Assessment of Microbiological Indoor Air Quality in Cattle Breeding Farms

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ABSTRACT

This study, which was conducted on a farm in central Poland from February till April 2019, aimed to evaluate microbiological threats on cattle breeding premises by counting and identifying the microorganisms (via culturing and high-throughput sequencing), assessing the endotoxin concentrations (via gas chromatography-mass spectrometry [GC-MS]) and analysing the secondary metabolites (including mycotoxins) in the air and sedimented dust (via liquid chromatography-tandem mass spectrometry [LC-MS/MS]) in six barns. In addition, the cytotoxicity of the dust to the human epithelial lung cell line was determined by using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. The concentrations of the total dust in the barns ranged from 0.175 to 0.351 mg m⁻³, with the dominant fraction (67.4–96.4%) displaying an aerodynamic diameter of < 1 µm. The bacterial and fungal concentrations fell between 7.14 × 10³ and 3.88 × 10⁴ CFU m⁻³, and 5.53 × 10¹ and 1.30 × 10⁴ CFU m⁻³, respectively. The microbial population measured in the air and that in the settled dust exhibited a linear relationship, with an R² of 0.8349–0.9471. We detected 172 and 210 bacterial/archaeal genera and 89 and 43 fungal genera in the dust and in the air, respectively. The dust samples were found to contain endotoxin concentrations of 98.98–178.31 nmol LPS mg⁻¹ and a cytotoxicity of 5.66–13.99 mg mL⁻¹. High-throughput sequencing revealed extensive biodiversity in the microorganisms, which hitherto has not been reported for cattle farms. Of the identified species, those belonging to the genera *Bacteroides*, *Corynebacterium*, *Staphylococcus*, *Ruminococcus*, *Aspergillus* and *Cladosporium* potentially affect human and cattle health. Furthermore, the dust contained 113 chemical compounds, including characteristically fungal metabolites, bacterial metabolites, phytoestrogens and antibiotics. Future research should focus on the epidemiology of farmer exposure to the harmful biological agents discovered in this study.

Keywords: Cattle breeding; Bioaerosol; Metagenomic analysis; Mycotoxins; Cytotoxicity.

INTRODUCTION

People working in professions such as farming, including cattle farming for milk and meat, are particularly susceptible to contact with harmful biological agents (Directive 2000/54/EC; Dutkiewicz *et al.*, 2011; Corrao *et al.*, 2012). These include allergenic and/or toxic agents, and those causing zoonoses and other infectious diseases (Rim and Lim, 2014). The former constitute biological particles (viruses, bacteria, endotoxin, fungi, secondary metabolites of moulds, and others), which

together with organic dust and water droplets are suspended in the air and form bioaerosol (Ławniczek-Wałczyk and Górny, 2010; Rim and Lim, 2014). The main source of harmful biological agents in cattle breeding premises are substances of animal origin (e.g., secretions, excrements, feather fragments or exfoliated epidermis) and vegetal matter used as feed or bedding. At least 40% of organic dust particles present in the bioaerosol have a diameter of 4 µm or less. At this size, they are capable of reaching the alveoli via the trachea and bronchi, and can have allergic or toxic effects (Hameed and Khodr, 2001; Lee *et al.*, 2006). The size of particles suspended in the air is one of the factors that determines how deep they can penetrate into the human respiratory system. Size also plays a role in retention time within the body, which affects the level of toxicity to humans. For this reason, division into size classes (fractions) is the most commonly used criterion

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for determining the highest allowable concentration of these particles at workplaces. In Europe, there are two types of occupational exposure limits (OELs): (i) EU limits set by the European Agency for Safety and Health at Work and (ii) national exposure limits set individually by the member states. However, EU and national OELs may differ from the limits set in non-European countries. For example, 8-h exposure limits can range from 4 mg m⁻³ (Germany, insoluble solid particles) to 15 mg m⁻³ (USA [OSHA]). For respirable dust, the 8-h exposure limits range from 1.25 mg m⁻³ (Germany, insoluble solid particles) to 6 mg m⁻³ (Hungary). OELs may also vary significantly depending on the particular type of dust. For instance, for organic grain dust the exposure limits range from 1 mg m⁻³ (Japan [JSOH]) to 10 mg m⁻³ (Ireland, USA [OSHA] and Great Britain; GESTIS, 2018).

The number of microorganisms in the air of cattle breeding farm premises amounts to 4.4×10^4 – 1.5×10^7 CFU m⁻³ (Larsson et al., 1988; Dutkiewicz et al., 1994; Seedorf et al., 1998).

Many species of bacteria can pose a health risk to people professionally involved in cattle farming. Among them the genus *S. suis* should be mentioned as an etiological factor of zoonotic meningitis, arthritis, pneumonia, endocarditis and deafness (Bartelink and van Kregten, 1995). In turn, *Campylobacter* sp. bacteria can cause campylobacteriosis in farmers resulting in enteritis and gastritis (Rim and Lim, 2014). Moreover, *Acinetobacter calcoaceticus*, *Alcaligenes faecalis*, *Pasteurella aerogenes*, *Pantoea agglomerans* and *Rahnella* are also responsible for farmers' infections (Ejlertsen et al., 1996; Milanowski et al., 1998). In addition, some of them (*A. faecalis* and *P. agglomerans* as well as *Arthrobacter globiformis* and *Agromyces ramosus* rods) are a source of highly biologically active allergens that may cause extrinsic allergic alveolitis (EAA), also known as hypersensitivity pneumonitis. Moreover, there are indications that *P. agglomerans* may induce occupational dermatitis. Non-tuberculosis bacilli from the genus *Mycobacterium*, present in dust, soil particles and animal products (meat, milk), are also dangerous for workers involved in cattle breeding. They may cause lung infection or lymph node inflammation in immunocompromised subjects (Rim and Lim, 2014).

Due to the presence of gram-negative bacteria in cattle breeding premises, there is also the presence of endotoxins in the bioaerosol. Endotoxins, together with respirable dust, enter the lungs and non-specifically activate lung macrophages, causing inflammation, fever, gas exchange disorders and bronchospasms. They also play an important role in the pathogenesis of organic dust toxic syndrome (ODTS) and may aggravate asthmatic reactions (Rylander and Peterson, 1994; Rylander, 2002).

Bioaerosol in cattle breeding rooms also contains actinomycetes. They are the etiological factor behind “farmer’s lung”, a well-known EAA. The most allergenic species include *Saccharopolyspora rectivirgula*, *S. viridis*, *Thermoactinomyces vulgaris* and *T. thalpophilus* that proliferate in moist, self-heating vegetal matter (grain, feed, hay and silage). Actinobacteria are an etiological factor in opportunistic infections in immunocompromised subjects or people with immune system dysfunction. This group

comprises invasive pathogens from the genera *Actinomyces*, *Corynebacterium*, *Gordonia*, *Mycobacterium*, *Nocardia* and *Tsukamurella* and opportunistic pathogens—*Rothia*, *Nocardiosis* and *Propionibacterium*—characterised by a special tendency to attack the lungs and the brain (Lacey and Crook, 1988; Schaal and Lee, 1992).

Likewise, moulds can also be present in cattle farms, which may be etiological factors for allergic or immunotoxic diseases. Moulds from the genera *Alternaria*, *Aspergillus*, *Penicillium*, as well as *Cladosporium*, *Fusarium*, *Mucor* and *Scopulariopsis* are most often isolated from the air in cattle breeding premises. *Candida*, *Rhodotorula* and *Cryptococcus* are the most common yeasts found in this environment. Workers, who are exposed to them, may suffer from allergic rhinitis, bronchial asthma, ODTS or skin mycoses (Sahin et al., 2005; Dave et al., 2015).

Many mould species (mainly from genera *Alternaria*, *Aspergillus*, *Fusarium*, *Penicillium*, and *Stachybotrys*) produce mycotoxins, of which aflatoxins, ochratoxin A, trichothecenes, satratoxin and zearalenone have the greatest clinical significance (Bennett and Klich, 2003). Mycotoxins are usually present in the air as components of spores or fragments of hyphae of moulds. Also these metabolites can be secreted directly into the air by toxigenic moulds. It was shown that mycotoxin inhalation may have up to ten times more of a toxic effect than dermal, gastrointestinal or intraperitoneal exposure. This fact may be due to greater bioavailability of mycotoxins or the ease with which they can penetrate the capillary walls in the alveoli (Soroka et al., 2008).

The problem of exposure of cattle breeding farm workers to biological agents is all the more important considering that amongst the most consumed meats in the world, beef comes in at third place after pork and poultry. In 2017, the cattle population amounted to 984.53 million heads, and the main cattle producers included India, Brazil, China, United States of America and European Union countries (Food and Agriculture Organization of the United Nations, 2012). In Poland as well, cattle farming is an important branch of agriculture, and its population in 2017 equalled 6.04 million heads. Therefore, there is a real need for detailed identification of microbiological threats in such facilities, considering that the research is limited (Dungan et al., 2010; Lecours et al., 2012). Therefore, the aim of the study was to evaluate the microbiological threats in cattle farms, which comprised assessing the number and types of microorganisms, and determining the concentrations of bacterial endotoxins and secondary mould metabolites (including mycotoxins) in the air and sedimented dust. High-throughput sequencing on the Illumina platform was used for the first time for the identification of harmful biological agents in this workplace. In addition, the cytotoxicity of sedimented dust against a human epithelial lung cell line was determined, and the premises studied were characterised in terms of microclimatic conditions and airborne dust concentration.

METHODS

Cattle Farm Studied

The study was performed at a farm that rears cattle for

dairy and beef. The majority of cattle at the farm were of the Holstein-Friesian breed with a black and white coat. The animals were reared in the barn system. The farm is located in central Poland (Lodz Voivodeship). The study was undertaken between February and April 2019 in six barns, which are described in Table 1.

Temperature, relative humidity and airflow rate were measured using a thermo-anemometer (VelociCalc® Multi-Function Velocity Meter 9545; TSI Inc., USA).

Airborne Dust Concentration Measurement

Airborne dust concentration was measured using a portable laser photometer (DustTrak™ DRX Aerosol Monitor 8533; TSI Inc., USA). The device simultaneously measured size-segregated mass concentrations of particulate matter (PM) corresponding to PM₁ (particles and droplets with diameters < 1 µm), PM_{2.5} (< 2.5 µm), PM₄ (< 4 µm), PM₁₀ (< 10 µm) and total PM (all particles from the measured diameter size

range) size fractions. The detection range of the instrument was between 0.001 and 150 mg m⁻³ for particles from 0.1 to 15 µm in size. Prior to each experiment, zero calibration was performed. The measurements were obtained at a height of 1.5 m from ground level in triplicates for each location, with a sampling rate of 3 L min⁻¹ and a sampling interval of 5 s.

Microbial Contamination Analysis

Microorganism numbers were determined both for the air and settled dust collected from the cattle breeding premises.

Microbiological contamination of the air was determined using a MAS-100 Eco® air sampler (Merck, Germany) according to the EN 13098 standard. Air samples of 10 L, 20 L and 50 L were collected onto media presented in Table 2. Samples of air were collected from three locations at a height of 1.5 m during routine work. Atmospheric air samples (external background) were collected at a distance of 0.5 km from the tested building.

Table 1. Description of tested premises in cattle farm.

Barn	Building material and room description	Dimensions* (m)	Cubature (m ³)	Ventilation	Feeding	Watering	Manure removal
1	Brick; barn; 11 dairy cows, 2 dairy calves	5.33 × 7.06 × 2.61	237.33	Natural (2 lockable doors and 4 windows)	Manual	Automatic	Automatic
2	Airbrick; barn; 18 dairy cows, 2 dairy calves	6.96 × 28.06 × 3.92	765.57	Natural and mechanical (2 lockable doors, 1 permanently open door, 1 window, 2 ventilators)	Half-automatic	Automatic	Automatic
3	Airbrick; barn; 3 dairy calves	4.69 × 4.64 × 2.30	50.05	Natural (1 lockable door, 1 permanently open door)	Manual	Automatic	Manual
4	Airbrick; barn; 4 dairy calves	4.69 × 4.64 × 2.30	50.05	Natural (1 lockable door, 1 permanently open door)	Manual	Automatic	Manual
5	Wood; barn; 11 beef cattle	13.24 × 7.93 × 5.12	537.57	Natural (2 lockable doors, 1 permanently open door)	Manual	Automatic	Manual
6	Brick; milking parlour; —**	2.67 × 3.98 × 2.39	25.40	Natural (2 lockable doors and 1 window)	Milking machine, milk tank, sink, cleaning products, table (animals absent)		

* Width × length × height. ** Premises without animals.

Table 2. Media used in microbial contamination analysis.

Medium	Manufacturer (country)	Microorganisms
Chapman Agar	Merck (Germany)	Mannitol-positive <i>Staphylococcus</i> sp.
Columbia Blood Agar	Oxoid (France)	Haemolytic staphylococcus
DG18 Agar (DG18 LAB-AGAR™)	Biocorp (Poland)	Xerophilic fungi
King B medium	Hi Media Laboratories (India)	<i>Pseudomonas fluorescens</i>
Malt extract agar (MEA) medium with (0.1%) chloramphenicol	Merck (Germany)	Fungi
Pochon's agar with (0.2%) nystatin	Labomix (Poland)	Actinomycetes
Tryptic soy agar (TSA) with (0.2%) nystatin	Merck (Germany)	Bacteria
Violet Red Bile Glucose Agar (VRBG LAB-AGAR)	Biocorp (Poland)	<i>Enterobacteriaceae</i>

Samples of settled dust from working environments were microbiologically analysed. For this purpose, 10–15 g of dust samples were collected in sterile containers, mixed, and 0.1 g of each of the mixed samples was suspended in 9.9 mL of saline solution (0.85% NaCl). The samples were diluted from 10^{-2} to 10^{-6} in triplicates and plated onto media described above.

All samples (air, dusts) were incubated at either $37 \pm 2^\circ\text{C}$ for 24–48 h (*Enterobacteriaceae*, mannitol-positive *Staphylococcus* sp.), $25 \pm 2^\circ\text{C}$ for 5–7 days (fungi, xerophilic fungi, actinomycetes), or $30 \pm 2^\circ\text{C}$ for 48 h (bacteria, *Pseudomonas fluorescens*).

After incubation, the colonies were counted, and the results were expressed in CFU m^{-3} for air and CFU g^{-1} for settled dust. The final result was calculated as the arithmetic mean of three independent repetitions.

Assessment of Microbial Diversity by High-throughput Sequencing

Air and dust samples for DNA extraction were collected from Barn 2. Air (2000 L) was passed through sterile gelatine filters (80 mm, 0.3 μL ; Sartorius, Germany) using AirPort MD 8 (Sartorius, Germany). DNA was extracted from dust samples (0.5 g) and the gelatine filters (0.5 g) using the FastDNA® SPIN Kit (MP Biomedicals, USA), according to the manufacturer's protocol. DNA concentration was determined using a Qubit 2.0 Fluorometer (Invitrogen/Life Technologies, USA).

The bacterial 16S rRNA gene was amplified using primers 341F and 785 R targeting the V3–V4 regions. The fungal ITS1 region was amplified with ITS1F12 (forward) (Schmidt *et al.*, 2013) and ITS2 (reverse) primers (White *et al.*, 1990). All polymerase chain reactions were performed in 50 μL volumes, containing 5 μL of DNA as a template, 25 μL of DreamTaq™ Hot Start DNA Polymerase (2 $\times$ master mix; Thermo Fisher Scientific, USA) and 10 pmol of relevant reverse and forward primers. The amplification of bacterial and fungal fragments was performed under the same conditions, with an initial denaturation at 95°C for 2 min, followed by 25 cycles of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min, with a final extension at 72°C for 5 min. DNA libraries were constructed using the Nextera Index Kit (Thermo Fisher Scientific, USA) according to the library preparation protocol for short amplicons (2 $\times$ 250 bp) provided by the manufacturer. Paired-end (PE; 2 $\times$ 250 nt) sequencing was performed on the Illumina MiSeq (MiSeq Reagent Kit v2) according to the manufacturer's protocol (Illumina, Inc., USA) at Genomed (Poland).

QIIME was used to determine different types of microbial communities in the analysed dust and air (Caporaso *et al.*, 2010). The raw reads were demultiplexed and quality-filtered on MiSeq using MiSeq Reporter (MSR) v2.4 (Illumina, Inc., USA). The sequences were clustered (8 taxonomic classifications) based on 97% identity using the uclust algorithm (Edgar, 2010), and operational taxonomic units (OTUs) were assigned to taxa employing the GreenGenes database v13.8 for bacteria and UNITE v8 database for fungi (Köljal *et al.*, 2013).

Analysis of Endotoxin in Dust Samples

3-Hydroxy fatty acids (3-OH FAs), which are unique compounds within the conserved portion of lipopolysaccharides (LPS), were used to identify endotoxins in settled dust samples from the cattle farm. Samples were subjected to hydrolysis prior to 3-OH FA release, and the subsequent steps were performed as described earlier (Sebastian and Larsson, 2003). Products of the reaction were measured using GC-MS, and the results were expressed in nmol of LPS per mg of dust.

Secondary Metabolites

For secondary metabolite analyses, air samples (2000 L) from gelatine filters and 1 g of dust were dissolved in 8 and 10 mL extraction solvent (acetonitrile:water:acetic acid = 79:20:1 v/v/v), respectively. Next dust and air samples were extracted for 90 min and diluted in the same volume of solvent prior to injection (Sulyok *et al.*, 2006). Secondary metabolite concentrations were analysed and quantified using LC-MS/MS, as described by Sulyok *et al.* (2007) with further modification. Parameters for liquid chromatography and mass spectrometry are described elsewhere (Malachová *et al.*, 2014). Briefly, LC-MS/MS screening of target microbial metabolites was performed with a QTrap 5500 LC-MS/MS System (Applied Biosystems, USA) equipped with a TurboIonSpray electrospray ionization (ESI) source and a 1290 Series HPLC System (Agilent, Germany). Chromatographic separation was performed at 25°C on a Gemini® C18 column (150 $\times$ 4.6 mm i.d., 5 mm particle size) equipped with a C18 (4 $\times$ 3 mm i.d.) security guard cartridge (Phenomenex, USA). ESI-MS/MS was performed in the time-scheduled multiple reaction monitoring (MRM) mode, both in positive and negative polarities, in two separate chromatographic runs per sample, by scanning two fragmentation reactions per analyte. The MRM detection window of each analyte was set to its expected retention times of ± 27 and ± 48 s in the positive and negative modes, respectively. The positive analyte was confirmed by the acquisition of two MRMs per analyte, with the exception of moniliformin, which exhibited only one fragment ion. This yielded 4.0 identification points according to European Union Commission Decision 2002/657 (Commission Decision 96/23/EC; European Commission, 2002). The LC retention time and the intensity ratio of the two MRM transitions agreed with the related values of an authentic standard within 0.1 min and 30% rel. Analysis was performed in three replicates per sample.

The limits of detection (LODs) of extrolites are presented in Table S1.

Cell Culture and Cytotoxicity Testing

For cell culturing (described later), 1.0 g of each dust sample was suspended in 10 mL of basal medium and mixed (stock dust concentration in each extract was 100 mg mL^{-1}). The samples were then extracted for 40 min (160 rev min^{-1}) at ambient temperature. Since alkaline or acidic pH of the test sample can negatively influence cell growth and adherence, the pH of each extract was adjusted to neutral (7.0 ± 0.2). Each extract was filtered twice using sterile

syringe filters (0.22 µm) (Membrane Solutions, USA). The final concentrations of the extracts analysed were 20, 10, 5, 2.5, 1.3, 0.6 and 0.3 mg mL⁻¹.

The cytotoxicity of the prepared water-soluble fraction of dust samples was assessed using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. The assay was performed on the human adenocarcinoma lung (alveolar) epithelial adherent cell line A-549 (Cell Line Service GmbH, Germany) from passage 33. The model cell line is often used for dust and air pollution cytotoxicity testing (Gutarowska *et al.*, 2018).

The cells were cultured as previously described (Gutarowska *et al.*, 2018). Briefly, they were cultured as a monolayer in Dulbecco's Modified Eagle's Medium: Ham's F12 basic (1:1 v/v, DMEM/Ham's F12; Cell Line Service GmbH, Germany) supplemented with 5% foetal bovine serum (FBS; Cell Line Service GmbH, Germany), 2 mM of glutamine (Cell Line Service GmbH, Germany), 25 mM of HEPES (Cell Line Service GmbH, Germany), and 100 µg mL⁻¹ streptomycin and 100 IU mL⁻¹ penicillin mixture (Sigma-Aldrich, USA). The cells were cultured for 3–5 days at 37°C in an incubator with 5% CO₂ atmosphere (Galaxy 48S, United Kingdom). After reaching 80% confluence, the cells were detached using Gibco TrypLETM Express (Thermo Fisher Scientific, USA), centrifuged (187 × g, 5 min) and the pellet was re-suspended in fresh culture medium. Subsequently the number of cells were counted using a haemocytometer. The viability of the cells was determined by trypan blue exclusion. The cells were only used if they showed a minimum of 80–90% viability.

For both MTT assays, 1 × 10⁴ A-549 cells were placed in each well of a 96 well plate (Greiner Bio-One GmbH, Germany) in the complete culture medium. The cells were incubated overnight at 37°C in 5% CO₂. The following day, the medium was gently aspirated, and 200 µL of each concentration of the tested extract in the culture medium was added to wells in the 96 well plate in four repeats. The negative controls (in eight repeats) consisted of cells without tested extracts. The cells were incubated in a CO₂ incubator at 37°C in 5% CO₂ for 48 and 72 h. After incubation, the samples were gently aspirated, 100 µL of MTT (0.5 mg mL⁻¹ in PBS,

pH: 7.2) (Sigma-Aldrich, USA) was added and they were further incubated at 37°C in 5% CO₂ for an additional 3 h. Then MTT was carefully removed and formazan precipitates were solubilised by adding 50 µL of DMSO (Sigma-Aldrich, USA). Absorbance was measured at 550 nm with a reference filter of 620 nm, using a microplate reader (TriStar2 LB 942; Berthold Technologies GmbH and Co. KG, Germany). The absorbance of the control sample (untreated cells) represented 100% cell viability. Cell viability (%) was calculated using the following equation: (sample OD/control OD) × 100%; the cytotoxicity (%) was calculated as: 100 – cell viability (%). The results are presented as mean ± standard deviation (SD). The mean error of these methods is up to 10%.

The IC₅₀ value (the concentration of the test compound required to reduce the cell survival rate to 50% of the control) is the degree of cellular sensitivity to a given treatment. IC₅₀ values were determined both from the plotted curves and according to the OECD Guidelines for the Testing of Chemicals (GESTIS, 2018; OECD, 2018).

Statistical Analysis

Statistical analyses were conducted using Statistica 13.1 (Statsoft, USA). Descriptive statistics for all variables of interest were calculated. Microorganism numbers in the air and dust samples as well as microclimatic conditions and dust concentrations were compared between the tested locations using one-way analysis of variance (ANOVA) at a significance level of 0.05 following Levene's test. When a statistical difference was detected ($p < 0.05$), means were compared using Tukey's *post hoc* procedure at 0.05 significance level.

Linear regression analysis was used to determine the correlation between the number of microorganisms in the air and settled dust at particular locations within the cattle farm. The significance tests were performed at the 0.05 significance level using a literature correlation scale (Hinkle *et al.*, 2003).

RESULTS AND DISCUSSION

Microclimatic Conditions

Microclimatic conditions present in the cattle farm are presented in Table 3.

Table 3. Microclimatic conditions during measurements at the tested workplaces.

Barn	Temperature (°C)	Relative humidity (%)	Air flow velocity (m s ⁻¹)
1	M: 11.6 ^{ab} SD: 1.0	M: 52.4 ^{ad} SD: 2.8	M: 0.15 ^a SD: 0.09
2	M: 10.9 ^b SD: 0.4	M: 57.8 ^b SD: 1.2	M: 0.40 ^b SD: 0.04
3	M: 12.5 ^a SD: 0.2	M: 56.7 ^{ab} SD: 1.3	M: 0.62 ^c SD: 0.12
4	M: 12.9 ^a SD: 0.2	M: 57.3 ^{ab} SD: 1.7	M: 0.03 ^a SD: 0.01
5	M: 14.6 ^c SD: 0.8	M: 49.7 ^{cd} SD: 1.6	M: 0.03 ^a SD: 0.01
6	M: 16.4 ^d SD: 0.2	M: 45.5 ^c SD: 2.6	M: 0.01 ^a SD: 0.01

M: mean; SD: standard deviation. Statistically significant differences (ANOVA: $p < 0.05$; Tukey's test: $p < 0.05$) occur for means marked with different letters (a, b, c, d) within the same parameter (column).

The air temperature in the barns ranged from 10.9°C (Barn 2) to 16.4°C (Barn 6, the milking parlour). We also found the highest (57.8%) and lowest (45.5%) relative humidity in these rooms compared to the rest of the farm. Airflow rate ranged from 0.01 m s⁻¹ (Barn 6) to 0.62 m s⁻¹ (Barn 3) (Table 3).

Microclimatic conditions in individual barns varied considerably; statistically significant differences were found even for contiguous areas (e.g., between Barns 3 and 4 as well as 2). Despite open doors and windows, low airflow rates were observed in the majority of the barns, which hampered air exchange with the surroundings. Statistically significant differences in this parameter were noted between Barns 2 and 3, and the rest of the farm complex, which was probably due to the presence of doors and windows along the lengths of the buildings. Different values of individual microclimatic parameters indicate that in individual barns there could be completely different conditions for microorganism growth.

Dust Concentrations at Workplaces

Total dust concentrations in the barns varied; high values were noted in Barns 1 and 6 (0.281 and 0.351 mg m⁻³, respectively), while the remaining barns had lower values (from 0.175 to 0.265 mg m⁻³) (Table 4). In all barns, the predominant dust fraction had an aerodynamic diameter < 1 µm. The share of the total amount of dust measured ranged from 67.4% in Barn 1 to 96.4% in Barn 5. The smallest fraction (0.4–3.4%) amongst the total dust collected consisted of particles of diameters ranging from 1 to 4 µm.

The dust concentration values shown in Table 4 did not exceed the threshold limits for workplaces according to national legislation. Hence, it is not a regulatory requirement to use respiratory protective equipment, and the use of such equipment is purely voluntary. Selecting appropriate equipment depends, in this case, on an individual user's comfort. Workers can decide to use any half mask, regardless of its protection class.

Microbial Contamination

The number of bacteria on cattle breeding premises ranged

from 7.14 × 10³ CFU m⁻³ (Barn 3) to 3.88 × 10⁴ CFU m⁻³ (Barn 2). The number of Actinobacteria were lower and ranged from 2.25 × 10¹ CFU m⁻³ (Barn 6) to 3.45 × 10⁴ CFU m⁻³ (Barn 4). The concentration of mannitol-positive Staphylococci ranged from 1.80 × 10² CFU m⁻³ (Barn 6) to 8.30 × 10³ CFU m⁻³ (Barn 1) (Table 5). In turn, the number of bacteria from the family *Enterobacteriaceae* was 5.75 × 10¹ CFU m⁻³ in Barn 6 and 7.05 × 10² CFU m⁻³ in Barns 1 and 2. Haemolytic Staphylococci were most abundant in Barn 2 (3.3 × 10² CFU m⁻³), while Barn 1 had the fewest (3.25 × 10¹ CFU m⁻³). *Pseudomonas fluorescens* bacteria in the barns was the lowest of all microorganism groups studied and ranged from 2.50 × 10⁰ to 1.00 × 10¹ CFU m⁻³. Fungi number in the barns studied ranged from 5.53 × 10¹ CFU m⁻³ (Barn 6) to 1.30 × 10⁴ CFU m⁻³ (Barn 2) and xerophilic fungi from 6.5 × 10² CFU m⁻³ (Barn 6) to 6.1 × 10³ CFU m⁻³ (Barn 1) (Table 5).

Statistically significant differences in microorganism numbers, with the exception of total bacteria number and the number of *Pseudomonas fluorescens*, were observed between the air of the barns and atmospheric air (control sample) sampled at a distance of 500 m from farm buildings. Thus, the highest microbiological air contamination was detected in Barns 1–4, and the lowest in Barns 5 and 6, where the number of microorganisms was most often comparable to atmospheric air. Barns 5 and 6 also had the lowest relative humidity (< 50%). Furthermore, Barn 6, as the milking parlour, was maintained under good hygienic conditions, where no animals were present; this probably explains the lower air contamination observed.

We found lower microorganism numbers in the air than previously observed by Dutkiewicz *et al.* (1994). These authors detected bacterial numbers ranging between 4.4 × 10⁴ and 2.8 × 10⁵ CFU m⁻³ in dairy barns and calf barns, and fungal numbers from 1.0 × 10³ to 6.1 × 10⁴ CFU m⁻³. The authors conducted the studies in premises housing 42–137 animals, which may have resulted in obtaining higher microorganism numbers in the air. Unfortunately, we do not know the dimensions of the premises investigated, which would have helped determine animal density per unit

Table 4. Airborne dust concentrations at tested premises.

Barn	Airborne dust concentrations corresponding to particle size fractions (mg m ⁻³)				
	PM ₁	PM _{2.5}	PM ₄	PM ₁₀	PM _{total}
1	M: 0.190 ^b SD: 0.297	M: 0.191 ^b SD: 0.298	M: 0.194 ^b SD: 0.304	M: 0.224 ^c SD: 0.403	M: 0.281 ^b SD: 0.676
2	M: 0.193 ^b SD: 0.099	M: 0.195 ^b SD: 0.104	M: 0.204 ^b SD: 0.130	M: 0.244 ^b SD: 0.263	M: 0.265 ^b SD: 0.276
3	M: 0.265 ^b SD: 0.276	M: .163 ^d SD: 0.033	M: 0.164 ^d SD: 0.034	M: 0.170 ^d SD: 0.036	M: 0.175 ^c SD: 0.045
4	M: 0.169 ^{cd} SD: 0.024	M: 0.170 ^{cd} SD: 0.024	M: 0.171 ^{cd} SD: 0.024	M: 0.171 ^{cd} SD: 0.024	M: 0.178 ^c SD: 0.039
5	M: 0.177 ^c SD: 0.013	M: 0.178 ^c SD: 0.013	M: 0.178 ^c SD: 0.013	M: 0.181 ^d SD: 0.017	M: 0.184 ^c SD: 0.028
6	M: 0.303 ^a SD: 0.023	M: 0.304 ^a SD: 0.023	M: 0.307 ^a SD: 0.024	M: 0.330 ^a SD: 0.038	M: 0.351 ^a SD: 0.059

M: mean; SD: standard deviation. Statistically significant differences (ANOVA: p = 0.05; Tukey's test: p = 0.05) occur for averages marked with different letters (a, b, c, d) within the same dust fraction.

Table 5. Microorganism number in air samples.

Barn	Microorganism number (CFU m ⁻³)					
	Bacteria	Actinomycetes	Mannitol-positive <i>Staphylococcus</i> spp.	<i>Enterobacteriaceae</i>	<i>Pseudomonas fluorescens</i>	Haemolytic <i>Staphylococcus</i>
1	M: 2.12×10^{4AB} SD: 4.84×10^3	M: 2.60×10^{2AB} SD: 1.02×10^2	M: 8.30×10^{3B} SD: 1.35×10^3	M: 7.05×10^{2B} SD: 1.98×10^2	M: 7.50×10^{0A} SD: 0.00	M: 3.25×10^{1A} SD: 9.57×10^0
2	M: 3.88×10^{4A} SD: 1.87×10^4	M: 2.63×10^{2AB} SD: 5.56×10^1	M: 7.24×10^{3B} SD: 1.51×10^3	M: 7.05×10^{2B} SD: 2.34×10^2	M: 5.00×10^{0A} SD: 0.00	M: 3.30×10^{2D} SD: 1.10×10^2
3	M: 7.14×10^{3BC} SD: 8.25×10^2	M: 6.25×10^{1A} SD: 2.99×10^1	M: 8.18×10^{3B} SD: 2.05×10^3	M: 4.25×10^{2BC} SD: 1.17×10^2	M: 1.00×10^{1A} SD: 0.00	M: 1.50×10^{2BC} SD: 5.29×10^1
4	M: 2.43×10^{4AB} SD: 9.92×10^3	M: 3.45×10^{2B} SD: 2.51×10^2	M: 1.32×10^{4C} SD: 4.77×10^3	M: 2.73×10^{2AC} SD: 1.55×10^2	M: 2.50×10^{0A} SD: 0.00	M: 1.38×10^{2ABC} SD: 2.06×10^1
5	M: 2.54×10^{4AB} SD: 2.26×10^3	M: 1.93×10^{2AB} SD: 6.40×10^1	M: 9.88×10^{2A} SD: 3.64×10^2	M: 7.00×10^{1A} SD: 8.16×10^0	M: 5.00×10^{0A} SD: 0.00	M: 1.83×10^{2C} SD: 4.65×10^1
6	M: 2.84×10^{4A} SD: 5.12×10^3	M: 2.25×10^{1A} SD: 1.50×10^1	M: 1.80×10^{2A} SD: 3.92×10^1	M: 5.75×10^{1A} SD: 5.00×10^0	M: 2.50×10^{0A} SD: 0.00	M: 5.50×10^{1AB} SD: 1.29×10^1
Control sample*	M: 3.93×10^{2A} SD: 1.03×10^2	M: 2.00×10^{1A} SD: 8.2×10^0	M: 2.75×10^{1A} SD: 2.36×10^1	M: 7.50×10^{0A} SD: 5.00×10^0	M: 2.50×10^{0A} SD: 5.00×10^0	M: 3.33×10^{2A} SD: 0.99×10^2
						M: 5.42×10^{3C} SD: 2.08×10^3
						M: 1.30×10^{4D} SD: 3.56×10^3
						M: 2.32×10^{3ABC} SD: 1.84×10^{3A}
						M: 3.71×10^2 SD: 8.31×10^2
						M: 4.68×10^{3BC} SD: 2.31×10^{3A}
						M: 8.54×10^2 SD: 2.61×10^2
						M: 3.61×10^{3ABC} SD: 1.87×10^3
						M: 2.57×10^3 SD: 6.50×10^{2A}
						M: 5.53×10^{2AB} SD: 9.31×10^1
						M: 3.86×10^1 SD: 4.90×10^{2A}
						M: 1.61×10^2 SD: 1.61×10^2

M: mean; SD: standard deviation. * Atmospheric air in distance 300 m. Means with the same capital letter (A, B, C, D) in the same column are not significantly different (ANOVA: $p < 0.05$; Tukey's test: $p < 0.05$).

volume. Moreover, the microclimatic conditions under which the study was conducted are also not known.

Our work was carried out during winter/early spring, which is not conducive to microorganism survival in the air. Like us, Lee *et al.* (2006) conducted studies in a dairy farm during winter, and found a similar number of microorganisms as in our study. They obtained 0.3×10^3 – 1.3×10^4 CFU m⁻³ for bacteria and 0.3×10^3 – 3.9×10^4 CFU m⁻³ for fungi.

Lange *et al.* (1997) studied bioaerosol concentrations in 48 dairy barns. They showed that concentrations of individual microorganism groups differed by two to three orders of magnitude between the barns. They demonstrated that, of all farm management practices, the type of fodder was the strongest correlate of microorganism concentration; feeds with a high moisture content were associated with lower concentration of bioaerosols. In addition, the authors proved that the type of ventilation influences dust, endotoxin and gram-negative bacteria concentrations in barns (Lange *et al.*, 1997).

In dust samples, bacteria prevailed (5.97×10^7 – 5.84×10^8 CFU g⁻¹), amongst which mannitol-positive Staphylococci (2.35×10^7 – 7.78×10^8 CFU g⁻¹) and bacteria from the family *Enterobacteriaceae* (9.08×10^4 – 1.72×10^5 CFU g⁻¹) were the most abundant (Table 6). Haemolytic Staphylococci (3.15×10^4 – 5.20×10^5 CFU g⁻¹) and Actinomycetes (1.40×10^3 – 4.45×10^4 CFU g⁻¹) were less abundant in dust. In all samples of dust, no *Pseudomonas fluorescens* was detected (below the method's threshold for detection). The overall fungal numbers in dust samples tested amounted to 5.20×10^5 – 4.44×10^7 CFU g⁻¹, with xerophilic fungal numbers between 1.22×10^6 – 1.53×10^7 CFU g⁻¹ (Table 6).

Statistical analysis found that the most microbiologically contaminated dust samples were from Barns 2 and 3, while the least was from Barns 4 and 5, which also had the lowest concentration of microorganisms in the air.

There are no data in the literature on microorganism numbers in sedimented dust from cattle breeding premises. In a previous study, we carried out an analogous analysis on sedimented dust from agricultural work environment (dust from poultry breeding premises and grain dust). We found similar bacterial numbers in dust from that work environment (3.33×10^7 – 1.57×10^8 CFU g⁻¹). There were less actinomycetes ($\leq 2.05 \times 10^3$ CFU g⁻¹), mannitol-positive Staphylococci (1.20×10^4 – 3.49×10^4 CFU g⁻¹), *Enterobacteriaceae* ($\leq 2.30 \times 10^4$ CFU g⁻¹), fungi (7.48×10^4 – 8.75×10^5 CFU g⁻¹) and xerophilic fungi (3.35×10^5 – 4.45×10^5 CFU g⁻¹). However, there were more *P. fluorescens* (1.00×10^5 – 5.73×10^5 CFU g⁻¹). The number of haemolytic Staphylococci was higher in poultry farm dust (8.50×10^5 CFU g⁻¹) and lower in grain dust (2.98×10^3 CFU g⁻¹) (Skóra *et al.*, 2016; Gutarowska *et al.*, 2018). Considering the above, dust from cattle breeding premises was more microbiologically contaminated than dust from poultry breeding premises and grain dust.

In the current study, we saw strong positive correlations ($R^2 = 0.8349$ – 0.9471) between microorganism numbers in the air and dust from the cattle farm (Fig. 1).

Diversity of Microorganisms from the Cattle Farm

High-throughput DNA sequencing data (Figs. 2 and 3;

Table 6. Microorganism number in dust samples.

Barn	Microorganism number (CFU g ⁻¹)						
	Bacteria	Actinomycetes	Mannitol-positive <i>Staphylococcus</i> spp.	<i>Enterobacteriaceae</i>	<i>Pseudomonas fluorescens</i>	Haemolytic <i>Staphylococcus</i>	Fungi
1	M: 7.39 × 10 ^{7A} SD: 3.29 × 10 ⁷	M: 2.53 × 10 ^{4B} SD: 1.02 × 10 ⁴	M: 4.51 × 10 ^{7A} SD: 7.62 × 10 ⁶	M: 1.19 × 10 ^{5A} SD: 7.54 × 10 ⁴	M: < 1.00 × 10 ² SD: 0.00	M: 5.15 × 10 ^{4A} SD: 9.67 × 10 ³	M: 2.00 × 10 ^{6A} SD: 7.48 × 10 ⁵
2	M: 8.88 × 10 ^{7A} SD: 7.53 × 10 ⁷	M: 4.45 × 10 ^{4B} SD: 1.97 × 10 ⁴	M: 3.14 × 10 ^{7A} SD: 6.87 × 10 ⁶	M: 1.16 × 10 ^{5A} SD: 1.12 × 10 ⁵	M: < 1.00 × 10 ² SD: 0.00	M: 5.20 × 10 ^{5B} SD: 3.27 × 10 ⁴	M: 4.43 × 10 ^{6A} SD: 3.58 × 10 ⁶
3	M: 5.84 × 10 ^{8B} SD: 4.85 × 10 ⁷	M: 1.40 × 10 ^{3A} SD: 4.90 × 10 ²	M: 7.78 × 10 ^{8B} SD: 1.23 × 10 ⁸	M: 9.08 × 10 ^{4A} SD: 8.22 × 10 ⁴	M: < 1.00 × 10 ² SD: 0.00	M: 3.15 × 10 ^{4A} SD: 1.01 × 10 ⁴	M: 4.44 × 10 ^{7B} SD: 3.43 × 10 ⁷
4	M: 1.63 × 10 ^{8A} SD: 1.28 × 10 ⁸	M: 3.35 × 10 ^{3A} SD: 5.21 × 10 ³	M: 5.21 × 10 ^{7A} SD: 4.04 × 10 ⁵	M: 1.72 × 10 ^{5A} SD: 9.69 × 10 ⁴	M: < 1.00 × 10 ² SD: 0.00	M: 7.50 × 10 ^{4A} SD: 4.08 × 10 ³	M: 1.32 × 10 ^{6A} SD: 3.48 × 10 ⁵
5	M: 5.97 × 10 ^{7A} SD: 4.96 × 10 ⁷	M: 2.28 × 10 ^{3A} SD: 9.14 × 10 ²	M: 2.35 × 10 ^{7A} SD: 2.42 × 10 ⁶	M: 1.65 × 10 ^{5A} SD: 2.89 × 10 ⁴	M: < 1.00 × 10 ² SD: 0.00	M: 4.50 × 10 ^{4A} SD: 5.05 × 10 ⁴	M: 5.20 × 10 ^{5A} SD: 1.50 × 10 ⁵

M: mean; SD: standard deviation. Means with the same capital letter (A, B) in the same column are not significantly different (ANOVA: $p < 0.05$; Tukey's test: $p < 0.05$).

Tables S2 and S3) shows the phylogenetic diversity of microorganisms that were present in the dust and air collected in Barn 2. We found a prevalence of 172 bacterial/archaeal genera, from 10 phyla, and 89 fungal genera from 6 phyla, in the dust. In addition, 210 bacterial/archaeal genera (10 phyla) and 43 fungal genera (6 phyla) were identified in the air.

Both samples (dust and air) were mainly contaminated with bacteria belonging to Firmicutes (85.8 and 46.2%), Proteobacteria (4.1 and 15.9%), Actinobacteria (4.7 and 13.8%), and Bacteroidetes (4.2 and 13.8%) (Fig. 2). Firmicutes from the dust sample mainly consisted of bacteria from the genera *Staphylococcus* (74.4%), *Ruminococcus* (3.3%), *Clostridium* (1.9%) and *Abiotrophia* (1.3%). Bacteria belonging to *Corynebacterium* (2.6%) dominated phylum Actinobacteria, while, in the case of Bacteroidetes, the most frequently occurring genus was *Bacteroides* (1.8%). Additionally, *Sphingomonas* (1.5%) was the most widespread genus among Proteobacteria. Amongst bacterial genera identified in the air, *Actinomyces* (2.8%), *Corynebacterium* (8.8%) and *Saccharopolyspora* (1.5%) belonging to Actinobacteria; *Bacteroides* (5.6%) and *Paludibacter* (1.2%) belonging to phylum Bacteroidetes; *Halomonas* (3.1%), *Sphingomonas* (1.8%) and *Ehrlichia* (1.3%) from phylum Proteobacteria; *Acholeplasma* (1.8%) from Tenericutes; and *Ruminococcus* (11.4%), *Clostridium* (6.2%), *Abiotrophia* (4.7%), *Facklamia* (4.0%), *Staphylococcus* (3.3%), *Psychrobacter* (2.3%), *Jeotgalicoccus* (1.2%), *Aerococcus* (1.4%), and *Acinetobacter* (1.2%) from Firmicutes were identified in abundance at a concentration > 1%.

Ascomycota and Basidiomycota were the most abundant fungi in both samples (Fig. 3). In the dust sample, Ascomycota (41.6% of phylum OTUs), *Aspergillus* (23.2%), *Cladosporium* (6.1%), *Mycosphaerella* (3.6%), *Alternaria* (1.9%), *Candida* (2.6%) and *Debaryomyces* (2.0%) had the highest OTUs. Additionally, members of *Wallemia* (38.8%) dominated among Basidiomycota (41.7% of phylum OTUs). Amongst fungi found in the air sample, DNA sequences associated with the genera *Mycosphaerella* (15.2%), *Alternaria* (5.6%), *Aspergillus* (7.8%), *Debaryomyces* (3.0%) and *Candida* (8.3%) were mainly detected. Simultaneously, *Fusarium* was identified both in the air (2.0% of all genera's OTUs) and dust (0.2% of OTUs). All of the above genera contaminating the air were from the phylum Ascomycota.

This is the first detailed study that describes the bacterial and fungal diversity in agricultural work environments. High-throughput sequencing analyses of air and dust samples, collected in Barn 2, found a variety of pathogens that may affect human and cattle health. Both dust and air were carriers of bacteria belonging to *Corynebacterium* sp. This genus was represented mostly by the species *C. bovis*, *C. durum*, *C. kroppenstedtii*, *C. pilosum* and *C. variabile*, which are responsible for infections of the respiratory tract, eye and soft facial tissue (Riegel et al., 1997; Berard, 2012; Chow et al., 2015). In addition, previous work found that patients suffering from mastitis, caused by *C. kroppenstedtii*, show symptoms of psychiatric illnesses. This fact was potentially associated with the infection promoted by this bacterium (Wong et al., 2017). The risk of airborne transmission of invasive multidrug-resistant strains is related to

Fig. 1. Correlation between number of microorganisms in the air and dust samples. 1–5: Barn number.

Fig. 2. Phylogenetic distribution of bacterial sequences assigned on phyla in dust and air samples.

Fig. 3. Phylogenetic distribution of fungal sequences assigned to phyla in dust and air samples.

the presence of bacteria belonging to *Staphylococcus* sp. Staphylococci are typical components of natural microflora of feathers, skin, hair and mucous membranes. Other species in this genus, such as *S. sciuri*, *S. equorum* and *S. epidermidis*, can be pathogenic to humans and are responsible for endocarditis, urinary tract infections, pelvic inflammatory disease, wound infections and septic shock (Chen *et al.*, 2007). *Bacteroides fragilis*, identified both in air and dust samples, is a known gram-negative rod involved in anaerobic infections (Wexler, 2007). *Ruminococcus* sp., identified in air, was reportedly found in the gastrointestinal tracts of animals and humans, and may be a factor for serious arthritis and bacteremia (Titecat *et al.*, 2014). What is more,

representatives of *Aspergillus* sp. (mainly *A. fumigatus*, *A. conicus*, *A. penicillioides*, *A. proliferans*, *A. quadrilineatus* and *A. restrictus*) were found in high relative abundance in dust and air. Amongst species of this genus, *A. fumigatus* is a serious human pathogen being the major cause of invasive aspergillosis (McCormick *et al.*, 2010; Kwon-Chung and Sugui, 2013). Moulds belonging to *Cladosporium* sp., which are common saprobic fungi or plant endophytes, are associated with human diseases and allergies (Sandoval-Denis *et al.*, 2016). Yeast *Debaryomyces* sp., which are considered a potential biocontrol agent against a number of moulds in food production, may cause human infections (Desnos-Ollivier *et al.*, 2008).

Endotoxin Concentrations

Endotoxin content in sedimented dust from the barns of cattle farms is shown in Fig. 4.

Endotoxin concentrations in sedimented dust ranged from 98.98 nmol LPS mg⁻¹ (Barn 3) to 178.31 nmol LPS mg⁻¹ (Barn 4) (Fig. 4). Similar values of LPS concentrations were previously reported in literature for sedimented dust from barns occupied by other farm animals. Pomorska *et al.* (2009) documented LPS concentration in horse stables between 11 and 110 nmol LPS mg⁻¹; in poultry houses from 65 to 176 nmol LPS mg⁻¹ and in sheep sheds between 61 and 226 nmol LPS mg⁻¹. For comparison, the endotoxin concentrations in sedimented dust from various domestic spaces (kitchen, bedroom and living room) is about 1000 times lower ranging between 0.092 and 0.155 nmol LPS mg⁻¹ (Park *et al.*, 2004).

In the current study, we found that endotoxin concentrations correlated with the number of bacteria from the family *Enterobacteriaceae* ($R^2 = 0.8495$). Thus, this family of gram-negative bacteria are likely to be mainly responsible for endotoxin presence in this environment. Similar correlation between endotoxin and gram-negative rod concentrations was found by Ławniczek-Wałczyk *et al.* (2013).

It was shown that bacterial endotoxin exposure through inhalation may lead to respiratory tract inflammation and toxic pneumonitis due to non-specific activation of alveolar macrophages, which release inflammatory mediators. Endotoxins can also cause fever, shivering, cough, and influenza-like symptoms (Ławniczek-Wałczyk and Górny, 2010; Degobbi *et al.*, 2011).

However, the role of endotoxins in the farming environment is still unclear. Endotoxins might act as a co-allergen facilitating sensitization to other allergens or they may increase the severity of allergies (Michel *et al.*, 1996; Ormstad *et al.*, 2003). Moreover, some data suggest that the inflammation caused by inhaled endotoxins may decrease the risk of atopic sensitisation amongst children, and can lead to lung cancer amongst workers exposed to organic dust (Rylander, 2002).

Secondary Metabolites

One-hundred-and-thirteen chemical compounds were detected in sedimented dust sampled on the cattle farm. The most dominant amongst them were metabolites characteristic of the genera *Fusarium* and *Penicillium*. Among the less abundant compounds those produced by moulds from the genera *Aspergillus*, *Alternaria* and other mould types were identified (Table 7). Interestingly, metabolites of bacterial origin and from lichens as well as phytoestrogens and antibiotics were also detected (Table 7).

The types and concentrations of secondary mould metabolites varied depending on which barn the sedimented dust originated from. Barns 1 and 5 had the lowest number of mould compounds (53 and 54, respectively), while Barns 2 and 4 had the highest (62 and 64, respectively). Mould metabolites that were present at high concentrations in all samples comprised siccanol (724–1560 ng g⁻¹), culmorin (126.5–569 ng g⁻¹), tenuazonic acid (435–540 ng g⁻¹), altersetin (38.5–457 ng g⁻¹), flavoglucin (69.5–1030 ng g⁻¹), cyclopenol (53.2–122 ng g⁻¹), viridicatol (54.9–367 ng g⁻¹), aurofusarin (118–226 ng g⁻¹), fusarinolic acid (120–357 ng g⁻¹), enniatin B1 (97.1–195 ng g⁻¹), enniatin B (65.2–206 ng g⁻¹), nivalenol (75.8–120 ng g⁻¹) and deoxynivalenol (104–176 ng g⁻¹) (Table 7).

The large number of metabolites specific to *Fusarium* mould and their high concentrations point to the potentially poor microbiological quality of vegetal matter used for feeding and bedding, e.g., haylage, silage, concentrated feed or straw and/or humidity or fungal contamination in the livestock building.

Phytoestrogens are one of many secondary metabolites produced by plants (e.g., legumes, soybeans, beans, nuts, cereals, flax seeds, sesame seeds, hops, and others) during photosynthesis (Graham, 1999). It was shown that legumes, commonly used for feeding farm animals, can contain 5–25% phytoestrogens. Phytoestrogen concentrations in these plants depend on environmental factors such as temperature, humidity, light, the age of the plant, fertiliser quantity and pathogens (Adams, 1995).

Fig. 4. Endotoxin concentration and correlation between endotoxin concentration and *Enterobacteriaceae* number in dust samples.

Table 7. Concentration of secondary metabolites in settled dust samples.

Secondary metabolite	Concentration in dust samples (ng g ⁻¹)				
	1	2	3	4	5
from <i>Alternaria</i> sp.					
Alternariol	28.8	19.4	26.3	16.6	23.7
Alternariol methyl ether	6.07	3.75	4.10	2.13	8.32
Altersetin	59.4	57.3	73.1	39.0	457
Infectopyron	438	678	501	614	915
Tentoxin	–	1.26	2.00	–	3.38
Tenuazonic acid	436	435	530	500	540
from <i>Aspergillus</i> sp.					
3-Nitropropionic acid	11.2	10.4	15.1	–	–
8- <i>O</i> -methyl averufin	–	1.52	–	–	–
Aspulvinone E	–	–	–	6,100,000*	51,500,680*
Averantin	–	1.43	–	–	–
Averufin	0.72	5.34	2.12	–	–
Fumigaclavine	–	3.45	–	–	–
Fumigaclavine C	–	12.1	–	–	–
Fumiquinazoline D	–	7.53	–	–	–
Methoxysterigmatocystin	6.34	17.2	2.03	3.67	–
Methyl sulochrin	1.82	6.26	2.99	3.17	–
Secosterigmatocystin	–	3.43	–	–	–
Sterigmatocystin	4.88	6.30	0.56	1.32	0.36
Sydowinin A	–	–	42.6	–	–
Versicolorin C	–	6.9	–	–	–
from <i>Fusarium</i> sp.					
15-Hydroxyculmorin	–	–	109	175	79.8
5-Hydroxyculmorin	–	–	187	–	138
Antibiotic Y	41.5	–	26.1	45.3	94.2
Apicidin	92.2	75.6	66.2	81.8	–
Aurofusarin	118	132	142	152	226
Beauvericin	86.9	70.3	58.4	78.7	35.8
Chrysogin	17.3	14.4	15.0	11.8	12.7
Culmorin	217	272	261	569	126
Deoxynivalenol	155	107	114	176	104
Enniatin A	1.44	1.23	0.93	0.71	2.01
Enniatin A1	39.1	30.7	30.0	21.5	53.3
Enniatin B	119	84.4	107	65.3	206
Enniatin B1	127	97.1	107	70.6	195
Enniatin B2	10.9	8.02	8.04	6.42	17.7
Enniatin B3	0.03	0.03	0.02	0.01	0.05
Epiequisetin	2.57	2.53	3.06	4.20	–
Equisetin	31.9	34.1	36.8	63.2	27.5
Fusaric acid	322	–	356	414	–
Fusarinolic acid	287	121	342	357	120
Fusapyrone	–	–	–	12.9	–
Moniliformin	–	–	7.38	8.81	21.2
Nivalenol	119	75.8	94.2	114	88.4
Siccanol	764	724	1220	1560	1480
T-2 toxin	–	–	–	–	2.95
W493	125	63.7	38.6	68.9	80.5
Zearalenone	17.1	14.0	15.3	18.0	–
Zearalenone sulphate	625,080	–	313,440	–	–
from <i>Penicillium</i> sp.					
7-Hydroxypestalotin	–	55.6	–	41.2	–
Agroclavine	–	0.46	–	–	–
Andrastin A	–	–	–	22.6	–
Aurantiamine A	–	–	–	29.0	5.64

Table 7. (continued).

Secondary metabolite	Concentration in dust samples (ng g ⁻¹)				
	1	2	3	4	5
Barceloneic acid	56.3	106	32.3	153	–
Citrinin	–	49.7	149	113	391
Chanoclavine	1.13	2.46	1.27	3.11	0.73
Curvularin	15.2	18.0	–	13.8	–
Cycloaspeptide A	2.31	1.39	3.48	1.64	–
Cyclophenin	3.07	3.87	–	15.0	1.64
Cyclophenol	77.9	122	53.2	115.7	88
Cyclopeptide	–	–	–	8.09	–
Dechlorogriseofulvin	–	–	–	11.6	–
Dihydrocitrinone	–	–	–	39.4	23.8
Flavoglucan	124	103	69.5	280	1030
Griseofulvin	–	–	–	32.0	–
Marcfortine A	1.17	0.56	0.65	1.55	–
Mycophenolic acid	7.22	14.0	82.1	17.5	3.92
Ochratoxin A	–	3.58	4.47	–	10.8
O-methyl viridicatin	1.50	4.71	1.48	7.50	3.35
Pestalotin	5.63	33.3	4.97	23.8	–
Questiomycin A	9.67	36.8	7.63	37.4	–
Quinolactacin A	1.57	2.05	0.95	1.84	0.56
Roquefortine C	4.41	–	2.80	19.6	–
Rugulovasine A	–	14.7	14.4	–	–
Secalonic acid D	6.87	6.67	6.34	7.47	7.45
Verrucofortine	–	–	0.63	–	–
Viridicatin	–	–	–	14.5	–
Viridicatol	54.9	200	94.9	367	76.9
from other fungi					
Abscisis acid	105	130	165	207	125
Ascochlorin	3.13	4.08	–	7.70	–
Bassianolide	31.2	22.2	9.34	16.6	8.40
Monocerin	3.03	8.47	–	4.53	–
Rubellin D	2.99	2.87	–	2.36	–
from bacteria					
Dinactin	–	4.52	–	–	–
Monactin	–	74.6	–	1.31	–
Nonactin	2.32	152	–	2.19	–
Valinomycin	–	16.3	–	–	–
from lichens					
Lecanoic acid	16.9	17.1	13.2	12.4	35.7
Usnic acid	5.27	–	–	3.89	–
phytoestrogens					
Biochanin	96.5	73.3	1670	42.3	105
Daidzein	439	733	1510	394	626
Daidzin	1420	487	4890	585	1940
Formonetin	336	236	2050	218	186
Genistein	377	903	1440	161	846
Genistin	2380	1140	5350	1020	3780
Glycitein	139	275	253	127	183
Glycitin	359	270	254	120	607
Ononin	79.8	14.9	50.0	39.4	26.6
antibiotics					
Lincomycin	–	3,230,000*	–	–	–
Oxytetracycline	2250000*	32,100,000*	73,600,000*	147,000,000*	962,000,000*
Tetracycline	–	404,640*	365,640*	622,200*	255,000*
unspecific					
Asperphenamate	5830	19,780	9600	16,400	15,300

Table 7. (continued).

Secondary metabolite	Concentration in dust samples (ng g ⁻¹)				
	1	2	3	4	5
Brevianamide F	–	–	23.4	27.4	–
cyclo(L-Pro-L-Tyr)	167	276	165	401	40.6
cyclo(L-Pro-L-Val)	511	285	397	887	57.8
Deoxyfusapyrone	–	–	–	12.0	–
Emodin	24.0	17.8	62.0	13.3	18.8
Endocrocin	–	548	519	329	263
Isorhodoptilometrin	4.27	6.20	6.08	3.29	1.72
Lotaustralin	116	41.3	43.2	63.6	55.3
N-benzoyl-phenylalanine	1150	6120	385	6010	2920
Neoechinulin A	488	982	451	536	372
Rugulusovin	6.24	–	15.0	–	–
Skyrin	1.15	–	–	–	3.02
Tryptophol	–	–	–	–	100

–: Below LOD. * Number denotes LC-MS peak area (lack of quantitative standard for this compound).

Genistin (1020–5350 ng g⁻¹) and daidzein (487–4890 ng g⁻¹) were detected in all sedimented dust samples at high concentrations. Together with glycitin, present in the dust (120–607 ng g⁻¹), these isoflavones are dominant compounds in soybeans. The presence of two isoflavones that are characteristic of clover, i.e., biochanin (42.3–1670 ng g⁻¹) and formonetin (186–2050 ng g⁻¹), was also confirmed in dust samples from all barns.

Phytoestrogens were found to have negative effects on the fertility and fecundity of grazing sheep and cows being fed with red clover (clover disease; Yildiz, 2005). It was proven that the consumption of phytoestrogens disturbs hormonal balance in the animal body leading to silent heat, progesterone deficiency, embryonic loss and low semen quality (Glover and Assinder, 2006; Piotrowska et al., 2006). In addition, phytoestrogens were shown to stimulate weight gain and increase growth rate in farm animals (Trenkle and Borroughs, 1978). Oestrogen activity of phytoestrogens depends on many factors such as its chemical structure, bioavailability, species of farm animal, target tissue and oestrogen receptor subtypes (ER α or ER β), and metabolites from the fermentation and digestion of the phytoestrogen.

Literature data indicate that high phytoestrogen consumption in humans is linked to a lower rate of breast and prostate cancer, cardiovascular diseases and osteoporosis (Cornwell et al., 2004). This may be due to the anti-oestrogen action of phytoestrogens in humans and/or their antioxidant activity (Adlercreutz et al., 1995; Siow and Mann, 2010).

Currently, there are no studies on the health consequences of phytoestrogen inhalation in both animals and humans.

Oxytetracycline and tetracycline were detected in the sedimented dust on all cattle breeding premises tested, while lincomycin was detected only in Barn 2 where the number of animals kept was the highest.

Tetracycline and oxytetracycline are used in the prevention and treatment of diseases, and to promote growth in animals such as cattle, pigs, sheep, poultry and others (Daghrir and Drogui, 2013). They are used to treat infections of the respiratory tract (pneumonia), urinary tract, soft tissues, and skin. They are used against infections caused by

a wide spectrum of bacteria, except that enteric gram-negative Bacilli and Staphylococci commonly develop resistance to these antibiotics. One of the most common uses in cattle is the treatment of bovine respiratory disease (BRD) caused by *Pasteurella multocida*, *Mannheimia haemolytica*, and *Histophilus somni* (formerly *Haemophilus somnus*) (Michalova et al., 2004; Papich, 2016).

Lincomycin is an antibiotic used for the treatment of staphylococcal, streptococcal, and *Bacteroides fragilis* infections in both humans and animals (Spížek and Řezanka, 2004).

Both lincomycin and tetracycline are naturally produced by Actinomycetes from the genus *Streptomyces* (Procópio et al., 2012); however, it is most likely that they originate mostly from the residues of medicines administered to animals. The presence of antibiotics in cattle breeding environments may contribute to the development of drug resistance by pathogenic microorganisms and cause difficulties in cattle treatment.

Much fewer secondary metabolites (22 compounds) were detected in the air of the barns compared to the sedimented dust (Table 8). Amongst them, like in the dust, were compounds characteristic of moulds from the genera *Fusarium*, *Aspergillus*, *Penicillium* and *Alternaria*, in addition to non-specific compounds. The air of Barn 6 (the milking parlour), where there were neither animals nor vegetal matter, had the lowest metabolite number (4 compounds). On the other hand, Barn 2 (21 compounds) and Barn 4 (10 compounds) had the highest number, which corresponded to the high number of metabolites found in the dust from these barns. In all livestock premises, we detected 3-nitropropionic acid (at a concentration of 19.7–27.9 ng m⁻³), asperglaucide (5.09–172 ng m⁻³), asperphenamate (2.55–89.4 ng m⁻³), cyclo(L-Pro-L-Tyr) (4.87–8.56 ng m⁻³), cyclo(L-Pro-L-Val) (6.34–57.0 ng m⁻³) and flavoglucan (11.4–79.4 ng m⁻³). Of these, 3-nitropropionic acid, cyclo(L-Pro-L-Tyr), and cyclo(L-Pro-L-Val) and, in addition, brevianamide F were also present in the milking parlour (Barn 6) (Table 8).

Mycotoxins are contaminants of commonly used dairy cattle feeds, including maize, wheat and soybeans, and the processed by-products of these crops, such as maize gluten

Table 8. Concentration of secondary metabolites in the air of tested premises.

Secondary metabolite	Concentration in the air (ng m ⁻³)					
	1	2	3	4	5	6
from <i>Alternaria</i> sp.						
Alternariol	–	4.38	–	–	–	–
Infectopyron	–	105	–	–	–	–
from <i>Aspergillus</i> sp.						
3-Nitropropionic acid	27.9	24.8	21.9	19.7	25.5	29.3
Festuclavine	–	0.08	–	–	–	–
Methyl sulochrin	–	2.07	–	–	–	–
Sterigmatocystin	–	0.07	–	–	–	–
from <i>Fusarium</i> sp.						
Culmorin	–	–	25.1	–	–	–
Enniatin A1	–	0.88	–	–	–	–
Enniatin B	0.44	4.41	–	0.95	–	–
Enniatin B1	0.28	4.15	–	0.31	–	–
from <i>Penicillium</i> sp.						
Andrastin A	–	5.25	–	–	–	–
Aurantine	–	6.14	–	–	–	–
Flavoglaucin	11.4	79.4	24.8	20.0	47.4	–
Quinolactacin A	–	0.16	–	–	–	–
unspecific						
Asperglaucide	23.4	172	5.09	21.6	5.5	–
Asperphenamate	21.1	89.4	5.48	28.9	2.55	–
Brevianamide F	0.69	1.97	–	1.01	2.08	1.75
cyclo(L-Pro-L-Tyr)	5.39	7.89	8.56	4.87	7.56	10.7
cyclo(L-Pro-L-Val)	34.8	57.0	43.5	6.34	50.9	37.4
Emodin	–	0.50	–	0.26	–	–
<i>N</i> -benzoyl-phenylalanine	–	1.01	–	–	–	–
Neoechinulin A	–	43.1	–	–	–	–

–: Below LOD.

meal, wheat grits and soybean husks. The most frequently identified mycotoxins present in animal feedstuffs are aflatoxin B1, deoxynivalenol, zearalenone, ochratoxin A, and fumonisins B1 and B2 (Placinta *et al.*, 1999; Driehuis *et al.*, 2008). Driehuis *et al.* (2008) used LC-MS/MS to identify mycotoxins in 140 maize silages, 120 grass silages and 30 wheat silages manufactured in the Netherlands between the years 2002–2004. They detected deoxynivalenol (DON) in 72% of maize and 10% of wheat silage at concentrations of 854 and 621 mg kg⁻¹, respectively. Zearalenone (ZEN) was identified in 49% of maize and 6% of grass silage at 174 and 93 mg kg⁻¹, respectively (Driehuis *et al.*, 2008). In the current work, DON occurred in all dust samples and ZEN in 4/5 samples.

Mycotoxicosis is an issue in both humans and animals. In cattle it often remains undiagnosed due to a lack of characteristic clinical signs (Obremski *et al.*, 2009).

According to American and Canadian data, DON causes a decrease in (or no) feed intake, decreased lactation and an increase in the number of somatic cells in milk (Charmley *et al.*, 1993; Diaz *et al.*, 2001). ZEN causes lower milk production, decreased appetite, diarrhoea, inflammatory lesions in the intestinal tract and a number of reproductive tract disturbances (irregular periods in oestrus cycle, silent oestrus, foetus death, abortions, placental retention, and uterus and mammary gland, inflammation) (Obremski *et al.*,

2009). Although ruminants have the ability to biodegrade mycotoxins, thanks to the presence of bacteria and protozoa in the rumen (presystemic degradation), such mechanisms are disrupted in many cases (high doses and/or high number of disparate mycotoxins). As a result, mycotoxins are not neutralised, and often new toxic compounds are formed that are absorbed in the duodenum, which can damage internal organs. DON and ZEN have a similar effect on human health. In addition, in the current study, other secondary metabolites were detected that may negatively impact the health of people employed in a cattle farm. Fumigaclavines and chanoclavines affect the central and peripheral nervous systems, while roquefortine C, rugulovasines, viridicatin and viridicatinol can cause neurohumoral and antibiotic activity (Kozlovsky *et al.*, 2013).

Importantly, the effects of mycotoxin ingestion by animals have been the subject of numerous studies, while still little is known about the health consequences of mycotoxin inhalation (Robbins *et al.*, 2000). It is currently believed that mycotoxin inhalation by humans can be up to ten times more toxic compared to skin, alimentary or intraperitoneal exposure, which is probably due to the ease of penetration across alveoli capillary walls (Soroka *et al.*, 2008). However, there is still a lack of data on mycotoxin concentrations in the air in agricultural work environments and epidemiological data on mycotoxin inhalation by agricultural workers.

Cytotoxicity of Dust

In vitro tests, using appropriate cell lines, are useful for characterizing the cytotoxicity of complex mixtures present in the air of different environments, such as organic dust from cattle breeding premises. This is the first report demonstrating cytotoxic effect of dust samples from a cattle farm.

Human epithelial lung cell line, A-549, were exposed to water-soluble fractions of dust samples for 48 and 72 h, with concentrations ranging from 0.3 to 20 mg mL⁻¹. The curves representing cytotoxicity of dust samples are presented in Fig. 5. The IC₅₀ values from the MTT assay are presented in Table 9.

Fig. 5. Cytotoxic activity of water-soluble fractions of dust samples in human lung A-549 cell line, evaluated in MTT assay. Each point represents the mean absorbance values from four repeats ($\pm$ SD). 1–5: Settled dust samples.

Table 9. Cytotoxicity (IC₅₀ values) of water-soluble fraction of dust samples from cattle breeding facilities estimated in MTT assay by using the human epithelial lung carcinoma cell line A-549.

Barn	IC ₅₀ (mg mL ⁻¹)	
	48 h	72 h
1	not detected	13.99
2	17.77	5.66
3	not detected	11.54
4	17.07	12.36
5	not detected	13.09

Generally, we observed that the cytotoxicity of the fractions increased with concentration. The highest tested concentration (20 mg mL⁻¹) induced the strongest cytotoxic effect, especially after 72 h exposure. Samples from Barns 2 and 4 exhibited the strongest cytotoxicity, which was always greater than 20%, even at the lowest tested concentrations, amongst all samples. It was only possible to estimate IC₅₀ after 48 h exposure for samples from these two barns; samples from all other barns required longer exposure times. For instance, the cytotoxicity of samples from Barns 1, 3 and 5 was too low to evaluate IC₅₀ after 48 h. After 72 h, dust from Barn 2 exhibited the strongest cytotoxicity, with an IC₅₀ value of 5.66 mg mL⁻¹ (Table 9). The remaining samples induced comparable results at both exposure times, and after exposure of the cells to 20 mg mL⁻¹ of the sample from Barn 3, the maximal cytotoxicity was about 86% (after 72 h).

The samples from Barn 2 had the highest cytotoxicity. This is in accordance with the concentration of secondary metabolites in the settled dust from this barn, which had the greatest number of fungal compounds. The cytotoxic effect of fungal metabolites on human health is indisputable (Zain, 2011). Barn 2 also had the highest concentration of secondary metabolites in the air, in addition to having antibiotics. Previous work reported the cytotoxic activity of antibiotics on human cell lines, e.g., gingival fibroblasts (FMM1) (Ferreira *et al.*, 2010), hepatoma (C3A), skin fibroblasts (HSF) (Zakrzewska *et al.*, 2014), osteosarcoma (MG63) and cervix carcinoma (HeLa) (Duewelhenke *et al.*, 2006). Antibiotics can disrupt mitochondrial function and induce oxidative stress (Xiao *et al.*, 2019). Oxidative stress to respiratory epithelial cells plays a crucial role in mediating respiratory diseases in farm animals and human workers (McClendon *et al.*, 2015).

CONCLUSIONS

Cattle breeding premises are diverse work environments where, additionally, many factors, including the animal population, volume of the premises, sanitary conditions, ventilation system and season, can influence the types and risk levels of biological threats. Our research was conducted during winter and early spring, when microorganisms are less likely to survive in the air. Therefore, it is highly probable that greater exposure to individual microbial groups occurs at higher temperatures. Future studies should verify the relationships between the various parameters of cattle breeding premises and of microbiological risks (e.g.,

the number of microorganisms, the airborne concentrations of the total dust and the levels of endotoxins and mycotoxins) for farms with different characteristics.

Our study found high and very high correlations between the number of microorganisms in the air and that in the dust of the cattle farm, indicating that deposited dust may constitute secondary contamination in such facilities. Therefore, monitoring sanitary conditions and regularly removing sedimented dust may reduce the risk of inhaling particles of microbial origin, including those with allergenic and toxic properties.

High-throughput sequencing revealed a large diversity of microorganisms in both the air and the sedimented dust on the farm, which has not previously been reported in the literature. Of the detected species, those belonging to the genera *Corynebacterium*, *Staphylococcus*, *Ruminococcus*, *Aspergillus* and *Cladosporium*, as well as *Bacteroides fragilis*, were identified as potentially harmful to human and cattle health.

The dust samples contained as many as 113 chemical compounds, including metabolites characteristic of fungal species from the genera *Fusarium*, *Penicillium*, *Aspergillus* and *Alternaria*; metabolites of bacterial origin; and phytoestrogens and antibiotics in high concentrations, which contributed to the cytotoxicity of the deposited dust to human lung cells. The most cytotoxic sample was collected from the barn with the highest secondary metabolite concentration as well as the worst microbiological contamination (Barn 2). Additionally, the presence of antibiotics (lincomycin, oxytetracycline and tetracycline) has been confirmed in cattle breeding environments, which may account for the development of drug resistance in pathogenic microorganisms.

The concentrations of the airborne dust in the barns did not exceed the threshold limits for workplaces according to national legislation. Therefore, the employees were not legally obligated to wear respiratory protective equipment, e.g., filtering half masks. However, the use of such equipment, with its anti-odour and anti-microbial properties, may enhance the comfort and safety of workers.

Our results demonstrate the complex microbiological risks on cattle farms. We recommend conducting epidemiological studies to determine the effects of exposure to these bioaerosols, endotoxins, mycotoxins, phytoestrogens and antibiotics. Currently, no legal guideline identifies safe inhalation exposure limits or specific health effects for these substances.

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SUPPLEMENTARY MATERIAL

Supplementary data associated with this article can be found in the online version at <http://www.aaqr.org>.

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