



Production of pro-inflammatory mediators stimulated by exposure of poultry house workers to airborne dust particulates

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A – Research concept and design, B – Collection and/or assembly of data, C – Data analysis and interpretation, D – Writing the article, E – Critical revision of the article, F – Final approval of the article

Górny RL, Cyprowski M, Gołofit-Szymczak M, Ławniczek-Wałczyk A, Stobnicka-Kupiec A, Wolska LA. Production of pro-inflammatory mediators stimulated by exposure of poultry house workers to airborne dust particulates. *Ann Agric Environ Med.* 2023; 30(4): 623–633. doi: 10.26444/aaem/174201

Abstract

Introduction and Objective. Poultry house employees spend a significant part of their work shift being exposed to airborne particulate pollutants. The aim of this study was to assess their exposure at different stages of chicken production cycle, based on quantification of pro-inflammatory mediators (IL-1 β , IL-6, IL-8, and TNF α) in nasal lavage (NAL) samples.

Materials and method. The concentrations of airborne dust at 3 different stages of the production cycle (i.e. empty poultry house, with 7- and 42-day-old chickens) were stationary measured using Grimm spectrometer, as well as CIS and Button samplers. The dust collected by the latter 2 samplers was analyzed for endotoxin and (1 \rightarrow 3)- β -D-glucan content. NAL samples were collected from employees after their work shift to determine the pro-inflammatory mediator levels.

Results. The maximum particulate aerosol, endotoxin, and (1 \rightarrow 3)- β -D-glucan concentrations at workplaces reached the levels of 4.12 mg/m³, 45.21 ng/m³, and 56.54 ng/m³, respectively. The IL-1 β , IL-6, and IL-8 concentrations in NAL samples ranged between 0.62–18.12 pg/mL, <0.70–25.37 pg/mL, and <3.50–259.5 pg/mL, respectively. All TNF α levels were below 4 pg/mL. There were no significant differences between these cytokine concentrations in NAL samples collected at different stages of chicken breeding in either 'winter' or 'summer' seasons.

Conclusions. Inhalation stimulation with poultry dust containing endotoxins and (1 \rightarrow 3)- β -D-glucans resulted in the production of pro-inflammatory mediators, which proves the course of immunological processes in the exposed employees that may lead to adverse effects. The use of nasal lavage fluid in the control of such exposure confirms that NAL analysis is a reliable laboratory tool for assessing the impact of poultry dust on exposed farm workers.

Key words

endotoxins, glucans, size distribution, nasal lavage, poultry house, particulate aerosol, pro-inflammatory mediators

INTRODUCTION

In recent years, the poultry industry has been revolutionized by the emergence of modern technologies and various systems that increase the profitability of chicken farming and at the same time provide animals with appropriate living conditions. With these technologies, farmers automate many processes from feeding and watering animals to controlling temperature and humidity levels inside the poultry house [1, 2]. Such automation allows for more precise supervision of the environment in which the poultry is reared; however, modern methods of poultry facility management still require that employees spend a significant part of their work shift being exposed to comparatively high levels of particulate and volatile pollutants [3].

Poultry dust, i.e. dust present in poultry houses and arising from work activities on the farms, is a complex mixture of organic and inorganic materials derived from soil, bedding, and other particles of vegetable origin (e.g. pollen grains, vegetal fibers, etc.), feed and feed components, chemicals

and therapeutic additives, faeces, feathers, epidermis and other animal origin particles, as well as microbiological and invertebrate contaminants [4]. Among microbial components of poultry dust, the most abundant and important are viable and non-viable microorganisms (bacteria, fungi, viruses, protozoa) and their metabolites, including endotoxins and (1 \rightarrow 3)- β -D-glucans [4–7].

There is broad scientific evidence that exposure to poultry dust, commonly understood as one of the types of organic dust, may exert numerous adverse effects on the human body from infections to diseases of both acute and chronic character. The latter effects include work-related respiratory symptoms and disorders (e.g. coughing, wheezing, sneezing, phlegm, sputum, chest tightness, nasal congestion and discharge, eye and throat irritation, dyspnea, distress, etc.), decline in lung function, increased airway responsiveness, pulmonary diseases, and a wide palette of allergic reactions [e.g. 4, 5, 8–14]. According to Viegas et al. (2013), for many of these adverse effects, the type of health response depends on the level of contamination and frequency of exposure [15]. Nevertheless, epidemiological studies show that regardless of how high and how frequent this type of exposure is, inhalation of poultry dust at the levels likely to be encountered in commercial poultry production could trigger allergic and exacerbate existing respiratory diseases [4, 13].

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Received: 07.09.2023; accepted: 16.10.2023; first published: 23.10.2023

As breathing provides us with the biggest interaction with our surroundings, the respiratory tract stands pre-eminent as the one of internal system most directly affected by the environment as well as the main pathway for particulate matter to enter the body [16]. Nearly three decades ago, Dr. Brain, summarizing the Aspen Lung Conference, made the following statement: 'I assert, and I expect no disagreement that more than 90% of lung disease is either initiated by or at least aggravated by the inhalation of particles and gases' [17]. Indeed, despite the passage of time since that conference, this statement still seems to be extremely accurate and up-to-date. The average adult human at rest, takes 12 – 16 breaths per minute [18, 19]. Taking into account an average tidal volume of 500 mL/breath, a total intake equals approximately 10,000 L of air per day. Increased physical activity can very easily double this volume to 20,000 L, which means that more than 20 kg of air enters our body each day [18, 20, 21]. The weight of the air inhaled is far greater than the weight of the food and water ingested [17]. Against this background, the respiratory tract is unique in that the relationship between it and the environment is profound.

Poultry farms dedicated to broiler chicken production, where birds are bred to reach slaughter weight in relatively short period of time (i.e. about 42–43 weeks), usually have an in house stocking rate (birds per usable area) of about 15–18 chickens per 1 m², which means that in one production cycle, about 30,000 chickens could be reared in the poultry house [6]. The farms holding such a large number of animals at high densities become intensive sources of emissions into the air of many harmful particles. Such environmental circumstances place farm workers at high health risk that is additionally shaped by the physicochemical characteristics of the particles (i.e. their sizes, shapes, charges, densities, hygroscopicity, solubility, and chemical reactivity) and by host factors, such as respiratory route (nasal versus mouth breathing), rate and tidal volume, and respiratory tract anatomy [22]. Since most of the air inhaled during normal breathing enters through the nose, the nasal mucosa acts as the main barrier that traps inhaled pollutants [23–25]. Hence, an analysis of nasal lavage (NAL) fluid may serve as a reliable analytical tool to control such inhalation exposure in occupational environment contaminated with organic dust [26].

The aim of this study was to assess the exposure of poultry house workers at different stages of chicken production cycle to airborne particulate (poultry dust, endotoxins, and (1→3)-β-D-glucans) pollutants, based on the quantification of pro-inflammatory mediators (i.e. interleukins IL-1β, IL-6, IL-8, and tumour necrosis factor α, TNFα) in NAL samples.

MATERIALS AND METHOD

Poultry farm characteristics. The studied poultry farm was located in north-eastern Poland. Three-quarters of the farm's surroundings are occupied by arable fields, and the remaining one-quarter of the area is devoted to residential premises and technical buildings of small companies not related to agricultural production, both located about 500 m from the farm. The investigated poultry house was a free-standing building, equipped with an automatically regulated ventilation system (its exhaust capacity was 4,000 m³ per hour). The operation of fans depended on the microclimate parameters inside the poultry house set at a given stage of

rearing, and related to the microclimate conditions outside the building. In the henhouse, broiler chickens were fattened in a bedding system. The poultry house also had automated watering and feeding systems. The production cycle of chickens lasted from the receipt of 1-day-old chickens to about 6 weeks of age and their slaughter weight of about 2.5 kg. About 6 rearing cycles are carried out on the farm annually. The in-house stocking rate (birds per usable area) was about 18 chickens per 1 m², which means that in one production cycle, about 30,000 chickens were bred in the studied poultry house. Manure (droppings with litter) was removed from building after completing the full rearing cycle but was not stored on the farm. For periodic disinfection of the henhouse between successive stockings with new chickens, the floor was washed with water, then sprayed (usually with ammonia water or sodium hypochlorite), and subsequently fogged with a bactericidal, virucidal and fungicidal preparation. During the production cycle, about 5 employees took care of the chicken flock. Workers were equipped with personal protective equipment, including goggles and gloves, but did not use respiratory protection.

Particulate aerosol analyzes. Altogether, 6 sampling campaigns were carried out during 'winter' (3 campaigns in the period February – March) and 'summer' (also 3 campaigns in the period June – September) seasons. In each sampling season, the bioaerosol measurement campaigns covered 3 different stages of the production cycle, i.e. in the clean and disinfected poultry house without chickens, as well as in the henhouse with 7-day-old (i.e. 1 week after flock stocking) and 42-day-old chickens (i.e. about one day before they departure to the slaughterhouse). In each sampling campaign, particulate measurements were simultaneously stationary performed by using a Grimm aerosol spectrometer (model 11A, Grimm Aerosol Technik GmbH, Ainring, Germany), as well as a Conical Inhalable Sampler (CIS) equipped with an APEX pump (both from Casella Measurements Inc., Bedford, UK) and a Button Aerosol Sampler (SKC Inc., Eighty-Four, USA) supplied with a GilAir5 pump (Sensidyne, LP, St. Petersburg, USA) operated at flow rates of 1.2 L/min as well as 3.5 L/min and 4 L/min, respectively. All particulate aerosol measurements were conducted for 3.5 hours in 5 replicates. All samplers (Grimm, CIS, and Button) were placed at the height of 1.5 m above floor or ground level (to simulate aspiration within the human breathing zone) [27] and at a distance of 1 m from each other to avoid possible interferences occurring between them.

A total of 75 workplaces (i.e. 25 Grimm, 25 CIS, and 25 Button) and 25 background (Grimm) samples were analyzed. In the case of the Grimm spectrometer, in addition to particulate mass concentration, the analyzer calculated the numbers of particulates in 31 size channels corresponding to their optical diameters from 0.25 μm – 32 μm, making readings at 10-min intervals. In the case of the CIS sampler, the particulates were collected on 37-mm Teflon filters with 2 μm pore size; regarding the Button sampler particulates were collected on 25-mm Teflon filters, 1 μm pore size (both from SKC, Ltd.). The collected particulates were gravimetrically determined by weighting the filters before and after sampling with ultra-microbalance (model XS105D, Mettler Toledo GmbH, Zürich, Switzerland), following in both cases a 24-hour equilibration period at constant air temperature and humidity (22±3 °C and 45±5%, respectively). After

gravimetric analysis, each filter was transferred into a 50 mL polypropylene tube filled with 10 mL of sterile pyrogen-free water (Lonza, Walkersville, USA) containing 0.05% Tween 20 (Sigma-Aldrich Sp. z o.o., Poznań, Poland) and vortexed on a platform shaker (model Promax 1020, Heidolph Instruments GmbH & Co., Schwabach, Germany) at room temperature for 15 min. After extraction, the resulting suspension was used to determine endotoxin and (1→3)-β-D-glucan concentrations.

Measurements of endotoxin and (1→3)-β-D-glucan concentrations. Endotoxin and (1→3)-β-D-glucan concentrations from particulate aerosol samples were quantitatively analyzed using kinetic QCL Limulus Amebocyte Lysate (LAL) (Lonza) and GlucateLL (Associates of Cape Cod, East Falmouth, MA, USA) assays, respectively. Briefly, after extraction and vortexing of the samples, the remaining particulate suspensions were centrifuged at $1,000 \times g$ for 15 min (model 5804 R, Eppendorf AG, Hamburg, Germany) and divided into 2 equal parts. From the first part of supernatant, 1.8 mL was spectrophotometrically analyzed (model Sunrise, Tecan Group Ltd., Männedorf, Switzerland) at the wavelength of 405 nm at 37°C for endotoxins using LAL assay with a potency of 9 endotoxin units, EU, per 1 ng against *E. coli* 055:B5 standard endotoxin. The concentration of airborne endotoxins was expressed in ng/m^3 .

The second part of supernatant was again vortexed (model BioVortex V1 Plus, Biosan, Riga, Latvia) for 2 more min, followed by additional 10 min agitation in an ultrasonic bath (model Sonic 5, Polsonic, Warsaw, Poland). Directly afterwards, 6 M NaOH was added in a volume to obtain its final concentration of 0.3 M NaOH, and the resulting suspension was additionally shaken for 10 min at 4°C temperature, and again centrifuged at $1,000 \times g$ for 15 min. The (1→3)-β-D-glucan concentrations were spectrophotometrically assayed (Tecan Group Ltd.) at wavelengths of 405 nm in 37°C using GlucateLL assay and expressed in ng/m^3 . For both endotoxins and (1→3)-β-D-glucans, their limits of detection were defined as the smallest concentrations that produced a signal in calibration curves (i.e. $0.08 \text{ ng}/\text{m}^3$ and $0.01 \text{ ng}/\text{m}^3$, respectively). All performed analyzes were duplicated. In total, 50 (25 CIS and 25 Button) workplace endotoxin samples and the same number (50) of workplace (1→3)-β-D-glucan samples were analyzed.

Nasal lavage sampling and analyzes. NAL samples were collected from 4 employees after their work shift according to the modified Greiff et al. method [28]. To begin with, 20 mL of physiological salt solution was forced into the nostril using a sterile syringe with soft rubber tip. The resulting fluid was carefully collected into a sterile Falcon-type tube and its volume was measured. The percentage of recovered NAL fluid ranged between 75–85% of the initial volume of saline. In the next step, NAL fluid was centrifuged at $1,200 \times g$ for 10 min at 4°C (Eppendorf AG) and the collected supernatant analyzed for pro-inflammatory mediators' content. For quantitative determination of interleukins IL-1β, IL-6, IL-8, and tumour necrosis factor TNFα in collected NAL samples, commercially available Human DuoSet ELISA kits (R&D Systems, Inc., Minneapolis, USA) were used.

In order to avoid microbial contamination, all materials in contact with the samples or reagents used for the test were sterile and pyrogen free. Before the test, NAL samples and reagents were brought to room temperature. According to the

test procedure, 100 μL of the capture antibodies in phosphate-buffered saline (PBS) was applied to the microplate and then incubated overnight at room temperature. After incubation, the microplate wells were washed 3 times with washing buffer using an automatic scrubber. Subsequently, 300 μL blocking solution (1% bovine serum albumin (BSA) solution in PBS) was applied to the wells and incubated at room temperature for a minimum of 1 h. The serial dilutions of both the analyzed NAL sample (ratios of 1:1, 1:5, 1:25, and 1:125) and standard (reference recombinant human cytokine; ratios of 1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, 1:512, and 1:1024) in 1% BSA solution in PBS were prepared, and, after one more washing of the microplates, added in 100 μL volumes into the wells and incubated for 2 h at room temperature. After another microplate washing, 100 μL of the detection antibody suspension in 1% BSA in PBS was added to each well and a 2-hour incubation performed. The wells were then washed again and 100 μL of streptavidin-HRP solution was added to each well. After 20 min incubation, the microplate was washed again and 100 μL of the substrate solution was applied to the wells and incubated for another 20 min. To stop the reaction, 50 μL of the inhibiting solution (2N H₂SO₄) was added. Subsequently, the optical density of each well was spectrophotometrically determined (Tecan Group Ltd.) using a primary wave length of 450 nm and reference wave length of 540 nm. Cytokine concentrations in NAL were expressed in pg/mL. The applied analytical kits had the following real quantification thresholds: 1 pg/mL for IL-1β, 0.7 pg/mL for IL-6, 3.5 pg/mL for IL-8, and 4 pg/mL for TNFα. For each studied cytokine, all collected samples were analyzed in duplicate. In total, 52 samples (13 for IL-1β, IL-6, IL-8, and TNFα, respectively) were analyzed.

Nasal lavage sampling was approved by the Bioethics Committee.

Measurement of microclimate parameters. The environmental conditions (temperature and relative humidity of the air) at workplaces in the poultry house and in the atmospheric (outdoor) air were recorded during every sampling session with thermohygrometer (model Omniport 20, E+E Elektronik GmbH, Engerwitzdorf, Austria). All microclimate parameter measurements were performed in triplicate.

Statistical analysis. After checking the normality of data distributions with Shapiro-Wilk test, the collected data were statistically elaborated by analysis of variance (ANOVA), *t*-test, and Pearson correlation analysis using Statistica (data analysis software system) version 10. (StatSoft, Inc., Tulsa, USA). Probability values were treated as statistically significant at $P < 0.05$.

RESULTS

Particulate aerosol. The concentrations (arithmetic means with standard deviations) of particulate aerosols at workplaces in the poultry house and in outdoor (atmospheric) air measured using CIS and Button Aerosol samplers, as well as the Grimm optical particle counter, are presented in Figure 1. The particulate aerosol concentrations at workplaces ranged from $0.07 \text{ mg}/\text{m}^3$ in the empty poultry house to $4.12 \text{ mg}/\text{m}^3$ in the poultry house with 42-day-old chickens in the 'winter' season, whereas in outdoor air it ranged from $0.01 \text{ mg}/\text{m}^3$ to

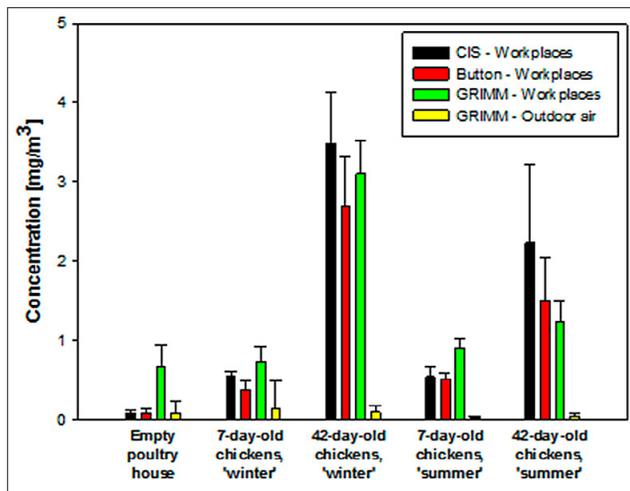


Figure 1. Concentrations of particulate aerosols at workplaces in the poultry house and in outdoor (atmospheric) air measured using CIS and Button Aerosol samplers, and a Grimm optical particle counter. Columns and whiskers represent mean concentrations and standard deviations, respectively

1.17 mg/m³. There were no significant differences between the particulate aerosol concentrations measured at workplaces with CIS, Button, and Grimm samplers (ANOVA: $P > 0.05$).

When chickens were introduced into the poultry house, independent of the sampling method used, the workplace concentrations of particulate aerosol always significantly augmented those measured in outdoor air ($P < 0.05$). The dynamics of production activities at particular stages of chicken breeding cycle in both seasons resulted in significant differences in particulate aerosol concentrations (ANOVA: $P < 0.05$). The highest concentrations were noted in the poultry house with 42-day-old chickens in the 'winter' season, and were significantly higher than those measured at workplaces with 7-day-old chickens in both seasons and in the empty henhouse (in all cases – Tukey tests: $P < 0.05$). In one case only, i.e. in the poultry house with 42-day-old chickens in the 'winter' season, particulate aerosol concentrations measured with CIS sampler crossed 4 mg/m³, which is the maximum permissible concentration for organic dust of animal and/or plant origin in a working environment [29]. Among all stationary samples collected using this aspirator, however, only 4% exceeded the above mentioned limit. This demonstrates that the appearance of such high pollution levels has the nature of temporary concentration peaks, which do not cover a significant part of the work shift.

Size distributions of particulate aerosol. The use of a Grimm spectrometer allowed obtaining data on size distribution of particulate aerosols at workplaces in poultry house and in outdoor (atmospheric) air (Fig. 2). The particulate aerosol concentrations at workplaces, as well as in outdoor environment in both submicron and micrometric size ranges, reached very high values of 2.6×10^9 #/m³ and 6.2×10^7 #/m³ as well as 3.9×10^9 #/m³ and 2.1×10^6 #/m³, respectively. The comparison of indoor and outdoor size distributions revealed significant differences between them. These differences became especially evident when 42-day-old chickens (t -tests: in 'winter' season – $P < 0.001$, in 'summer' season – $P < 0.05$) and 7-day-old chickens (t -tests: in 'winter' season – $P = 0.0588$, in 'summer' season – $P < 0.05$) were present in poultry house. The increasing activity of the birds, along with their physical

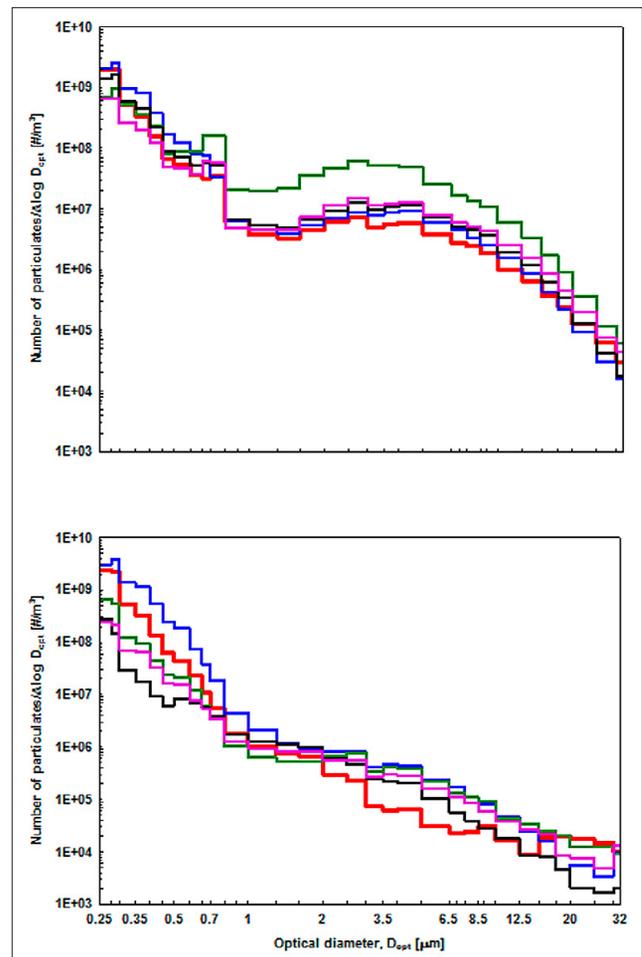


Figure 2. Size distributions of particulate aerosols at workplaces in the poultry house (top) and in outdoor (atmospheric) air (bottom). The colours of step plots indicate: red – empty poultry house (averaged for 'winter' and 'summer' seasons), blue – 7-day-old chickens in 'winter' season, green – 42-day-old chickens in 'winter' season, black – 7-day-old chickens in 'summer' season, and violet – 42-day-old chickens in 'summer' season

growth in both size and weight, was a significant source of particulate emissions into the poultry house air.

Endotoxins and (1→3)-β-D-glucans in aerosol samples. The concentrations (arithmetic means with standard deviations) of airborne endotoxins and (1→3)-β-D-glucans measured with CIS and Button samplers at workplaces in poultry house are presented in Figure 3. There were no significant differences between their levels determined using CIS and Button samplers (t -tests: in both cases – $P > 0.05$). The endotoxin concentrations measured using the CIS sampler ranged from 1.16 ng/m³ in the empty poultry house to 45.21 ng/m³ in the henhouse with 42-day-old chickens in 'summer' season, whereas the levels obtained with the Button sampler ranged from 0.16 ng/m³ to 26.02 ng/m³ in respective environmental circumstances. In turn, the (1→3)-β-D-glucan concentrations measured using the CIS sampler ranged from 3.32 ng/m³ in the poultry house with 42-day-old chickens in 'winter' season, to 56.54 ng/m³ in henhouse with 42-day-old chickens in 'summer' season, while determined with Button sampler ranged from 1.18 ng/m³ to 30.39 ng/m³ under the same environmental conditions as previously mentioned. There were also no significant differences between the endotoxin and (1→3)-β-D-glucan levels measured at workplaces in different

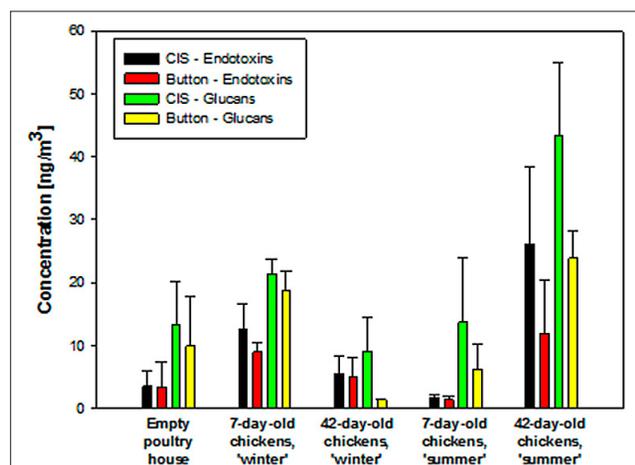


Figure 3. Concentrations of endotoxins and (1→3)-β-D-glucans in aerosol samples from workplaces in the poultry house measured using CIS and Button Aerosol samplers. The columns and whiskers represent mean concentrations and standard deviations, respectively

stages of chicken breeding and in different seasons (in both cases – ANOVA: $P > 0.05$). Correlation analyzes revealed that, regardless of the sampler used to measure the concentrations of particulate aerosols, their levels positively and significantly influenced the noted concentrations of endotoxins ($r^2 = 0.08$ at $P < 0.05$). Also, for both CIS and Button samplers, endotoxin concentrations determined with their use positively and significantly correlated with the levels of (1→3)-β-D-glucans measured with the same samplers ($r^2 = 0.67$ at $P < 0.05$).

The comparison of measured endotoxin and (1→3)-β-D-glucan concentrations with the existing proposals for occupational exposure limit (OEL)/threshold limit values (TLV), revealed ambiguity in the assessment of the environmental situation described in this way. The endotoxin concentrations measured in the poultry house with both CIS aspirator and Button sampler were lower than the OEL/TLV proposed by expert committees (i.e.: 200 ng/m³ proposed by the Expert Group on Biological Agents at the Polish Interdepartmental Commission for Maximum Admissible Concentrations and Intensities for Agents Harmful to Health in the Working Environment [30]; 1,000 EU/m³ recommended by the Swiss SUVA occupational committee [11] and the French Health Insurance Network–Occupational risks consists of INRS, Carsat, Cramif, and CGSS [31]; and OEL/TLV proposed by different scientist or groups (e.g.: Donham et al. (2000) [32] – 614 UE/m³; Haglind and Rylander (1984) [33] – 80 ng/m³; Clark (1986) [34] – 100 ng/m³; Rylander (1987) [35] – 100 ng/m³; Malmros et al. (1992) [36] – 100 ng/m³).

On the other hand, 23% as well as 6% of the measurements obtained using both CIS and Button samplers were higher than 90 EU/m³ (i.e. the OEL proposed by the Dutch Expert Committee on Occupational Safety (DECOS), based on Castellan et al. studies [37] as a not observed effect level for both chronic and short-term exposure to inhalable endotoxins [38]), as well as higher than 25 ng/m³ (i.e. OELs recommended by Laitinen et al. (2001) [39] – 25 ng/m³ and Palchak et al. (1988) [40] – 30 ng/m³).

In turn, regarding (1→3)-β-D-glucan concentrations in the air of the studied poultry house, their comparison with OEL proposed by Parker et al. (150 ng/m³) as value protecting workers against inflammatory and immunosuppressive reactions and possibly development of respiratory diseases

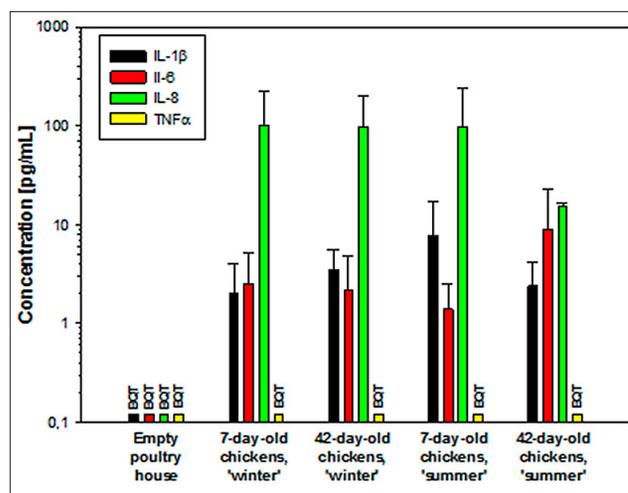


Figure 4. Cytokine (interleukins IL-1β, IL-6, IL-8, and tumour necrosis factor TNFα) concentrations in nasal lavage samples collected from workers exposed to aerosol particulates in the poultry house. Columns and whiskers represent mean concentrations and standard deviations, respectively. BQT – below quantification threshold

[41], revealed that all (1→3)-β-D-glucan concentrations measured with both CIS and Button samplers were below this occupational exposure level.

Cytokines in NAL samples. The levels of pro-inflammatory mediators in nasal lavage samples collected from poultry house employees are presented in Figure 4. The concentrations of IL-1β, IL-6, and IL-8 ranged between 0.62–18.12 pg/mL, <0.70–25.37 pg/mL, and <3.50–259.5 pg/mL, respectively. All measured TNFα concentrations in NAL samples were below quantification threshold, i.e. <4 pg/mL. After occupational activities carried out in empty poultry house, in all tested samples from employees, the concentrations of all studied cytokines were below their quantification thresholds. Their levels in NAL samples, taking into account the different stages of chicken breeding (7- and 42-day-old chickens) in both 'winter' and 'summer' seasons, revealed no significant differences (in both cases – ANOVA: $P > 0.05$). Correlation analysis showed that only the concentrations of IL-6 from among all tested cytokines in NAL samples showed positive relationships with the concentrations of particulate aerosols, endotoxins, and (1→3)-β-D-glucans obtained using CIS and Button samplers; however, statistical significance of these dependences were confirmed for endotoxin and (1→3)-β-D-glucan concentrations measured using the CIS sampling head only (in both cases: $r^2 = 0.90$ at $P < 0.05$). All other factors measured in this study, including temperature and relative humidity of the air, did not significantly influence the concentrations of tested cytokines. When analyzing pro-inflammatory mediator levels in the NAL fluid, it is also worth mentioning that in all tested samples, the level of IL-6 significantly negatively correlated with the concentration of IL-8 ($r^2 = 0.98$ at $P < 0.05$).

Influence of microclimate parameters on particulate aerosol, endotoxin, (1→3)-β-D-glucan, and cytokine concentrations. The mean values (and ranges) of air temperature at workplaces in the poultry house and in the background (outdoor) environment in 'summer' as well as in 'winter' seasons, were as follows: 25.5°C (24.8–26.3) and 28.2°C (28.1–28.4), as well as 33.7°C (32.8–34.7) and -3.9°C (-3.7–-4.1) at the beginning of the rearing period;

22.1 °C (21.3–23.8) and 16.5 °C (16–17.1), as well as 18.9 °C (16.6–23) and 2.8 °C (2.6–3.1) at the end of the rearing period. In turn, the respective mean values (and ranges) of relative humidity of the air were as follows: 48.4% (45.5–52.4) and 36% (34.5–37.1), as well as 28.8% (26.1–31.5) and 18.7% (18.5–18.9) at the beginning of the rearing period; 69.4% (65.5–70.8) and 73% (70.4–75.2), as well as 66.1% (60.5–71.8) and 86.2% (85.3–87.1) at the end of the rearing period. Neither temperature nor relative humidity of the air had statistically significant effects on particulate aerosol, endotoxin, (1→3)- β -D-glucan concentrations measured at different stages of chicken breeding (in the empty poultry house as well as with 7- and 42-day-old chickens in the poultry house), and in both seasons ('winter' and 'summer'), as well as on cytokine concentrations in NAL samples from poultry farm workers.

DISCUSSION

Poultry dust is very heterogeneous and may be composed of particles of agriculture products and plants, feed including grain, hay and silage, animal particulates derived from dander, feathers, urine and faeces, microbiological particulates, such as bacteria, fungi, their by-products (including endotoxins and glucans), pollens and mites, as well as mineral components from soil sources [4, 7, 42–44]. Broiler farm workers can be exposed to dust during routine bird care, including feeding, cleaning, handling chickens, replacing bedding, etc. This dust is especially problematic when generated and subsequently suspended for a long time in the air of enclosed spaces, such as confinement buildings. Despite the use of increasingly modern poultry farming systems, its presence in farm buildings is still associated with significant human pulmonary exposure to dust-derived microorganisms and their by-products. In the present study, particulate aerosol concentrations (understood here as inhaleable fraction of dust) determined in poultry house, did not differ in their levels from the concentrations determined in this type of work environment by other researchers (proper value ranges are given in parentheses) in: Spain (0.03–15.2 mg/m³) [6, 15], Switzerland (0.42–21.75 mg/m³) [45], The Netherlands (4–4.4 mg/m³) [46], Croatia (1.8–4.8 mg/m³) [47], Sweden (1.76–5.17 mg/m³) [48], USA (0.03–5.58 mg/m³) [49–52], China (0.17–9.61 mg/m³) [53], Korea (0.53–31.5 mg/m³) [54–56], Pakistan (0.66–1.56 mg/m³) [13], Iran (2–5.4 mg/m³) [57], Saudi Arabia (2.11–18.11 mg/m³) [58], Egypt (0.63–3.13 mg/m³) [43], South Africa (1.16–57.52 mg/m³) [44], and were almost on the same level as in an earlier study performed by the authors of the current study in poultry houses in southern Poland (0.03–4.51 mg/m³) [58]. However, some measurements carried out in the USA [59] and China [60] revealed much higher inhalable poultry dust levels in chicken farms, reaching 92.4 mg/m³ and 230 mg/m³, respectively.

In occupational settings, bacteria and fungi can induce adverse health responses via inhalation of endotoxins and (1→3)- β -D-glucans [6]. Both of these microbial by-products have been recognized for their potent ability to induce pro-inflammatory reactions [21, 61, 62]. Endotoxins are a permanent component of poultry dust. Work on poultry farms with high endotoxin contamination levels may lead to lung function changes and the development of acute and chronic respiratory diseases [e.g. 7, 13, 63, 64]. Based on several studies, it can be hypothesized that an increase

in airborne concentration of endotoxins may lead to well-defined adverse effects on human health, starting from decrease in lung functions at 53 EU/m³, through pulmonary impairment at 90 EU/m³, airway inflammation and mucous membrane irritation at 200 EU/m³, over-shift decline in forced expiratory volume in the first second (FEV₁) at 2,000 EU/m³, chest tightness at 3,000 EU/m³, to organic dust toxic syndrome at 10,000–20,000 EU/m³ [35, 37, 65, 66].

The hitherto obtained endotoxin concentrations in the poultry farm environment present a rather consistent picture of such pollution which in individual countries on different continents reaches the following values (adjusted from EU/m³ to ng/m³ using 10 EU equal to 1 ng as conversion factor): in Switzerland (18.99–1,634.8 ng/m³) [45], United Kingdom (0.02–76.2 ng/m³) [67, 68], The Netherlands (45.22–469.3 ng/m³) [46, 67], Denmark (116.6–139.7 ng/m³) [67], Sweden (10–1,003 ng/m³) [48, 66], France (3.5–315.6 ng/m³) [64], Germany (1.98–6,434.7 ng/m³) [67, 69], Korea (0.16–251 ng/m³) [54–56, 70], Iran (5.4–23.6 ng/m³) [57], and the USA (13.5–1,360 ng/m³) [49, 51, 67]. Endotoxin concentrations measured in earlier research by the authors of this study, performed in poultry houses in southern Poland, were at much higher levels (0.04–8,364 ng/m³) [58] than those noted in the current study. They were also much higher than the endotoxin concentrations measured in broiler farms in South Africa (21.2–308,100 ng/m³) [44].

From the perspective of occupational exposure in agricultural settings, endotoxin is probably the most relevant parameter identified so far with airborne particulates associated with lung function impairment [46]. Its microbial origin, ubiquity, persistence, and capacity to attach to substances and particulates make it a challenging material to control in agricultural operations [71]. Numerous studies have underlined that the concentrations of inhaled endotoxins are associated with the development of airflow obstruction among poultry workers, as well as with increased levels of IL-1 β , IL-6, IL-8, and TNF α in lavage fluid (see below) [72].

Another biologically-potent agents in poultry dust are (1→3)- β -D-glucans which are able to initiate a wide range of biological responses in vertebrates inducing cytokine release [73], pulmonary and gastrointestinal symptoms, and diseases [68, 74]. They also have a well-documented epidemiological potential to play a crucial role in the development of respiratory tract diseases (including airway inflammation and farmer's lung) and exacerbation of some disorders (e.g. dry cough or nose irritation) [6, 58, 66, 75].

The hitherto measured (1→3)- β -D-glucan concentrations in poultry farms revealed the following contamination levels, in Sweden (0.01–870 ng/m³) [66], Germany (2–972 ng/m³) [76], USA (11.4–272.4 ng/m³) [51], and South Africa (15–2275 ng/m³) [44]. The (1→3)- β -D-glucan concentration measured in the poultry house in the current study was on a much lower level than in earlier measurements by the authors performed in poultry houses in southern Poland (0.8–6,886 ng/m³) [58].

In the case of the tested poultry house, the highest concentrations of particulate aerosol were observed in 'winter' at the end of the rearing period and regardless of the measurement technique used, were higher than those in 'summer' for the same rearing stage (Fig. 1). Such season-dependent concentration differences are related to the microclimatic conditions prevailing in a given period of the year. In 'winter', when the atmospheric air is cold, the indoor spaces of the poultry house are much more

airtight than in 'summer' season to maintain appropriate breeding parameters. In the warmer period of the year, the operation intensity of the poultry house ventilation system increases, which results in an increased removal efficiency of particulate contaminants from its interior. In contrast to particulate aerosol concentrations, the highest endotoxin and (1 \rightarrow 3)- β -D-glucan concentrations in the air of poultry house were noted in 'summer' season at the end of the rearing period (Fig. 3). Moreover, while in the 'summer' season an increase in the concentrations of these substances in the air was observed, along with an increase in the number of days of chicken rearing, in the 'winter' season this trend was the opposite. The warmer season favoured the development of microorganisms which (at relatively high values of air temperature and humidity inside the hen house) support the development of microbiota, which are the source of both of these biologically-active substances. On the other hand, the recorded decrease in endotoxin and (1 \rightarrow 3)- β -D-glucan concentrations in 'winter' season with the increase in the number of days of chicken rearing may be the result of changes in the bedding replacement system. In 'winter' season, when breeding conditions are more airtight, removing old litter and adding new bedding is more frequent than in 'summer' season, when increased ventilation does not cause such intense overheating of plant matter. More frequent replacement of the litter removes microorganisms growing on the plant matter, and thus also reduces the emission intensity of endotoxins and (1 \rightarrow 3)- β -D-glucans into the air.

As shown above, the mass concentration measurements of poultry dust rarely exceeded the threshold limit value; however, analysis of size distribution of this dust showed very high fine fraction concentrations, especially in the submicrometric size range (Fig. 2). From the scientific literature it is known that endotoxins are particularly willing to form biological-dust aggregates with particulates of fine diameters and in this particular mode are transported in the environment [77–80]. If this principle can equally apply to (1 \rightarrow 3)- β -D-glucan particles, then in the case of the poultry house under study, one can talk about significant intoxication of the respiratory system of poultry workers by both these microbial by-products. Inhalation studies involving direct nasal deposition of pure (1 \rightarrow 3)- β -D-glucan preparations demonstrate no inflammatory reaction, which suggests that (1 \rightarrow 3)- β -D-glucan acts synergistically with other airborne microbial particulates, such as endotoxins, to produce an inflammatory response [81, 82]. The positive correlation between endotoxin and (1 \rightarrow 3)- β -D-glucan concentrations found in the studied poultry house seems to make this concept very probable.

Although the respiratory system is designed to maintain a sterile gas exchange, it is not only responsible for oxygen delivery, humidification or conditioning of the air, olfaction, vocalization, and metabolism. As the largest surface area of the body, it also plays a key role as an immune defence system protecting against adverse effects of the external environment [18]. Agricultural workers generally tend to have a much higher incidence of occupational lung diseases than average employees and appear to be particularly at risk in this context [12]. People employed in work with poultry often report a wide range of respiratory symptoms, including cough, eye irritation, breathlessness, chest tightness, nasal congestion, and wheezing. Poultry production workers also have an increased risk of developing respiratory diseases, such as asthma-like syndrome, rhinosinusitis, hypersensitivity

pneumonitis, organic dust toxic syndrome, chronic obstructive pulmonary disease (COPD) and chronic bronchitis, as a result of chronic inhalation exposure to both infectious and noninfectious airborne particulates present in farm animal production buildings [83, 84]. Viegas et al. studying particle contamination in 7 poultry farms, noted a high prevalence for asthmatic (42.5%) and nasal (51.1%) symptoms in poultry workers [15]. This raises concerns about poultry dust as a respiratory hazard that may trigger harmful airway responses. The longer employees have worked on a poultry farm and experienced repetitive exposure to particulate pollution, the more likely it is that they may develop chronic respiratory problems [12, 84]. Taluja et al. (2019) studying occupational exposure of 66 poultry workers to airborne dust, revealed that such exposure adversely affects the respiratory function, and this impairment is associated with the duration of exposure to poultry dust [14]. The pulmonary functions started deteriorating gradually in employees after 5–10 years of exposure to poultry dust, with maximum decrease after 20 years. There was a statistically significant decrease in forced vital capacity (FVC), forced expiratory volumes over fixed time intervals given in seconds (FEV_{0.5}, FEV₁, FEV₃) with normal FEV₁/FVC, suggesting restrictive ventilatory changes in poultry workers. There was also a significant decrease in forced expiratory flow at 25% and 75% of expiration (FEF_{25–75%}), as well as in FEF between 0.2–1.2 liters of volume change (FEF_{0.2–1.2}) and peak expiratory flow (PEF), suggesting an early small, as well as large airway obstruction, respectively. Such a significant decrease of pulmonary functions, namely FVC, FEV₁, FEV₁/FVC, and FEF_{25–75%}, were also observed among poultry workers by Morris et al. [85], Donham et al. [32], Viegas et al. [6], Yasmeen et al. [86], Younis et al. [43], and Neghab et al. [87]. Studies by Rylander and Carvalheiro confirmed these dependencies for FEV₁ only [66]. According to Zuskin et al., the reason for that may be the development of an inflammatory reaction, thereby releasing inflammatory cytokines such as TNF α and IL-1 β [8]. On the other hand, however, even repetitive pulmonary exposure to low dust concentrations containing propagules of microbial origin that fail to elicit an immediate response, may distract the normal functioning of the pulmonary immune system and render the host more susceptible to infectious diseases [88].

As mentioned above, nasal mucosa is the main barrier that traps inhaled particulate contaminants [23–25]. The respiratory system uses ciliated and mucosal cells, mucus, antibodies, and alveolar macrophages in an attempt to protect this big surface area [89, 90]. The main entry for aerosol particulates into the respiratory tract is the nasal cavity. Its anatomical shape resembling the conchae creates turbulent airflow causing impaction of aspirated particulates on nasal surfaces [90, 91]. In inhalation exposure, nasal lavage (NAL) fluid can be of great analytical importance as a material that is easy to collect by applying a practically non-invasive method [26]. NAL fluid provides the possibility to obtain a cytological picture of the processes (e.g. inflammation) occurring in the nasal mucous membrane of persons exposed to harmful microbial agents [24, 25, 74, 92, 93]. NAL fluid can also be used for inflammatory cell quantification after exposure to organic dusts from animal farms [94–97]. In general, exposure of workers to organic dust induces their immune system to secrete IL-1 β , IL-6, IL-8 and TNF α , and respiratory epithelial cells are among the major sources of these cytokines following such exposure [98, 99].

IL-1 β is one of the main regulators of the immune and inflammatory response. Almost all cells in the body have receptors for IL-1 and can respond to its effects; it is also essential for the host-response and resistance to pathogens, and exacerbates damage during chronic disease and acute tissue injury [100, 101]. This master regulator via controlling a variety of innate immune processes plays a role in resolving acute inflammation. Thorne [102] stated that based on inhalation studies, a dose-dependent increase in IL-1 β levels is clearly visible. This point of view also seems to be shared by Kaur and Sethi [103], who studying mice exposure to poultry barn air containing endotoxin lipopolysaccharide (LPS), suggest that long-term exposures with or without LPS caused lung damage and altered the pulmonary expression of Toll-like receptor 4 (TLR-4) and IL-1 β . Increased expression of IL-1 β has been associated with chronic inflammation and plays an important role in various lung inflammatory diseases, including COPD, asthma, and even lung cancer. In the poultry house in the current study, IL-1 β levels were significant and similar to those measured for IL-6 although they did not correlate significantly with the concentrations of endotoxins and (1 \rightarrow 3)- β -D-glucans in the air.

IL-6 is a powerful cytokine essential, among others, for the inflammatory acute phase response induced by tissue damage [104], and has been reported to exert direct pro-inflammatory actions in lymphocytes and other cell types [105]. In the early phase of inflammation, IL-6 is produced by monocytes and macrophages immediately after the stimulation of Toll-like receptors with distinct pathogen-associated molecular patterns (e.g. endotoxins). However, it is not clear that IL-6 is a key molecule of severe inflammation like other cytokines, such as TNF α and IL-1. There are several reports advocating both the pro-inflammatory and anti-inflammatory potentials of IL-6 against acute inflammatory responses, including acute respiratory distress syndrome [106]. Nevertheless, as shown by Hoffmann et al., even a single exposure of organic dust in an animal confinement building may result in an inflammatory response, documented by an increase in IL-6 concentrations in both bronchoalveolar lavage and serum samples [94]. Similar observations were noted by Larsson et al. [107] and Wang et al. [24] when studying inhalation exposure to swine dusts. In turn, Larsson et al. investigating acute health effects from exposure in poultry houses to airborne dust, discovered that inhalation of dust at levels between 2–4 mg/m³ containing endotoxins at the levels of ~100 ng/m³ resulted in increase of IL-6 concentration in NAL fluid as a result of such exposure [108]. In the studied poultry house, only the concentrations of IL-6 revealed positive relationships with poultry dust, endotoxin, and (1 \rightarrow 3)- β -D-glucan concentrations. This clearly suggests that the observed level of aerosol stimulation had an influence on the health of the exposed workers.

IL-8 is a pro-inflammatory protein, the primary effect of which is the active recruitment of neutrophils to the site of inflammation [109, 110]. Increases in IL-8 expression directly correlate with an increase in cellular immune response, both *in vivo* and *in vitro*. Therefore, IL-8 is commonly used as a measurable indicator to assess immune response to exposure events. Research of Redente and Massengale on the influence of different (including poultry) dusts on IL-8 production by human respiratory epithelial cells, revealed that IL-8 induction varies between agricultural dust types and does not always correlate with endotoxin levels present in the dust [98]. The described relationship was also observed in the current study.

This finding is also in line with the observations by Natarajan et al. (2019), suggesting that endotoxins in poultry dust extracts are weak stimulators of epithelial cells to produce IL-8 [99].

TNF α is a pleiotropic cytokine playing critical role in host defence and acute and chronic inflammation. TNF α has a chemotactic effect on monocytes and neutrophils and activates them in a similar way to macrophages. It enhances the cytotoxicity of monocytes and macrophages, being at the same time one of the mediators of this cytotoxicity. TNF α also activates neutrophils, increasing their phagocytic properties, production of reactive oxygen species, and enhancing their bactericidal and cytotoxic properties [111]. Studies carried out by Poole et al. showed that exposure of monocytes to organic dusts extracts that contained endotoxins and were markedly depleted of endotoxins, in both cases resulted in significant secretion of TNF α [112]. These results demonstrate that the endotoxin component of such dusts does not completely explain the inflammatory cytokine release in airway epithelial cells, and that other potent microbial substances (e.g. peptidoglycans) may play also an important role.

Nevertheless, endotoxins are one of the most immunologically reactive macromolecules, especially abundant in the farming environment where organic material is handled [113]. Endotoxin-associated inflammatory lung disease has also been documented in employees working with poultry [e.g. 44, 58, 114]. After inhalation, endotoxins (or dust containing endotoxins) are deposited in the airways and the lipid A part of endotoxin is opsonized by a lipopolysaccharide binding protein (LBP) present in the fluid on the airway surface produced by epithelial cells. This protein transports endotoxins to attach them to reactor cell surface, i.e. to macrophages and epithelial cells, through the surface protein CD-14. To initiate activation leading to metabolizing and destruction of such foreign substance, the TLR-4 is needed for cellular activation by endotoxins. When the endotoxin is internalized, nuclear factor kappa B (NF- κ B) initiates the production of a variety of inflammatory cytokines, particularly IL-1 β , IL-6, and TNF α [5, 63, 102, 115–117]. However, not every reaction under the influence of organic dust stimulation is the same. Although Brown and Donaldson [118] suggest that exposure to wool and grain dusts stimulated TNF α secretion by alveolar macrophages, in the presented study poultry dust did not show such stimulation on a significant scale. Even though the endotoxin concentrations in the studied poultry house were above 10 ng/m³, which is considered as contamination level initiating respiratory disorders among poultry farm workers, respiratory symptoms might not be apparently shown [56].

Recent reviews on the relationship between agricultural dust exposure and occurrence of respiratory illness in farmers are inconclusive, revealing only that organic dust exposure may have both protective as well as adverse effects [5, 99, 119]. This may be due to the complexity of organic dust components, polymorphism of related genes, such as TLR-4, or endotoxin tolerance phenomenon [56]. Endotoxin tolerance hypothesis suggests that prior exposure of innate immune cells, such as monocytes/macrophages, to minute amounts of endotoxin cause them to become refractory to subsequent endotoxin challenge. Hence, the production of pro-inflammatory cytokines, such as TNF α , could be suppressed following a subsequent endotoxin exposure [120, 121]. This phenomenon could at least partially explain the situation in the poultry house under study where all TNF α concentrations in NAL samples were below the quantification threshold.

CONCLUSIONS

The poultry dust associated with broiler production can pose serious health hazards to exposed workers. Inhalation stimulation with such dust containing endotoxins and (1→3)-β-D-glucans causes the production of pro-inflammatory mediators, proving that the course of the immunological processes in the exposed employees may lead to adverse effects. The use of nasal lavage fluid in the control of this type of exposure confirms that the NAL analysis is a reliable (and easily obtainable) laboratory tool for assessing the impact of poultry dust on exposed farm workers; however, when analyzing NAL samples it should be remembered that stimulation with organic dust does not always trigger the production of all key cytokines. In cases where such exposure can occur, efficient preventive measures need to be implemented to reduce the risk of adverse health outcomes. As in many cases (as also observed in this study) poultry farm workers avoid wearing respiratory protection, their exposure should be limited through all other available technical and organizational actions, such as adequate ventilation of animal housing, decreased stocking density, structural and regular management of manure, etc. [71]. Being aware of the potential health consequences for poultry workers, all possible efforts should be intensified in the future to better understand the mechanisms of occupational diseases, and to elaborate methods for mitigating the adverse effects of exposure caused by inevitable contact with poultry dust.

Acknowledgement

The study was funded by the National Science Centre, Kraków, Poland (Contract No. 2019/35/B/NZ7/04394, Intensive rearing of poultry – identification of changes occurring in the environment and their impact on human health).

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