26 farms and sawdust or shredded straw on the other three.

3.2 Exposure to thoracic dust, endotoxins and microbial agents.

Table 3 shows the mean amounts of thoracic dust, endotoxins, bacteria and fungi collected on the 29 farms. Quantifiable amounts of Gram-negative bacteria were found in only two samples and are thus not shown. The exposure to the three microbial contaminants varied according to the season. The exposure to thoracic dust in winter and spring were very similar but exposure was higher in summer. Most endotoxins were inhaled in winter, with less in spring and the least in summer; it thus tended to decrease as the temperature increased. In contrast, exposure to Gram-positive bacteria and fungi was highest in summer and lowest in spring. The numbers of values below the LOQ were also greatest in spring.

3.3 Univariate statistical analysis

The results of the univariate statistical analysis assessing the effects of tasks and farm features on the exposure of farmers are summarized in Tables S1 and S2. Only results with *p*-values below 0.25 were used for multivariate analysis as explained in Materials and Methods.

3.4 Determinants of exposure to thoracic dust, endotoxins and Gram-positive bacteria

The results of the multivariate linear mixed-effect models for exposure to thoracic dust, endotoxins and cultivable Gram-positive bacteria are shown in Table 4. The hierarchical sampling design produced three components of variance: "between-farms", "between-workers" and "within-worker" (day-to-day). The "within-worker" variance was the dominant variance of exposure to thoracic dust, endotoxins and Gram-positive bacteria, accounting for 69%, 60% and 76% of their respective variations. The "between-farms" and "between-workers" components accounted for 5%, 15% and 24%, and 26%, 25% and 0% of the variations in exposure to thoracic dust, endotoxins and bacteria, respectively.

The multivariate models used five tasks, of which grain or feed handling and mechanical bedding spreading were significant exposing tasks common to the three models. The 10-min mechanical bedding spreading increased exposure to thoracic dust by 60%, to endotoxins by 120%, and to Gram-positive bacteria by 46%. Ploughing and hay handling increased exposure to dust and Gram-positive bacteria by over 90%. More generally, handling grain, silage, bedding and slurry were all major contributors to exposure to airborne contaminant. The models also contained three to four farm features depending on the contaminant. The only characteristic that reduced exposure to all three contaminants was the systematic use of grass for feed. Exposure to Gram-positive bacteria was 70% lower in cowsheds with cubicles than in those with deep litter pens. Main cowsheds with large wall openings (> 0.36 m^2/m^2) reduced exposure to dust, but the reduction was not statistically significant. In contrast, automated milking systems increased exposure to endotoxins and gram positive bacteria with over 150%. In addition, storing manure in the cowshed and using an automatic scrapper in alleyways increased exposure to endotoxin by 126% and to Gram-positive bacteria by 149%. Increasing

the number of cows housed in cowsheds also significantly enhanced exposure to Gram-positive bacteria. Finally, environmental parameters influenced exposure to thoracic dust and endotoxins. A 15% increase in relative humidity (corresponding to the interquartile range value) reduced exposure to thoracic dust (by 21%), as did a 10°C increase in temperature (corresponding to the interquartile range value) for exposure to endotoxin (by 45%).

Their respective multivariate models explain almost half the variations in exposure to thoracic dust (46%), endotoxin (48%) and Gram-positive bacteria (40%) (Table 4). The "between farms" variability of the three airborne contaminants was totally explained by the multivariate models, as was the "between workers" variance for exposure to dust. These models accounted for 22% (dust), 45% (endotoxin), and 22% (bacteria) of "within farmer" variations in exposure.

3.5 Determinants of exposure to fungi

The determinants affecting exposure to fungi (> 1000 CFU/m^3) were assessed using a multivariate logistic mixed-effect model because so many of these values were below the LOQ. Many determinants increased the probability of exposure to fungi on dairy farms (Table 5). Handling grain, silage, bedding and slurry/manure probably promoted exposure, as for the other contaminants. The 10-min mechanical spreading of bedding had the greatest effect; it should increase chance of exposure to fungi by an OR of 3.16. Mechanical manure scraping and manual spreading of bedding also increased exposure to fungal contaminants but only marginally.

Workers who mainly fed cows with corn silage were more likely to be at risk of exposure to fungi/mould (OR = 3.81) than their counterparts who used a fodder system (grass or mix grass + corn silage). In contrast, workers on farms with cowsheds with cubicles were less likely to be exposed to moulds (OR = 0.25) than those on farms with other housing systems. Lastly, a 100 m² increase in wall opening was linked to much lower exposure to fungi (OR= 0.24).

Finally, the area under the ROC curve, used to determine the accuracy of the multivariate logistic mixed-effect model, was 0.86, indicating that the model was highly discriminatory.

4. Discussion

Our Breton dairy farmers were exposed to thoracic dust containing endotoxins, cultivable Gram-positive bacteria and fungi. We measured the types and durations of occupational activities to describe precisely the factors determining exposure and analysed the findings with mixed-effect linear and logistic regression models to show that exposure to thoracic organic dust was mainly controlled by specific occupational tasks and farm features.

These Breton dairy farmers were exposed to concentrations of thoracic dust (GM = 240 µg/m^3) three to four times lower than the dust (GM = 812 to 1000 µg/m^3) inhaled by American or other European dairy farmers (Basinas et al., 2014; Garcia et al., 2013; Samadi et al., 2012). Thus, thoracic dust might not account for most of the particles inhaled by Breton dairy farmers during their work. However, their levels are substantially higher than respiratory dust ($PM_{2.5}$) levels (GM = 35.3 to 48 µg/m^3) found in Californian dairies (Garcia et al., 2013). The particles in thoracic dusts may thus be small enough for them to be deposited in the bronchial region but not in the alveolae. This is supported by the results of our study showing that spreading straw bedding in Breton dairy farms mainly released organic dust particles with diameters of 3 to 10 µm, which are unlikely to enter the alveolae (Pfister et al., 2017). The endotoxin concentrations (GM = 128 EU/m^3) in the thoracic dust collected on Breton dairy farmers were also lower than those in inhalable dusts in Californian, Dutch or Danish barns (GM = 329-453 EU/m^3) (Basinas et al., 2012, 2014; Garcia et al., 2013; Samadi et al., 2012). Nevertheless, thoracic dust collected on Breton dairies contained more endotoxin than that calculated for inhalable dusts from other dairy farms when the endotoxin concentrations are normalized to mg/dust. We found 512 EU/mg endotoxins in our thoracic dust, while it was 360 EU/mg inhalable dust from Danish dairy farms (Basinas et al., 2014). These results agree with those of Madsen and Nielsen (2010) who reported that thoracic dust from straw storage halls at power plants contained more endotoxins than did inhalable dusts (Madsen and Nielsen, 2010). In addition, the concentrations of cultivable Gram-positive bacteria (9.6E+02 CFU/m^3) and fungi (6.9E+02

CFU/m^3) in thoracic dust were about one order of magnitude lower than those in inhalable dust from Dutch dairy barns (1.11E+02 and 2.37E+03 CFU/m^3) (Samadi et al., 2012). These differences may be linked to the type of bedding and/or feed used by Breton and other workers (Samadi et al., 2012). The concentrations of both cultivable Gram-positive bacteria and fungi also varied widely, perhaps due to the poor survival of some microorganisms. Gram-negative bacteria were detected in only two of our 110 dust samples. This is not surprising since cultivable Gram-negative bacteria collected in cow and calf sheds have short survival periods (Clark et al., 1983; Zucker et al., 2000). However, we recently sequenced bacterial ribosomal 16S-RNA and found that two Gram-negative phyla *Proteobacteria* and *Bacteroides* accounted for the majority of bacteria in thoracic dust released from straw bedding in four of five Breton dairy farms (Pfister et al., 2017). We also measured significant amounts of endotoxins in most samples. Thus, Gram-negative bacteria were widespread in thoracic dust collected on Breton dairy farms, although they probably could not be cultivated.

Our results showed that the variability in exposure to thoracic dust was mainly due to the "within-worker" variance component (Table 4), as observed by Basinas et al. (2014). However, the variance components accounting for the variations in total dust (69%) and endotoxin (60%) in our models are higher than those measured by Basinas et al. (2014). These differences may arise because the activities of Breton farmers frequently changed from day to day, while Danish dairy farmers perform similar tasks every day. The variability in exposure to Gram-positive bacteria is also mainly due to "within-worker" variance. In contrast, the model on dust exposure explained all (100 %) the "between workers" variability. This variability was however small and accounts for only 26 % of the total variance in the naïve model for dust. This indicates that there was little difference in the exposure of workers to dust on our 29 dairy farms. Our mixed-effect linear model describes 40% to 48% of the overall variability in exposure of the investigated airborne contaminants. The accuracy of our predictive model is probably due to the methodology used. The nature and duration of the tasks performed by the

farmers were recorded directly during dust sampling, unlike the protocols of previous studies in which task information was recorded retrospectively during a post-sampling interview. Our direct on-farm observations may have provided more accurate measurements of task times. Nevertheless, a substantial part of the overall variability remained unexplained due to unrecorded parameters like specific feeding practices or stable management, physicochemical characteristics of grain or straw, and weather conditions. Another factor may be the mathematical model misspecification inherent to the use of log TWA-exposure in the present statistical analysis (Burstyn, 2009). Indeed, this model implied that the duration of each task had an exponential effect on the untransformed TWA-exposure. Since it is admitted that the mean exposure level during a task is constant, the impact of the duration of a task on the TWA-exposure is thus linear and not exponential (Burstyn, 2009). Consequently, this model may not correctly adjust to some measurement values.

The mixed-effect linear and logistic model results suggest that specific tasks and farm features markedly increased exposure to dust and microorganisms. They confirm the effects of grain handling and bedding-related tasks on exposure to thoracic dust and endotoxins. These tasks were previously identified as the greatest contributors to inhalable dust and endotoxins in dairy and pig farms (Garcia et al., 2013; Basinas et al., 2013; Basinas et al., 2014). Our data also show that they contributed to exposure to the Gram-positive bacteria and fungi in the thoracic fractions of inhaled dust. Silage handling was particularly associated with increased probability of exposure to fungi, which suggests that silage is an important source of fungal contaminants.

We found that a higher outdoor temperature was associated with a lower exposure to thoracic endotoxins, in agreement with the findings for inhalable endotoxins on dairy and pig farms (Preller et al., 1995; Basinas et al., 2013, 2014, 2017). This is probably because thoracic dust contains more endotoxins in cold weather (winter) than when it is warm (summer). The endotoxin content in thoracic dust were 714 EU/mg in winter, 547 EU/mg in spring and 393

EU/mg, in summer (data not shown). And exposure to dust varied inversely with the outdoor humidity, perhaps because a high humidity seems to limit the aerosolisation of particles released during work, so reducing exposure to thoracic dust. A similar association was found on Estonian dairy farms (Kaasik and Maasikmets, 2013). Greater areas of open walls, which favour stable ventilation, was associated with a much lower probability of exposure to fungi (OR = 0.24) and better ventilation is also likely to reduce indoor humidity, producing less favourable conditions for mould growth.

Our data also show a positive, very significant association between the use of an automatic milking system and exposure to endotoxins (change factor = 3.50, $p < 0.007$), in agreement with the report for inhalable endotoxins on Danish farms (Basinas et al., 2014). These authors suggest that working with milking robots makes it possible to have more cows per farm, so increasing the time spent by the farmer doing more exposing work. This is supported by our finding of a significant positive correlation between the presence of an automatic milking system and the time spent manually spreading straw ($r = 0.63$, $p < 0.001$, data not shown). The presence of milking robots also required cows to be kept in the main cowshed, which probably increased the volumes of manure or slurry and the production of particles heavily contaminated with endotoxins when the cows moved.

The mechanical scraping of slurry and manure also had a major influence on exposure to both endotoxins and Gram-positive bacteria, while having a manure storage pit in the cowshed significantly enhanced exposure to endotoxins. As most of the endotoxins in manure are thought to come from anaerobic Gram-negative bacteria (Zucker et al., 2000), the solid structure of manure and the unstirred nature of the manure in storage pits could foster the growth of anaerobic Gram-negative bacteria, so increasing the endotoxin concentration. Having an automatic scrapper in alleyways also seems to enhance exposure to Gram-positive bacteria, while the repeated scraping of alleyways could help produce small particles of fresh slurry contaminated with Gram-positive bacteria. In contrast, cowsheds divided into stalls had

much lower (70-75%) Gram-positive bacteria and mould concentrations. Cows housed in cubicles were usually cleaner and their movements should result in fewer suspended particles contaminated with bacteria and mould than those of cows in deep litter pens.

But the factors determining increased thoracic dust and exposure to endotoxins in our study were not always the same as those identified in previous similar studies. Basinas et al. (2014) showed that milking was positively correlated with exposure to inhalable endotoxins, whereas our multivariate linear mixed-effect model did not identify milking. Forcing the duration of milking in our thoracic endotoxins model resulted in a very low, non-significant correlation ($\beta = 0.0002$, $p = 0.91$), without substantially modifying the impact of other determinants (data not shown). Basinas et al. (2014) also reported that using an automatic scrapper in alleyways reduced exposure to inhalable dust by 40%, while we find that this device had no effect on exposure to thoracic dust on Breton dairy farms and increased exposure to Gram-positive bacteria. These differences are probably due to the different particle fractions (thoracic/ inhalable) sampled. Inhalable dust may contain mostly extra-thoracic particles that cannot be collected by thoracic samplers, as reported recently in a study showing that most of the particles produced during milking are extra-thoracic particles (Schaeffer et al., 2017). Farm tasks or features that selectively increase the production of extra-thoracic dust are not selected by multivariate models investigating the determinants of exposure to thoracic dust. Moreover, extra-thoracic particles are unlikely to enter the bronchio-tracheal regions where COPD and asthma are believed to develop. Thus, the results of studies investigating the risk factors for lower airway diseases like COPD and asthma may be misinterpreted if the proportions of extra-thoracic and thoracic fractions in inhalable dust are not determined after sampling, especially when the inhalable dust is mainly made up of extra-thoracic dust. In contrast, studying the thoracic fraction may strengthen the specificity and relevance of determinants correlated with exposure to organic dust. Therefore, it may be essential to compare directly the

factors determining exposure to inhalable and thoracic dusts collected on a particular dairy farm. The main limiting factor of this work may be that we did not do these comparisons.

5. Conclusions

Our study of the exposure of dairy farmers to the thoracic fraction of inhalable organic dust and their quantitative and qualitative determinants shows that the mechanical handling of bedding, grain and dry feed markedly increased the exposure of farmers to thoracic dust, endotoxins, Gram-positive bacteria and fungal material. However, certain farm features, such as the presence of cubicles in the main cowshed and large open wall areas can result in much lower exposure to these harmful agents. Further studies are required to confirm the roles of these determinants and to identify strategies that protect dairy farmers from exposure to organic dusts. Lastly, it is essential to determine whether exposure to thoracic dust is correlated with a dairy farmer's respiratory status.

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Table 1a : Quantitative characteristics of the 29 diary farms

Stable characteristics	Mean	Median (min-max)
Dimension		
Length (m)	48.93	45.5 (23 - 87)
Width (m)	20.23	19.25 (11 - 37)
Surface (m^2)	935	870 (207 - 2554)
Number of cows	77.77	75 (20 - 160)
Surface area per cow (m^2/cow)	11.9	10.61 (6.5 - 23)
Ventilation estimate		
Surface of wall opening (m^2)	161	131 (15 - 340)
SWON (m^2/m^2) [a]	0.24	0.18 (0.02 - 1.2)
Environmental parameters		
Outdoor temperature (°C)	12.42	13 (-2.7 - 26)
Outdoor relative hygrometry (%)	76.65	77.3 (42.5 - 100)

[a] SWON = Surface of wall opening normalized on stable surface

Table 1b : Occurrence of qualitative farm characteristics based on 112 measurements from workers of main stables

Stable characteristics	N[a]
Fodder	
Fodder = mainly corn silage	59
Fodder = mix of corn silage + haylage	47
Fodder = only haylage or grass	6
Feeding system	
Presence of an automatic rail dispenser	4
Grazing	
Cows going to pasture during measurement	65
Milking	
Milking parlor directly connected to stable	67
Automatic milking	11
Usage of high pressure washing at least once a week	75
Stable	
Concrete + deep litter pens	52
Concrete + cubicle	60
Bedding	
Straw	97
No bedding material	6
Saw dust	5
Shredded straw	4
Flooring	
Presence of mattresses	24
Ventilation	
Natural	112
Natural with control device	0
Mechanical without control device	0
Mechanical with control device	0
SWON > 0.36 m^2/m^2 (75^{th} percentile value) [b]	31
Slurry/manure	
Automatic scrapper in alleyway	54
Solid manure pit in stable	48

[a] N = number of observations among the 112 measurements
[b] SWON = Surface of wall opening normalized on stable surface

Table 2 : Occurrence and median duration of tasks performed by dairy workers based on 112 measurements

Tasks	Occurrence	Time (range) in min [c]
Milking (including gathering cows)	73	60 (10 - 195)
Milking parlor washing	57	15 (3 - 39)
Calf feeding with milk	25	13 (2 - 150)
Moving animals to meadow	13	10 (2 - 36)
Moving animals in stable	25	10 (4 - 35)
Calving	1	60 (60 - 60)
Ear marking/inseminating/cleaning cow feet/injecting	16	14 (2 - 215)
Displacing dead animals	0	-
Manual loading/unloading of hay [a]	18	4.5 (2 - 17)
Mechanical loading/unloading of hay [b]	1	8 (8 - 8)
Manual loading/unloading of silage [a]	29	5 (2 - 120)
Mechanical loading/unloading of silage [b]	44	14.5 (3 - 145)
Manual loading/unloading of grain or dry feed [a]	57	5 (1 - 56)
Mechanical loading/unloading grain or dry feed [b]	11	6 (1 - 43)
Grain grinding	4	5.5 (2 - 19)
Manual spreading of bedding materials [a]	38	10 (1 - 56)
Mechanical spreading of bedding materials [b]	33	10 (2 - 28)
Manual scrapping of slurry/manure [a]	19	10 (3 - 120)
Mechanical scrapping of slurry/manure [b]	18	14 (5 - 65)
Manual scrapping of stable corridor [a]	12	9 (1 - 28)
Truck maintenance	13	14 (5 - 30)
Harvesting	0	-
Spreading fertilizer or pesticide on field	6	110.5 (22 - 185)
Soil preparation	6	136.5 (112 - 160)
Displacement: walking inside building [d]	80	25 (5 - 310)
Displacement: walking outside building [d]	75	20 (4 - 206)
Displacement: by truck [d]	36	26 (5- 205)

[a] manual = using a shovel or a bucket or both. Do not include transport time
[b] mechanical = using truck + adequate trailer. Do not include transport time
[c] median, min and max time of positive values.
[d] refers to times spent by the farmers between their tasks. Include transport time.

Table 3: Arithmetic Mean (AM), Geometric Mean (GM) and Geometric Standard Deviation (GSD) of personal exposure levels to the thoracic fraction of dusts, endotoxins, Gram-positive bacteria and mould. Limit of quantification (LOQ)

Period	**Dust (mg/m³)**				**Endotoxins (EU/m³)**			
	n (n<LOQ)	AM	GM (GSD)	Range	n (n<LOQ)	AM	GM (GSD)	Range
Overall	**112 (25)**	**0.42**	**0.24 (2.8)**	**LOQ - 5.13**	**110 (0)**	**318**	**128 (4.0)**	**2 - 8672**
Winter	37 (4)	0.38	0.22 (2.9)	LOQ - 3.54	37 (0)	304	159 (3.7)	4 - 1136
Spring	38 (14)	0.37	0.23 (2.6)	LOQ - 2.24	37 (0)	239	125 (3.7)	2 - 1184
Summer	37 (7)	0.53	0.27 (3.0)	LOQ - 5.13	36 (0)	413	104 (4.6)	3 - 8672
Period	**Gram-positive bacteria (CFU/m³)**				**Mould (CFU/m³)**			
	n (n<LOQ)	AM	GM (GSD)	Range	n (n<LOQ)	AM	GM (GSD)	Range
Overall	**110 (29)**	**1.1E+04**	**9.6E+02 (6.3)**	**LOQ - 5.5E+05**	**110 (47)**	**6.1E+03**	**6.9E+02 (5.4)**	**LOQ - 1.9E+04**
Winter	37 (11)	2.7E+03	8.7E+02 (4.9)	LOQ - 3.0E+04	37 (17)	4.8E+03	7.3E+02 (5.3)	LOQ - 6.4E+04
Spring	37 (12)	1.0E+04	7.6E+02 (6.8)	LOQ - 2.8E+05	37 (23)	5.2E+02	3.2E+02 (2.1)	LOQ - 4.9E+03
Summer	36 (6)	1.9E+04	1.3E+03 (7.4)	LOQ - 5.5E+05	36 (7)	1.3E+04	1.4E+03 (7.7)	LOQ - 1.9E+04

Table 4: Multivariate mixed-effect linear model analysing the effects of tasks and farm characteristics on the log-transformed personal exposure levels to the thoracic fraction of dusts, endotoxins and Gram-positive bacteria. Blank spaces indicate that the determinant was not selected in model.

Determinants	Dusts (n = 112)			Endotoxins (n = 110)			Gram-positive bacteria (n = 110)		
	β (se)	Change factor [a]	*P*-value	β (se)	Change factor [a]	*P*-value	β (se)	Change factor [a]	*P*-value
Background level	-0.478 (0.61)	0.62	0.435	4.314 (0.234)	75	<0.001	5.14 (0.489)	171	<0.001
Tasks (min)									
Manual loading/unloading of hay				0.042 (0.032)	1.23 [b]	0.198	0.132 (0.054)	1.93 [b]	0.016
Manual loading/unloading of grain or dry feeds	0.068 (0.010)	1.40 [b]	<0.001	0.075 (0.013)	1.45[b]	<0.001	0.085 (0.022)	1.53 [b]	<0.001
Mechanical loading/unloading of silage	0.010 (0.005)	1.16 [b]	0.030						
Manual spreading of bedding materials	0.021 (0.007)	1.37 [b]	0.004						
Mechanical spreading of bedding materials	0.047 (0.011)	1.60 [b]	<0.001	0.079 (0.015)	2.20 [b]	<0.001	0.038 (0.021)	1.46 [b]	0.07
Mechanical scrapping of slurry/manure				0.024 (0.012)	1.40 [b]	0.047	0.056 (0.019)	2.32 [b]	0.005
Manual scrapping of slurry/manure				0.012 (0.007)	1.13 [b]	0.068			
Soil preparation	0.005 (0.003)	1.97[b]	0.105				-0.009 (0.006)	0.29 [b]	0.141
Farm characteristics (qualitative)									
fodder = only haylage or grass	-0.648 (0.366)	0.523	0.079	-0.876 (0.444)	0.42	0.052	-1.527 (0.746)	0.22	0.043
SWON [d] >0.36m²/m²	-0.292 (0.170)	0.747	0.089						
Cow going to pasture during measurement	-0.240 (0.170)	0.787	0.161						
Automatic milking				1.148 (0.408)	3.15	0.007	0.947 (0.598)	2.58	0.118
Solid manure pit in stable				0.816 (0.242)	2.26	0.001			
Floor = concrete + cubicle							-1.205 (0.370)	0.30	0.002
Presence automatic scrapper in alleyway							0.912 (0.373)	2.49	0.019
Farm characteristics (quantitative)									
Cows number (increment for one cow)							0.015 (0.006)	2.02 [c]	0.017
Environmental parameters									
Temperature (°C)				-0.059 (0.016)	0.55[c]	<0.001			
Hygrometry (%)	-0.016 (0.008)	0.79[c]	0.036						

Table 4: continued

Variance components of naive models [e]	**Dusts**	**Endotoxins**	**Gram-positive bacteria**
$_{bf}\sigma^2$	0.05 (5%)	0.29 (15%)	0.99 (24%)
$_{bw}\sigma^2$	0.28 (26%)	0.49 (25%)	0 (0%)
$_{ww}\sigma^2$	0.73 (69%)	1.16 (60%)	3.10 (76%)
$_{total}\sigma^2$	1.05 (100%)	1.94 (100%)	4.09 (100%)
Variability explained by complete model [f]			
Between farms	100%	100%	100%
Between workers	100%	24%	-
Within worker	22%	45%	22%
Overall	**46%**	**48%**	**40%**

[a] change factor in exposure was calculated as exp(β) if not specified otherwise
[b] change factor in exposure was calculated as exp (β x median time of the task duration).
[c] change factor in exposure for an increment of the interquartile value of the determinants was calculated as exp(βx interquartile value). Interquartile values are 15%, 10°C and 45 cows for relative hygrometry, temperature and cow numbers, respectively
[d] SWON = Surface of wall opening normalized on stable surface
[e] models without fixed effects (bf : between-farm, bw: between-worker, ww: within-worker)
[f] explained variability was calculated as 1-[variance component (complete model)] / [variance component (naive model)]

Table 5: Multivariate mixed-effect logistic model analysing the effects of performed tasks and farm characteristics on the probability of mould exposure > 1000 CFU/m^3 with n = 112 and 32 measurements > 1000 CFU/m^3 (28.5%)

Determinants	**β (se)**	**OR (CI 95%)[a]**	***P*-value**
Background level	-1.840 (0.717)		0.010
Tasks (min)			
Manual loading/unloading grain of dry feed	0.131 (0.053)	1.93 (1.31 - 3.24) [b]	0.014
Manual loading/unloading of silage	0.059 (0.029)	1.34 (1.02 - 3.18) [b]	0.040
Manuel spreading of bedding materials	0.054 (0.029)	1.72 (0.97 - 3.03) [b]	0.065
Mechanical spreading of bedding materials	0.115 (0.038)	3.16 (1.50 - 6.65) [b]	0.003
Mechanical scrapping of slurry/manure	0.054 (0.03)	2.13 (0.94 - 4.85) [b]	0.073
Farm characteristics (presence of absence)			
Fodder = mainly corn silage	1.337 (0.651)	3.81 (1.84 - 13.64)	0.040
Floor = concrete + cubicle	-1.387 (0.667)	0.25 (0.07 - 0.92)	0.038
Farm characteristics (quantitative)			
Surface of wall opening (m²)	-0.006 (0.003)	0.24 (0.3 - 0.99) [c]	0.036
Model evaluation			
Area under ROC curve	0,89		

[a] Odd Ratio was calculated as exp(β) if not specified otherwise
[b] Odd Ratio was calculated as exp (β x median time of task duration).
[c] Odd Ratio for an increment of 100 m² was calculated as exp(β x 100).

Table S1: Univariate effects of task duration on log-transformed personal exposure levels to the thoracic fraction of dusts, endotoxins and Gram-positive bacteria.

Determinants	Dust[a]		Endotoxins[a]		Gram-positive bacteria[a]		mould[b]	
Tasks (min)	**β**	***P*-value[c]**	**β**	***P*-value[c]**	**β**	***P*-value[c]**	**β**	***P*-value[c]**
Milking	-0.006	0.006	-0.005	0.087	-0.005	0.178		
Milking room washing	-0.014	0.212	-0.018	0.21				
Manual loading/unloading hay			0.065	0.119			0.102	0.163
Manual loading/unloading of grain or dry feed	0.057	<0.001	0.056	<0.001	0.077	<0.001	0.105	0.004
Manual loading/unloading of silage							0.072	0.021
Mechanical loading/unloading of silage	0.011	0.047					0.018	0.201
Manual spreading of bedding materials	0.017	0.061	0.034	0.005	0.036	0.056	0.049	0.070
Mechanical spreading of bedding materials	0.032	0.027	0.075	<0.001	0.043	0.080	0.093	<0.001
Manual scrapping of slurry/manure			0.019	0.036				
Mechanical scrapping of slurry/manure			0.025	0.119	0.048	0.023	0.058	0.077
Truck maintenance	-0.018	0.227					-0.086	0.207
Spreading of fertilizer or pesticides							0.023	0.022
Soil preparation	0.006	0.082			-0.009	0.181		

[a] effect assessed in mixed-effect linear model. *P*-values are associated to likelihood ratio test (naïve model vs bivariate model)
[b] effect assessed in mixed effect logistic model. *P*-values are associated to likelihood ratio test (naïve model vs bivariate model)
[c] only *P*-values < 0,25 are shown

Table S2 : Univariate effects of farm characteristics on log-transformed personal exposure levels to the thoracic fraction of dusts, endotoxins, Gram-positive bacteria and mould.

Farm characteristics	Dust[a]		Endotoxins[a]		Gram-positive Bacteria[a]		Mould[b]	
	β	*P*-value[a]	β	*P*-value[a]	β	*P*-value[a]	β	*P*-value[a]
Qualitative								
Floor = concrete + cubicle					-0.678	0.167	-0.852	0.085
Floor = concrete + mattresses					-0.737	0.234	-1.07	0.085
Automatic milking	0.80	0.032	0.998	0.059 .	2.34	< 0.001	2.215	0.001
Milking parlor directly connected to stable	0.34	0.143						
Usage of high pressure washing							-0.671	0.134
Automatic scrapper in alleyways					0.71	0.132		
Manure storage in stable			0.829	0.011				
Cows pasturing at least a part of the day	-0.297	0.114	-0.516	0.045			-0.665	0.04
Fodder = only haylage or grass	-0.814	0.069	-1.962	0.002	-1.671	0.035		
Fodder = mainly corn silage							-0.931	0.014
SWON > 0.36	-0.410	0.114						
Quantitative								
Cow number					0.012	0.129		
Surface of wall opening (per $10m^2$)							-0.044	0.055
SWON > (m^2/m^2)[d]	-0.555	0.241						
Environmental parameters								
Hygrometry (%)	-0.007	0.243					0.195	0.006
Temperature (°C)			-0.044	0.014				

[a] effect assessed in linear mixed effect model. *P*-value associated to likelihood ratio test (naïve model vs bivariate model)
[b] effect assessed in logistic mixed effect model. *P*-value associated to likelihood ratio test (naïve model vs bivariate model)
[c] only *P*-values < 0.25 are shown
[d] SWON = Surface of wall opening normalized on stable surface