MYCOTO·XIM

ORIGINAL ARTICLE

Aflatoxin production and in vitro toxicity of Aspergilli section *Flavi* isolated from air samples collected from different environments

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Abstract

Aspergilli section *Flavi*, originally isolated from air samples collected from inhabited apartments (AP), unoccupied basements (BS), and processing facilities of a grain mill (GM), were analyzed for their potential to produce aflatoxin B_1 (AFB_1) on solid media. The isolates were further characterized with regard to their cytotoxic, genotoxic, and pro-inflammatory properties in vitro. Aspergilli were identified based on partial calmodulin (*CaM*) gene sequencing; the producing capacities of isolates were analyzed by HPLC/FLD and confirmed by genes in biosynthesis (*aflR*, *norA*, *omtA*). In the grain mill, the Aspergilli section *Flavi* (up to 1.3×10^6 cfu/m^3) dominated by AFB_1-producing *Aspergillus flavus* (71%, 4.5–5254 ng/ml) which showed a serious health risk for workers. Living environments were not relevant sources of exposure. After 24 h, AFB_1 (1–100 μmol/l) reduced cell viability (MTT test) in both A549 cells and THP-1 macrophage-like cells without reaching IC_{50}. In A549 cells, the extract of the AFB_1-producing *A. flavus* significantly decreased cell viability but not below 50%. THP-1 macrophage-like cells were more sensitive to both extracts, but IC_{50} was obtained only for the AFB_1-producing strain (0.37 mg/ml; AFB_1 2.78 μmol/l). AFB_1 (1 and 10 μmol/l) induced significant DNA damage (tail intensity, alkaline comet assay) in A549 cells in contrast to Aspergilli extracts. AFB_1 elevated IL-6 and IL-8, while Aspergilli extracts increased IL-1β, TNF-α, and IL-17 release in THP-1 macrophages (ELISA). Chronic exposure to AFB_1 and/or other metabolites in airborne *A. flavus* from occupational environments may stimulate epithelial damage of airways accompanied by lowered macrophage viability.

Keywords Airborne fungi · Aflatoxin B_1 · Cytotoxicity · DNA damage · Cytokines

Introduction

The *Aspergillus* section *Flavi* comprises several species widely distributed in the environment. These species can be separated into two groups based on their economic importance and impact on human and animal health. The first group includes the non-producing aflatoxin species *Aspergillus oryzae*, *Aspergillus sojae*, and *Aspergillus tamarii* used in oriental food fermentation (Campbell-Platt 1994). Genetically modified *A. oryzae* strains are used for the production of enzymes, including lactase, pectin esterase, lipase, protease, and xylanase (Pariza and Johnson 2001). The second group includes aflatoxin-producing species *Aspergillus flavus*, *Aspergillus parasiticus*, and *Aspergillus nomius*, which contaminate and damage various pre-harvested and stored agricultural products (Perrone et al. 2014). In the past decade, the production of aflatoxins was also reported in newly described species *Aspergillus arachidicola*, *Aspergillus bombycis*, *Aspergillus minisclerotigenes*, *Aspergillus parvisclerotigenus*, *Aspergillus pseudocaelatus*, *Aspergillus pseudonomius*, *Aspergillus pseudotamari*, *Aspergillus togoensis*, *Aspergillus mottae*, *Aspergillus sergii*, and *Aspergillus transmontanensis* (Varga et al. 2011; Soares et al. 2012).

Aflatoxins are known hepatotoxic mycotoxins with carcinogenic, genotoxic, and teratogenic properties in both humans and animals (Bennett et al. 2003). Among these mycotoxins,

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aflatoxin B_1 (AFB_1) is the most toxic as well as the most frequently occurring aflatoxin in agricultural products (Varga et al. 2011).

In general, aflatoxins are principally produced by *A. flavus* and *A. parasiticus*; *A. flavus* mainly produces AFB_1 and AFB_2, while most *A. parasiticus* isolates produce AFG_1 and AFG_2 in addition to AFB_1 and AFB_2. However, *A. flavus* is considered more invasive and out-competes *A. parasiticus* when both species are present in soil (Perrone et al. 2014). Aflatoxigenic strains of *A. flavus* are most widespread in tropical and subtropical areas. However, in recent years, a more frequent occurrence of aflatoxin producers has been detected in temperate-climate regions, associated with current climate change. Air humidity changes (abundant rain, droughts), increase in temperature, and increased CO_2 concentration directly affect the expression of regulatory (*aflR*) and structural genes (*aflD*) involved in the biosynthesis of AFB_1 and, thus, correlate with its biosynthesis (Paterson and Lima 2010; Medina et al. 2014).

Next to *A. fumigatus*, *A. flavus* is the second most frequent cause of invasive and non-invasive aspergillosis in humans; it may also cause sinusitis, cutaneous and wound aspergillosis, as well as otitis and keratitis (Hedayati et al. 2007; Manikandan et al. 2013).

Taking into account the aflatoxigenic ability of the listed Aspergilli section *Flavi*, particularly *A. flavus*, as well as its role in the etiology of several diseases in humans, an accurate identification of the species would provide fundamental information concerning their aflatoxigenic and pathogenic properties (Godet and Munaut 2010). Several molecular genetic techniques have been developed to distinguish the *Aspergillus* species in the section *Flavi*; Varga et al. (2011) proposed a polyphasic approach, including morphological characters, extrolite data, and partial calmodulin (*CaM*), β-tubulin (*BenA*), and ITS sequences as well as the presence of genes involved in aflatoxin biosynthesis.

Since the discovery of aflatoxins, the hepatotoxic action of AFB_1 has been mainly studied in various experimental models through AFB_1-contaminated food consumption (Bennett et al. 2003). However, a link between AFB_1 inhalation in an industrial setting and liver or lung cancer incidence and mortality has been suggested by several investigations (Olsen et al. 1988; Viegas et al. 2012). Taking into account the lack of data on human exposure to fungal burden in urban homes and occupational settings in our country, recently, we conducted a year-round investigation of airborne fungi in living (apartments, basements) and occupational (grain mill) indoor environments in Croatia with special attention to *Aspergillus* sections *Flavi*, *Nigri*, and *Versicolores* due to their known pathogenic and/or mycotoxin-producing properties (Jakšić Despot and Šegvić Klarić 2014,b). Based on morphological methods, Aspergilli sections *Flavi* and *Nigri* were recovered in the highest frequencies (50–100% and 15–55%, respectively)

from samples taken in the grain mill, while Aspergilli section *Versicolores* was more abundant in apartments and basements (10–65%) (Jakšić Despot and Šegvić Klarić 2014,b). Before appropriate molecular identification methods for species assigned to these sections, indoor air isolates were usually reported as *A. flavus*, *A. niger*, or *A. versicolor* (Samson et al. 2014). As we previously noted, accurate identification of the *Aspergillus* species is fundamental with regard to their toxigenic and/or pathogenic properties (Godet and Munaut 2010). Thus, herein, we described the species diversity of *Aspergillus* sections *Nigri* (Jakšić et al. 2018) and *Versicolores* (Jakšić Despot et al. 2016) using calmodulin sequence-based methods, their ability to produce fumonisin B_2 and sterigmatocystin, respectively, and to explore toxic potential of Aspergilli extracts in comparison with these mycotoxins alone. To further extend knowledge on *Aspergillus* section *Flavi* biology and toxicology, the present study aimed to explore the species diversity of isolated strains using a polyphasic approach (morphological methods, *CaM* sequence, and presence of genes taking part in aflatoxin biosynthesis) and confirm aflatoxin-producing abilities in Aspergilli extracts by HPLC/FLD.

Some studies showed that AFB_1 alone induces several toxic events in human airways, including damage of airway epithelial cells, decrease of the ciliary beat frequency (Lee et al. 2016), DNA damage due to formation of exo-AFB1-8,9-epoxide by human lung microsomes (Kelly et al. 1997) as well as takes a part in inflammation (Lee et al. 2016; Luongo et al. 2014). However, the effects of inhaled aflatoxin-producing fungi on the airway epithelium have not been well-characterized so far.

As *A. flavus* was among the most abundant species in the grain mill, it was justified to explore the cytotoxic, genotoxic, and immunomodulatory properties of AFB_1-producing and AFB_1-non-producing *A. flavus* extracts in contrast to AFB_1 alone in order to clarify whether particular toxic properties of Aspergilli could be attributed to AFB_1. This was carried out using human lung adenocarcinoma A549 cells and macrophages derived from human leukemic monocyte THP-1 cells as experimental models.

Materials and methods

Chemicals and media

Methanol, acetonitrile, methylene chloride, ethyl acetate, and n-hexane for sample preparation and eluents were purchased from VWR (Debrecen, Hungary). Formic acid, trifluoroacetic acid, and aflatoxin B_1 standard were obtained from Sigma-Aldrich (Budapest, Hungary). Deionized water for both sample preparation and high-performance liquid chromatography (HPLC) runs was produced by Merck Millipore Milli-Q

Gradient A10 water purification equipment (Budapest, Hungary). Dichloran 18% glycerol agar (DG-18), Czapek Yeast Agar (CYA), and Malt Extract Agar (MEA) were purchased from Oxoid (Hampshire, UK). Media for cell culture maintenance, including RPMI 1640, fetal bovine serum (FBS), trypsin-EDTA, phosphate-buffered saline (PBS; Ca^{2+} and Mg^{2+} free), penicillin, and streptomycin were from Lonza (Basel, Switzerland). MTT reagent [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], phorbol 12-myristate 13-acetate (PMA) ethidium bromide, acridine orange, normal melting point (NMP) agarose, low melting point (LMP) agarose, Triton X-100, Tris buffer, and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (Deisenhofen, Germany). Dimethyl sulfoxide (DMSO) ethanol HCl, NaCl, Na_2EDTA, and NaOH were from Kemika (Zagreb, Croatia).

Sampling areas

The samples of airborne fungi were collected over a one-year period (2012) in two-month intervals at the processing facilities of a grain mill (GM) situated near Zagreb, Croatia, as well as in residential locations in the city which included two inhabited apartments (AP) and two unoccupied basements (BS) as well as outdoor air (ODA) as described in detail in Jakšić Despot and Šegvić Klarić (2014,b). In each period of sampling in GM, twenty samples were collected during milling operations at the site of grain/flour exchange, site of flour storage, site of sieving, and site of milling. In each AP (approximately 70 m^2), samples were taken at five locations, including the kitchen, dining room, living room, bedroom, and bathroom, in duplicate. BSs were located in the same buildings as the AP, and residents used these spaces to store various items (e.g., wooden, metal, plastic, or glass materials). In each BS, samples were taken at five locations in duplicate. Samples of outdoor air were taken in proximity to indoor locations, and, during each sampling period, ten ODA samples were collected.

The sampling approach included collecting 20 indoor samples and 10 outdoor samples per each location and at a given time point. Altogether, 420 individual samples were taken.

Sample collection

Airborne fungi were sampled using an Airsampler Mas-100 Eco (Merck, Berlin, Germany) with 400 holes (hole to agar impactor, impaction velocity 10.8 m/s, and airflow rate 100 l/min) and DG-18 agar plates (Samson et al. 2010). Because of the high contamination level, a volume of 10 l was chosen for sampling in the GM, while a volume of 50 l was applied at the other sampling locations. The plates were incubated at 25 °C $\pm$ 0.2 °C in the dark for five days, after which the developed fungal colonies were counted, and results were expressed as colony-forming units per cubic meter (cfu/m³). Aspergilli

were isolated on CYA and MEA agar plates (Samson et al. 2010). Isolates assigned to the *Aspergillus* section *Flavi* were purified and cultivated on CYA and MEA plates at 25 °C in the dark for seven days. Morphological identifications were carried out according to literature (Samson et al. 2010).

Determination of Aspergilli section *Flavi* genotypes

Isolation of genomic DNA from mycelia grown in liquid YPD medium (1% Bacto yeast extract, 1% Bacto peptone, 1% D-glucose) for five days was performed by Masterpure™ yeast DNA purification kit (Epicentre Biotechnologies, Madison, WI, USA) according to the manufacturer's instructions. The isolated DNA was diluted to < 1.000 ng and used as template DNA in PCR reactions. A fragment of the calmodulin gene (*CaM*), part of the transcriptional regulator of aflatoxin biosynthesis (*aflR*), norsolorinic acid reductase (*norA* or *aflE*), and O-methyltransferase gene (*omtA*, *aflP*) (Yu et al. 2004) were amplified using the primers specified in Table 1. Each PCR reaction mixture (20 μl) contained 0.2 mmol/l deoxyribonucleotide triphosphate (dNTP), 0.2 μmol of primers, 1 U DreamTaq DNA Polymerase (Thermo Scientific, Madison, WI, USA) with the respective buffer (with 1.5 mmol/l $MgCl_2$) (Thermo Scientific, Madison, WI, USA), nuclease-free water, and template DNA. Amplifications were performed using a Thermocycler T-100 (BioRad, Budapest, Hungary). The PCR cycling protocol consisted of 35 cycles, including an initial denaturation at 95 °C for 2 min in the first and 20 s in the following runs, annealing temperature as specified in Table 1 for 20 s, elongation at 72 °C for 40 s, and 2 min for the final elongation. The amplification products were analyzed by electrophoresis on 2% agarose gels using fluorescent dye GR Green (Excellgen, Budapest, Hungary), and the banding patterns were visualised under ultraviolet light (254 nm). In case of PCR products assigned to *aflR*, *norA*, and *omtA*, the bands were scored as either present or absent for each *A. flavus* isolate. Partial *CaM* sequences were determined at the LGC Genomics GmbH (Berlin, Germany). Sequence analysis was performed by nucleotide–nucleotide BLAST similarity search at the website of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST), and sequences were, also, compared with our own sequence database. Species identification was determined from the lowest expected value of the BLAST output (Altschul et al. 1990).

Preparation of Aspergilli section *Flavi* extracts and aflatoxin analysis

Extracts of *A. flavus* isolates ($n = 65$) were prepared and analyzed as described previously (Baranyi et al. 2015). Briefly explained, following a microextraction procedure according to Frisvad et al. (2007), three agar plugs (6 mm in diameter

Table 1 The primers used in PCR

Primer	Sequence (5′–3′)	Annealing temperature (°C)	Gene	Reference
Cmd-5	CCGAGTACAAGGARGCCTTC	52–55	*CaM*	Hong et al. (2005)
Cmd-6	CCGATRGAGGTCCATRACGTGG			
Afl-F	GGGATAGCTGTACGAGTTGTGCCAG	57–59	*aflR*	Varga et al. (2011)
Afl-R	TGGKGCCGACTCGAGGAAYGGGT			
Nor-1	ACCGCTACGCCGGCACTCTCGGCA	64–66	*norA*	Criseo et al. (2001); Varga et al. (2011)
Nor-2	GTTGGCCGCCAGCTTCGACACAGC			
Omt-1	GTGGACGGACCTAGTCCGACATCAC	59–61	*omtA*	Geisen (1996); Varga et al. (2011)
Omt-2	GTCGGCGCCACGCACTGGGTTGGGG			

each) were extracted using a mixture of organic solvents methanol/dichloromethane/ethyl-acetate (1/2/3, *v/v/v*) supplemented with 1% of formic acid. The extracts were ultrasonicated, and the organic phases were separated, filtered through, and evaporated to dryness under a slight stream of nitrogen. Dry extracts were derivatized with trifluoroacetic acid (Fazekas et al. 2005). HPLC (Shimadzu, Japan) equipped with a fluorescence detector set at an excitation wavelength of 365 nm and an emission wavelength of 430 nm was used for AFB_1 analysis. Separations were achieved on a LiChroCART 250 × 4 mm, 5 μm (Merck, Hungary) column coupled with a LiChrospher 100 RP-18 (Merck, Hungary) guard column and an injection volume of 5 μl. The isocratic mobile phase composition was water/methanol/acetonitrile 65/17.5/17.5 (*v/v/v*), and the flow rate was maintained at 1 ml/min, while the column temperature was 40 °C. For the quantification of AFB_1, the linear calibration curve was used in a concentration range of 6.25 to 100 ng/ml and the equation of resulted calibration curve was $y = 4056.2x − 7321.2$ ($R^2 = 0.9994$).

Cytotoxicity, genotoxicity, and immunomodulation of AFB_1 vs Aspergilli section *Flavi* extracts

Cell cultures

Human lung adenocarcinoma cells A549 and human leukemia monocytes THP-1 (European Collection of Cell Cultures, Salisbury, UK) were grown in 75 cm² flasks in RPMI supplemented with 2 mmol/l glutamine, 10% heat-inactivated FBS, penicillin (100 IU/ml; 1 IU 67.7 μg/ml), and streptomycin (100 μg/ml). Cultures were maintained in a moisturized atmosphere with 5% CO_2 at 37 °C and 95% relative humidity.

Preparation of tested compounds

A stock solution of AFB_1 (0.01 mol/l) was prepared in DMSO/70% ethanol mixture 80/20 *v/v*. Weighed dried fungal extracts were dissolved in 100% DMSO. The final concentrations of AFB_1 and fungal extracts as well as DMSO/ethanol used in the treatments of cells were obtained by dilution with the culture medium. The highest concentration of fungal

extract applied as treatment was limited by the content of DMSO (up to 1%) that showed no significant effect on cell viability. Mass concentrations of fungal extracts used in MTT assay ranged from 0.05 to 0.6 mg/ml, while the corresponding content of AFB_1 in the extract of toxin-producing *A. flavus* was 0.75 to 4.5 μmol/l. Concentrations of the extracts used in other assays were selected according to the results of MTT and corresponded to subcytotoxic concentrations.

MTT proliferation assay

Viability of A549 cells and macrophage-like THP-1 cells was estimated using MTT assay, as described previously (Jakšić Despot et al. 2016). The differentiation of THP-1 cells into macrophages was performed using PMA (20 nm/well). The A549 (10⁴ cells/well) and macrophage-like THP-1 cells (5 × 10⁴ cells/well) were grown in a 96-well flat-bottom microplate in RPMI 1640 medium supplemented with 10% of FBS. Upon 24 h treatment with AFB_1 (0.1 to 100 μmol/l) or with Aspergilli extracts (0.05 to 0.6 mg/ml) of AFB_1-producing and non-producing isolates, the concentration that inhibits growth in 50% of cells (IC_{50}) was determined. Following treatment, the medium was removed and 100 μl of MTT-tetrazolium salt reagent diluted in RPMI 1640 medium without FBS (0.5 mg/ml) was added (V = 100 μl per each well). After 3 h of incubation, the medium was replaced with 100 μl of DMSO to dissolve formazan (product of metabolised MTT reagent), and cells were incubated at room temperature on a rotary shaker for 15 min. The absorbance was measured using a microplate reader (Labsystem iEMS, type 1404) at a wavelength of 540 nm. All tests were performed in five replicates, and results were expressed as percentage of control.

Alkaline comet assay

The extent of primary DNA damage produced after 24 h treatment with AFB_1 and *Aspergilli* extracts was assessed in A549 cells using the alkaline comet assay. A standard protocol, proposed by Singh et al. (1988), with minor modifications was used. Before treatment, A549 cells were seeded in 6-well plates (3 × 10⁵ cells per well) in RPMI 1640 medium

supplemented with 10% of FBS. After 24 h of growth, the cell medium was discarded and cells were treated with AFB$_1$ (at 1 and 10 μM), Aspergilli extracts (at 0.05 and 0.1 mg/ml for non-producing and 0.1 and 0.2 mg/ml for AFB$_1$-producing isolate). Control cell cultures were grown in parallel (with and without 0.1% DMSO/ethanol) for 24 h. After 24 h of treatment, the cells were washed with 1 ml cold PBS, scraped with rubber, and resuspended in 300 μl of PBS. Aliquots (V = 20 μl) of this suspension were used to prepare slides for the comet assay. Briefly, cell samples were mixed with 100 μl 0.5% LMP agarose (in Ca- and Mg-free PBS) and spread onto fully frosted slides, previously pre-coated with 1% and 0.6% NMP agarose. The microgels were allowed to solidify on ice for 10 min. Then, the slides were subjected to lysis at 4 °C in a buffer containing 2.5 mol/l NaCl, 100 mmol/l Na$_2$EDTA, 10 mmol/l Tris (pH 10), supplemented with 1% Triton-X 100. The lysis step lasted for 1 h and was followed by denaturation in alkaline buffer (10 mmol/l NaOH, 200 mmol/l Na$_2$EDTA, pH 13), for 20 min. Electrophoresis lasted for 20 min in the same buffer at 25 V and 300 mA. Microgels were, then, neutralised with three changes of 0.4 mol/l Tris/HCl buffer (pH 7.5), for 5 min each. The slides were kept in a humid atmosphere in a dark box at 4 °C until further analysis. Before image analysis, DNA was stained for 10 min with ethidium bromide (20 μg/ml). Slides were scored using an image analysis system (Comet assay IV, Instem-Perceptive Instruments Ltd., UK) connected to a fluorescence microscope (Olympus, Japan). All of the experiments were performed in duplicate, and, in each experiment, images of 200 randomly selected nucleoids (100 nucleoids from each of the two replicate slides) were measured. Only comets with a defined head were scored. As indicators of DNA damage, tail length (presented in micrometres) and tail intensity (i.e., percentage of DNA in the comet tail) were selected (Collins 2004).

Determination of cytokine levels

To establish cytokine levels after 24 h treatment with AFB1 and Aspergilli extracts, THP-1 cells were selected as an appropriate cell model. Cells were first seeded on 24-well cell culture plates (1×10^6 cells per well) in RPMI 1640 medium supplemented with 10% of FBS and differentiated into macrophages for 24 h with addition of 40 nmol/l LM PMA for 24 h. After that, the medium was discarded, and cells were treated with AFB$_1$ (at 1 and 10 μmol/l LM) and Aspergilli extracts (at 0.05 and 0.1 mg/ml for non-producing and 0.1 and 0.2 mg/ml for AFB$_1$-producing isolate) for 24 h. Control cell cultures were grown in parallel in RPMI 1640 medium supplemented with 10% of FBS (with and without 0.1% DMSO/ethanol) for 24 h. Following treatment, the cell medium was harvested and frozen at − 80 °C until analysis. Concentrations of TNF-α, IL-1β, IL-6, IL-8, and IL-17 were

determined in harvested cell medium by DuoSet ELISA kits (R&D Systems, Minneapolis, USA) according to instructions provided by the manufacturer and as described elsewhere (Hulina et al. 2018). Cytokine concentrations were calculated from measured optical densities determined at 450 nm, using a microplate reader (En Vision® Multilabel Plate Reader, Perkin Elmer) and by standard calibration curves, and results of the three replicates were expressed as percentage of control.

Statistics

Data obtained using the MTT test, comet assay, and cytokine concentration were first evaluated using descriptive statistics. The results were presented as mean ± standard error of mean (SEM). Normality of data distribution was tested by Kolmogorov–Smirnov test; one-way ANOVA was applied for normally distributed data (MTT test and cytokine concentrations) followed by Sidak's post-test, while for non-normally distributed data (comet assay), Kruskal–Wallis test was applied followed by Dunn's multiple-comparison test. A value of $P < 0.05$ was considered statistically significant for all calculations. To obtain the IC$_{50}$ from the results of the MTT assay, non-linear dose–response fitting was applied using the equation y = A1 + (A2 − A1) / (1 + 10^((LOGx0-x) ∗ p)).

Results and discussion

Occurrence of aflatoxigenic Aspergilli section *Flavi* in air samples

The abundance of airborne *Aspergillus* species section *Flavi* in the occupational environment (GM) in contrast to inhabited urban homes (AP) and urban home basements (BS) as well as outdoor air (ODA) in the six periods of sampling is presented in Table 2. Only in GM were these Aspergilli present throughout the whole year, comprising 1–12% of total viable airborne fungi, with mean absolute concentrations approximately 100 to 2000 times higher than in other indoor, as well as outdoor locations. In the GM, the mean absolute concentrations of Aspergilli were highest in March and September (about 2000 cfu/m^3), with a maximum of 2×10^4 cfu/m^3 in September. Looking at the estimated concentrations (probable viable number), the airborne Aspergilli section *Flavi* were present at concentrations between 1.7×10^3 cfu/m^3 (January) and 1.3×10^4 cfu/m^3 (March) from January to September and dropped below 1000 cfu/m^3 only in November. Comparing the results of the present study with our previous reports, the Aspergilli section *Flavi* occurred in significantly higher mean absolute concentrations in the GM (405–2035 cfu/m^3) than both Aspergilli from the subclade *Versicolores* (6.5–44 cfu/m^3) and Aspergilli section *Nigri* (20–240 cfu/m^3) (Jakšić Despot et al. 2016; Jakšić et al. 2018). Mean concentrations

Table 2 Abundance of Aspergilli from the section *Flavi* in indoor and outdoor air for each sampling period

	Grain mill (GM)					Apartment (AP)			Basement (BS)			Outdoor air (ODA)		
	cfu/m³					cfu/m³			cfu/m³			cfu/m³		
	Absolute Mean ± SD	Estimated*	Absolute Range	Estimated* Range	%	Mean ± SD	Range	%	Mean ± SD	Range	%	Mean ± SD	Range	%
January	290 ± 218	1743 ± 1576	0–600	0–3942	1.06	–	–	–	–	–	–	–	–	–
March	2035 ± 2486	13,370 ± 16,330	0–5000	0–32,850	5.57	–	–	–	1 ± 4.5	0–20	0.13	–	–	–
May	405 ± 369	2579 ± 2495	0–1000	0–6570	1.51	–	–	–	1 ± 4.5	0–20	0.09	–	–	–
July	1335 ± 751	8771 ± 4932	100–3000	657–19,710	3.34	2 ± 6	0–20	0.20	–	–	–	2 ± 6	0–20	0.10
September	1839 ± 4446	8091 ± 29,129	0–20,000	0–131,400	12.85	1 ± 4.5	0–20	0.22	1 ± 4.5	0–20	0.10	–	–	–
November	150 ± 176	167 ± 199	0–600	0–690	2.39	–	–	–	1 ± 4.5	0–20	0.08	6 ± 10	0–20	2.5

* The probable number of viable particles calculated from Feller's formula (Pr = N 1/N + 1/N-1 + 1/N-2 + 1/N-r+1), given by the manufacturer (Merck KgaA, Darmstadt, Germany; Pr=probable statistical total; r=number of cfu counted; N=total number of holes in the sampling head)

%, – Mean concentration of Aspergilli section *Flavi* / mean concentration of total airborne fungi × 100. (Mean concentration of total airborne fungi adopted from Jakšić Despot and Šegvić Klarić (2014))

of viable Aspergilli section *Flavi* were also much higher than those reported in poultry farms, soybean and cotton mills, and the food industry (17–137.6 cfu/m³) in India, Italy, and Egypt (Sharma et al. 2010; Abdel Hameed et al. 2012; Cafarchia et al. 2014). Maximum values of statistically estimated concentrations of Aspergilli section *Flavi* obtained in July (1.9 × 10⁵ cfu/m³) and (1.3 × 10⁶ cfu/m³) were 10–100-fold higher than concentrations of airborne fungi (> 10⁴ cfu/m³) considered as a health hazard in non-sensitized subjects (Oppliger et al. 2005). Contrary to the GM, Aspergilli section *Flavi* were rarely present or were in low concentrations (maximum 20 cfu/m³) in urban apartments and basements (AP and BS) and outdoors which is in agreement with recently conducted studies in homes of low-income areas in Syracuse (NY, USA) as well as in homes of hematologic patients in Germany (Crawford et al. 2015; Schweer et al. 2016). In most periods, these Aspergilli comprised between 0.1 and 0.2% of total viable airborne fungi recovered from AP, BS, and ODA.

Based on *CaM* gene region sequencing, among a total of 67 Aspergilli section *Flavi* isolates, 65 were identified as *A. flavus* and 2 as *A. parasiticus* (Table 3) (data on *A. parasiticus* are not shown; results were published in Baranyi et al. 2015). The majority of *A. flavus* strains (89%) were recovered from the GM. Among these strains, 76% produced AFB_1; the highest amounts of AFB_1 (10–30 μg/ml) were produced by strains isolated in March and November. For five strains of *A. flavus*, isolated from the GM environment, production of low amounts of AFB_2 (0.03 ± 0.02 μg/ml) was detected, in addition to AFB_1 (13.4 ± 9.8 μg/ml). These five strains represented 10% of all *A. flavus* isolates from the GM environment. The production of AFB_1 and AFB_2 correlated with genes detected for the transcriptional regulator of aflatoxin biosynthesis (*aflR*), norsolorinic acid reductase (*norA*), and O-methyltransferase gene (*omtA*); 15 strains were positive for two or all three genes for aflatoxin biosynthesis, but the amounts of the toxin were below the LOD. Several earlier studies have suggested the significance of respiratory exposure to *A. flavus* in biowaste-handling facilities (up to 10⁵ cfu/m³) and AFB_1 in grain dust ranging from 0.04 to 107 ng/m³ (Fischer et al. 2000a,b; Fischer and Dott 2003). Hardin et al. (2009) reviewed the significance of exposure to airborne fungi and mycotoxins in light of concentration of no toxicologic concern-CoNTC (30 ng/m³), which showed that in occupational environments, such as grain, handling the concentration of AFB_1 in the facilities can exceed the CoNTC. Recently, in poultry farms in Portugal, *A. flavus* was found in three of seven poultry units, and this Aspergilli was the third most frequently found fungal species among indoor airborne fungi (Viegas et al. 2012). In the same study, the presence of aflatoxigenic strains was only confirmed in an outdoor air sample from one of the poultry units but direct evidence of the poultry workers' professional exposure to AFB_1 was found in 58% of positive serum

Table 3 AFB$_1$-producing abilities of indoor *A. flavus* isolates from different sampling periods and locations

Month of collection	Location	Isolate ID[a]	*aflR*	*norA*	*omtA*	AFB$_1$ (ng/ml)
January	GM	AF10527 I.	+	+	+	4.9
		AF10529 I.	+	+	+	4.3
		AF10523 I.	+	+	+	4.1
		AF10525 I.	+	+	+	4.4
March	GM	AF10869B III.	+	+	+	< LOD
		AF10873 III.	+	–	–	< LOD
		AF10868A III.	+	+	+	4.7
		AF10868B III.	+	+	+	12,000.0
		AF10869A III.	+	+	+	4.8
May	GM	AF10923A V.	+	+	+	8.5
		AF10926A V.	+	+	+	5.6
		AF10926B V.	+	+	+	11.0
		AF10923B V.	+	+	+	4.6
		AF10925B V.	+	+	+	11.5
		AF10927A V.	+	+	+	4.3
		AF10930B V.	+	–	–	< LOD
	BS	AF10959B V.	+	+	+	5.0
July	GM	AF10989B VII.	+	+	+	4.6
		AF10992B VII.	+	+	+	4.9
		AF10993A VII.	+	+	+	< LOD
		AF10993B VII.	+	+	+	5.6
		AF10994A VII.	+	+	+	5.0
		AF10995A VII.	+	+	+	4.4
		AF10997A VII.	+	+	+	4.3
		AF10988A VII.	+	+	+	4.9
		AF10988B VII.	+	+	+	4.4
		AF10989A VII.	+	+	+	< LOD
		AF10990B VII.	+	+	+	< LOD
		AF10991A VII.	+	+	+	4.1
		AF10992A VII.	+	+	+	< LOD
		AF10994B VII.	+	+	+	5.0
		AF10996A VII.	+	+	+	204.0
		AF10996B VII.	+	+	+	< LOD
		AF10997B VII.	+	+	+	5.5
		AF11012B VII.	+	+	+	4.6
	AP	AF11008B VII.	+	+	+	< LOD
September	GM	AF11045A IX.	+	+	+	15.6
		AF11046A IX.	+	+	+	< LOD
		AF11048B IX.	+	+	+	4.1
		AF11050A IX.	+	+	+	4.2
		AF11149B IX.	–	–	–	–
		AF11046B IX.	+	+	+	< LOD
		AF11047B IX.	+	+	+	< LOD
		AF11049A IX.	+	+	+	4.71
		AF11049B IX.	+	+	+	657.0
		AF11050B IX.	+	–	+	4.3
		AF11051A IX.	+	+	+	6.2
		AF11053A IX.	+	+	+	5.9
		AF11054A IX.	+	+	+	4.7

Table 3 (continued)

Month of collection	Location	Isolate ID[a]	*aflR*	*norA*	*omtA*	AFB$_1$ (ng/ml)
November	GM	AF11110A XI.	+	+	+	11,000.0
		AF11112A XI.	+	+	+	5.0
		AF11113B XI.	+	+	+	4.5
		AF11117B XI.	+	+	+	31,000.0
		AF11118A XI.	+	+	+	4.4
		AF11119A2 XI.	+	+	+	11,000.0
		AF11119B XI.	+	+	+	5.5
		AF11110B XI.	+	+	−	< LOD
		AF11111B XI.	+	−	+	< LOD
		AF11116A XI.	+	+	+	5.0
		AF11119A1 XI.	+	+	+	16,000.0
	BS	AF11144B XI.	+	+	+	< LOD
		AF11140A XI.	+	+	+	5.9
		AF11140B XI.	+	+	+	4.7
	ODA	AF11146B XI.	+	+	+	4.5
		AF11148A XI.	+	+	+	6.1

[a] Isolate ID, isolates of *A. flavus* are deposited under their designated ID in the microbial collection (MFBF) at the Department of Microbiology Faculty of Pharmacy and Biochemistry, University of Zagreb

GM, grain mill; AP, apartment; BS, basement; ODA, outdoor air

samples (mean 2 ng/ml) in contrast to the AFB$_1$-negative serum sampled from control individuals. On the other hand, in living environments, AFB$_1$ does not seem to be relevant since its formation on building materials during fungal growth has not been detected (Rao 2016; Ren 1999). Our results suggest that a serious threat to human health due to exposure to airborne *A. flavus* could come only in occupational environments, such as grain mill facilities, but not from exposure in urban apartments and basements.

Cytotoxicity of AFB$_1$ vs *A. flavus* extracts

The cytotoxic potential of Aspergilli extracts and AFB$_1$ applied alone on A549 cells and THP-1 macrophage-like cells is presented in Table 4 and Fig. 1. Although AFB$_1$ alone significantly reduced the viability of both A549 and THP-1 macrophage-like cells at concentrations up to 100 μmol/l, the reduction did not fall below 50% and the IC$_{50}$ was not calculated. Our results are consistent with previous studies on A549 cells and human bronchiolar epithelium BEAS-2B cells (Palanee et al. 2001; Vleet et al. 2002; McKean et al. 2006). Van Veelt

et al. (2002) showed that CYP 1A2 and 3A4 enzymes are responsible for the bioactivation of AFB$_1$ into metabolites (AFB$_1$ 8,9-epoxide and aflatoxin Q$_1$) that evoked cytotoxicity in CYP-transfected cells of the bronchiolar epithelium (B-CMV1A2 and B3A4). On the contrary, BEAS-2B and A549 have limited CYP activity, which was in agreement with their CYP gene expression profile (Garcia-Canton et al. 2013) and may explain the weak cytotoxicity of AFB$_1$ obtained in both A549 and THP-1 macrophage-like cells.

The extract of AFB$_1$-producing *A. flavus* induced a significant drop in the viability of A549 cells, while the non-producing strain was not cytotoxic to them. THP-1 macrophage-like cells were more sensitive to both extracts, but the AFB$_1$-producing strain exerted stronger cytotoxicity with IC$_{50}$ (0.37 ± 0.024 mg/ml; the corresponding AFB$_1$ concentration is 2.78 ± 0.18 μmol/l AFB$_1$). Thus, we may suggest that AFB$_1$ contributed to the *A. flavus* extract cytotoxicity but only in THP-1 macrophage-like cells. Piecková and Wilkins (2004) showed that endo- and exometabolite extracts of dust-borne *A. flavus* were able to stop chicken tracheal cilia beating after the first 24 h of activity. Recently, it was shown that exposure

Table 4 The calculated IC$_{50}$ of AFB$_1$ negative and positive *A. flavus* extracts (0.05–0.6 mg/ml) in A549 and THP-1 macrophage-like cell lines using non-linear curve fitting (0.9914)

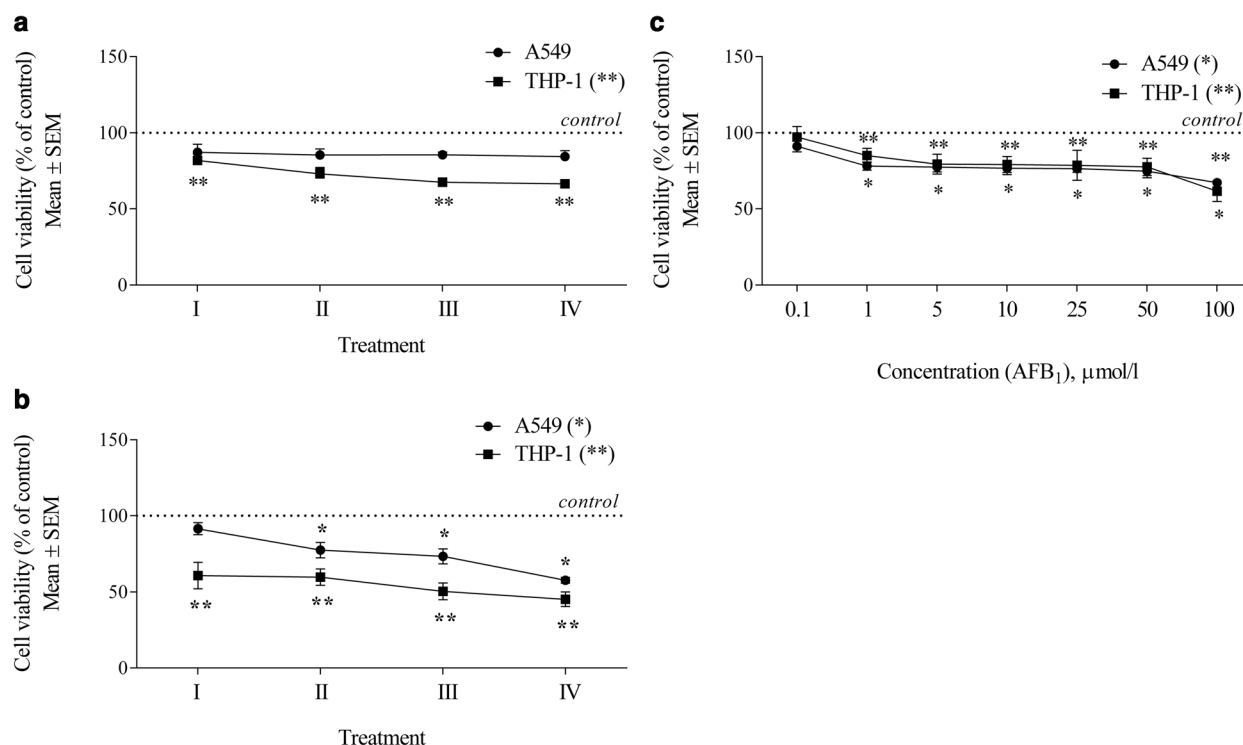| Treatment	IC$_{50}$ (A549) ($\overline{x} \pm$ SEM)	IC$_{50}$ (THP-1) ($\overline{x} \pm$ SEM)
A. flavus (AFB$_1$-negative)	> 0.6 mg/ml	> 0.4 mg/ml
A. flavus (AFB$_1$-positive)	> 0.4 mg/ml	0.37 ± 0.024 mg/ml (AFB$_1$ 2.78 μM)
AFB$_1$	> 100 μM	> 100 μM

Fig. 1 Survival of THP-1 macrophage-like cells and A549 cells after 24 h of the treatment with the extract prepared from AFB$_1$-negative (**a**) and AFB$_1$-positive (**b**) isolate of *A. flavus* and single AFB$_1$ (**c**). Mass concentrations of fungal extracts prepared from AFB$_1$-negative isolate of *A. flavus* were I 0.05, II 0.1, III 0.25, and IV 0.4 mg/ml (**a**), in the extract of toxin-producing *A. flavus* I 0.1, II 0.2, III 0.4, and IV 0.6 mg/ml, while the corresponding content of AFB$_1$ was 0.75, 1.5, 3.0, and 4.5 μmol/l, 1–4 respectively (**b**). Each data point represents the mean ± SEM of cell viability (% of control, control = 100% of cell viability). *, **Significantly different as compared to control in A549 and THP-1 macrophage like cells, respectively ($P < 0.05$)

of primary human sinonasal and bronchial cell cultures to aflatoxins (0.1 to 10 μmol/l) as well as to conditioned media from *A. flavus* reduced the ciliary beat frequency. The effect was blocked by an anti-aflatoxin antibody suggesting that the aflatoxin was responsible for the reduction of ciliary beat frequency (Lee et al. 2016). The same study revealed that AFB$_2$ activates protein kinase C in A549 cells, which has been linked to inflammation and apoptosis (Diaz-Meco and Moscat 2012; Zhao et al. 2012). Taken together, our results suggest that although AFB$_1$ alone exerted weak cytotoxic properties in THP-1-like macrophages and A549 lung cells, it significantly contributes to the cytotoxicity of *A. flavus* extracts. Therefore, chronic exposure to aflatoxins in the mixture of *A. flavus* metabolites in an occupational environment may stimulate epithelial damage in airways accompanied by lowered macrophage viability contributing to the pathogenesis of respiratory diseases.

Genotoxicity of AFB$_1$ vs *A. flavus* extracts

Genotoxic effects were evaluated in A549 cells using the alkaline comet assay (Fig. 2). AFB$_1$ alone evoked significant concentration-dependent DNA damage measured as tail intensity but not in terms of tail length as compared to control.

Contrary to AFB$_1$, Aspergilli extracts provoked a significant concentration-dependent increase of tail length but not tail intensity. The difference in the responses at DNA level observed after the treatments could be explained by the intrinsic differences in the mechanisms behind DNA lesions. Without conducting additional experiments, we cannot establish the exact mechanism(s) responsible for the observed effects. However, as a comet's tail length is proportional to the number of relaxed DNA loops (Collins et al. 2008), it seems that treatment with Aspergilli extracts led to an increased number of relaxed DNA loops, but without a concomitant increase in the amount of DNA breaks. This could be, at least in part, associated with the chemical composition of the extract, which—besides AFB$_1$—contains other active compounds as well, which possess different potentials to damage DNA or even modulate the DNA damaging effects of AFB$_1$ itself.

When discussing the significance of the comet assay parameters evaluated in this study, it has to be stressed that tail intensity has been deemed the most useful because as the level of damage increases so does the relative intensity of DNA staining in the tail, rather than tail length (Collins et al. 2008). Therefore, the significantly increased tail intensity we recorded at both of the tested concentrations represents an important piece of information regarding AFB$_1$ genotoxicity,
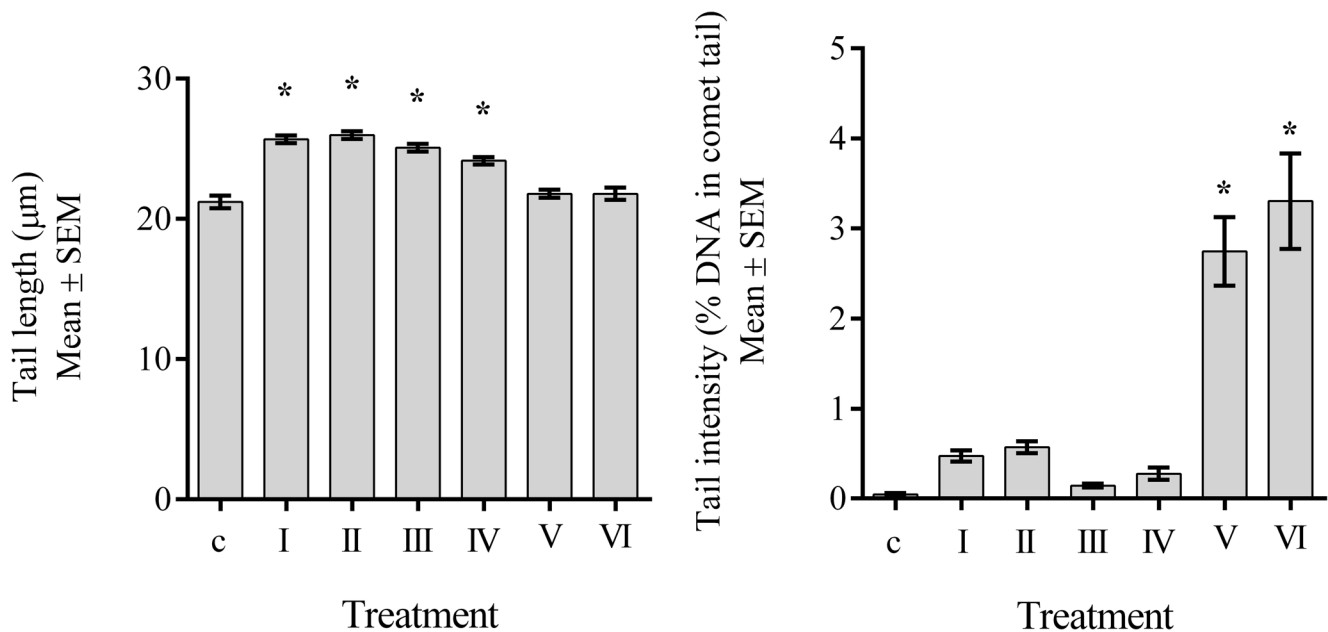
Fig. 2 Genotoxicity of AFB$_1$-negative and AFB$_1$-positive extracts of *A. flavus* isolates as well as AFB$_1$ applied in subcytotoxic concentrations determined by alkaline comet assay as tail length and tail intensity in A549 cells. C control (0.1% DMSO), I AFB$_1$-negative extract of *A. flavus* 0.05 mg/ml, II AFB$_1$-negative extract of *A. flavus* 0.1 mg/ml, III AFB$_1$-positive extract of *A. flavus* 0.1 mg/ml, IV AFB$_1$-positive extract of *A. flavus* 0.2 mg/ml, VAFB$_1$ 1 μmol/l, VI AFB$_1$ 10 μmol/l. The statistical significance of the treatment compared to control is marked with an asterisk (*, $P < 0.05$) above each histogram

which will be useful for planning future experiments with other cell types and exposure scenarios.

The underlying mechanism of AFB$_1$ genotoxicity was previously studied in the liver in vivo as well as in liver cells and several human bronchial cell lines with good expression of CYP 1A2 and 3A4 enzymes responsible for AFB$_1$ biotransformation into reactive AFB$_1$ 8,9-epoxide that binds to DNA (reviewed in Marchese et al. 2018). An association between lung cancer development following AFB$_1$ exposure was described in workers occupationally exposed to grain dust contaminated with this compound (Hayes et al. 1984). It was demonstrated that AFB$_1$ exposure induced the production of a DNA binding metabolite (epoxide) by lung cytosols which was correlated with lipoxygenase and prostaglandin H synthase and increased human pulmonary susceptibility to AFB$_1$ (Massey et al. 2000). Also, oxidative DNA damages in mouse lung cells were correlated to AFB$_1$ genotoxicity, owing to the induction of 8-hydroxy-20-deoxyguanosine (8-OHdG) formation (Guindon et al. 2008). Although A549 cells possess a limited expression of CYP enzymes (Garcia-Canton et al. 2013), our results suggest that it was sufficient to induce DNA damage upon exposure to low concentrations of AFB$_1$. In our previous study, AFB$_1$ (at 5 μmol/l) also evoked significant DNA damage and mutagenic activity, as revealed by the alkaline comet assay, and led to micronuclei formation (Jakšić et al. 2012). Since both the AFB$_1$-positive and the AFB$_1$-negative Aspergilli extracts exerted similar genotoxic action in A549 cells, we may hypothesize that other metabolites present

in the extract might antagonise AFB$_1$'s genotoxic action in AFB$_1$-producing strains.

Immunomodulatory effects of AFB$_1$ vs *A. flavus* extracts

Many macrophage and epithelial cells produce cytokines and chemokines after challenges from various inflammatory stimuli. Thus, we explored the differences in the secretion of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6, IL-8, IL-17) by THP-1 macrophage-like cells upon exposure to single AFB$_1$ or Aspergilli extracts (Fig. 3). TNF-α is an endogenous pyrogen and immunoregulatory cytokine responsible for the production of IL-1, IL-6, and IL-8. Deficiency of TNF-α in experimental animals promoted cancer (Suganuma et al. 1999). TNF-α stimulates polymorphonuclear leukocytes to damage *Aspergillus* hyphae enhancing phagocytosis (Roilides et al. 2002). IL-1β is a potent pro-inflammatory cytokine secreted by macrophages and monocytes which has a stimulatory effect on CD4+ T cells promoting differentiation into the T helper cell. It has a beneficial role of mediating an immune response against pathogenic infiltration, but it can also promote the pathogenesis of tissue damage that leads to chronic inflammation (Eder 2009; Turner et al. 2014). Similarly to TNF, IL-6 is a pleiotropic cytokine showing both pro-inflammatory and anti-inflammatory activities, but, in chronic inflammation, it is rather pro-inflammatory. The synthesis of this cytokine is induced by IL-1 and TNF-α (Duque and
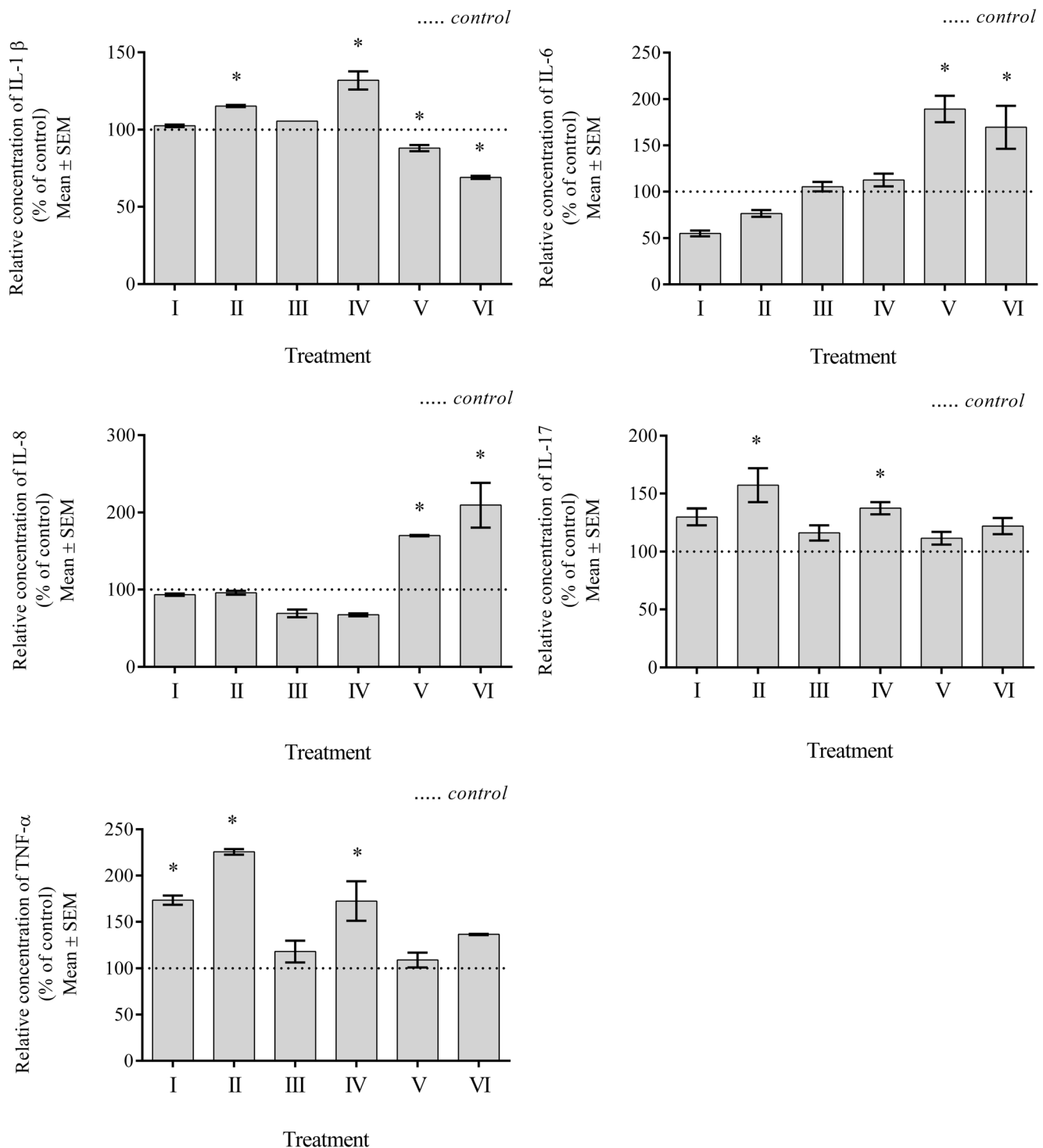
Fig. 3 Relative concentration of cytokines measured in the supernatant of THP-1 macrophage-like cells upon treatment: I AFB$_1$-negative extract of *A. flavus* 0.05 mg/ml, II AFB$_1$-negative extract of *A. flavus* 0.1 mg/ml, III AFB$_1$-positive extract of *A. flavus* 0.1 mg/ml, IV AFB$_1$-positive extract of *A. flavus* 0.2 mg/ml, V AFB$_1$ 1 μmol/l, VI AFB$_1$ 10 μmol/l. The statistical significance of the treatment compared to control (0.1% DMSO) is marked with an asterisk (*, $P < 0.05$) above each histogram

Descoteaux 2014). IL-8, also known as chemokine CXCL8, is a monocyte- and macrophage-derived cytokine that serves as a chemoattractant of neutrophils to the site of infection or injury and whose secretion from macrophages can be stimulated with TNF-α, IL-1β, or a lipopolysaccharide (Carré et al. 1991). IL-17 is a pro-inflammatory cytokine that plays an essential role in the host's defense against microbial infections and is implicated in various inflammatory

conditions, such as autoimmune diseases, metabolic disorders, and cancer (Gu et al. 2013).

AFB$_1$ alone vs Aspergilli extracts showed differences in the immunomodulatory pattern. AFB$_1$ has the most pronounced concentration-dependent effect on IL-6 and IL-8 excretion. Although the release of TNF-α and IL-17 was also increased by AFB$_1$ in a concentration-dependent manner, it was not significantly different from controls. Additionally, a difference in IL-1β release by THP-1 macrophage-like cells upon treatment with single AFB$_1$ vs Aspergilli extract was also observed; AFB$_1$ decreased IL-1β levels, while both Aspergilli extracts increased the release of this cytokine in a concentration-dependent manner. Several in vivo studies have also shown that AFB$_1$ stimulates the expression of IL-6 (Hinton et al. 2003; Meissonnier et al. 2008; Qian et al. 2014; Abbès et al. 2016). The same in vivo studies suggested that secretion of TNF-α significantly depends on the administered dose of AFB$_1$; e.g., a dose of 1.8 mg/kg AFB$_1$ during four weeks of administration resulted in an increased expression of TNF-α (Meissonnier et al. 2008), while at a significantly lower dose (80 μg/kg), AFB$_1$ decreased TNF-α (Abbès et al. 2016). In primary alveolar pig macrophages, AFB$_1$ (1–100 ng/ml) did not have an effect on the expression of both TNF-α and IL-1β but decreased phagocytosis efficiency (Liu et al. 2002). The inhibition of macrophage phagocytic activity was also established in vivo in a study on pigs which found that the inhalation of 16.8 μg/kg of AFB$_1$ inhibited the phagocytic activity of alveolar macrophages without recovery of function two weeks after inhalation (Jakab et al. 1994). Similarly to our study, AFB$_1$ evoked an increased expression of IL-8 mRNA in human lymphoblastoid Jurkat T-cells. The activation of an inflammatory response and over-active IL-8-induced recruitment of neutrophils to specific tissues can result in extensive tissue damage (Luongo et al. 2014). As in our study, IL-17 levels increased as the dose increase of AFB$_1$ in mice liver did, but no differences were detected between the treated and control groups (Ishikawa et al. 2017). Opposite to AFB$_1$, both Aspergilli extracts provoked a significant increase of TNF-α, IL-1β, and IL-17 in THP-1 macrophage-like cells, while not inducing a release of IL-6 and IL-8. The excretion of TNF-α, IL-1β, and IL-17 was probably affected by the other metabolites in the extracts of A. flavus that could have acted in synergy with AFB$_1$. At the same time, the metabolite mixtures in the extract could have had an antagonising activity on the induction of IL-6 and IL-8. Additionally, IL-17 appeared to play a central role in eosinophil extravasation from the blood into the lungs of mice upon intranasal exposure to A. fumigatus conidia (Murdock et al. 2012), which also contains metabolite mixture. Taken together, AFB$_1$ elevated IL-6 and IL-8 while Aspergilli extracts increased IL-1b, TNF-a, and IL-17 release in THP-1 macrophage-like cells suggesting that AFB$_1$ alone and both AFB$_1$-positive and AFB$_1$-negative Aspergilli extracts could potentially impart adverse effects on

innate immunity but with different mechanisms that could be influenced by the metabolite mixture composition in the extracts.

Considering the limitations of in vitro experiments, we may only suggest that chronic inhalatory exposure to AFB$_1$ and/or other A. flavus metabolites in occupational environments can stimulate epithelial damage of airways accompanied by lowered macrophage viability but with a different underlying mechanism depending on the extrolite profile of the airborne A. flavus.

Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflict of interest.

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References

Abbès S, Ben Salah-Abbès J, Jebali R, Younes RB, Oueslati R (2016) Interaction of aflatoxin B$_1$ and fumonisin B$_1$ in mice causes immunotoxicity and oxidative stress: possible protective role using lactic acid bacteria. J Immunotoxicol 13:46–54. https://doi.org/10.3109/1547691X.2014.997905

Abdel Hameed AA, Ayesh AM, Abdel Razik Mohamed M, Abdel Mawla H (2012) Fungi and some mycotoxins producing species in the air of soybean and cotton mills: a case study. Atmos Pollut Res 3:126–131. https://doi.org/10.5094/APR.2012.012

Altschul S, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403–410

Baranyi N, Jakšić Despot D, Palágyi A, Kiss N, Kocsubé S, Szekeres A, Kecskeméti A, Bencsik O, Vágvölgyi C, Šegvić Klarić M, Varga J (2015) Identification of Aspergillus species in Central Europe able to produce G-type aflatoxins. Acta Biol Hung 66:339–347. https://doi.org/10.1556/018.66.2015.3.9

Bennett JW, Klich M, Mycotoxins M (2003) Mycotoxins. Clin Microbiol Rev 16:497–516. https://doi.org/10.1128/CMR.16.3.497

Cafarchia C, Camarda A, Iatta R, Danesi P, Favuzzi V, Di Paola G, Pugliese N, Caroli A, Montagna MT, Otranto D (2014) Environmental contamination by Aspergillus spp. in laying hen farms and associated health risks for farm workers. J Med Microbiol 63:464–470. https://doi.org/10.1099/jmm.0.065946-0

Campbell-Platt G (1994) Fermented foods—a world perspective. Food Res Int 27:253–257. https://doi.org/10.1016/0963-9969(94)90093-0

Carré PC, Mortenson RL, King TE, Noble PW, Sable CL, Riches DW (1991) Increased expression of the interleukin-8 gene by alveolar macrophages in idiopathic pulmonary fibrosis. A potential mechanism for the recruitment and activation of neutrophils in lung fibrosis. J Clin Invest 88:1802–1810. https://doi.org/10.1172/JCI115501

Collins AR (2004) The comet assay for DNA damage and repair: principles, applications, and limitations. Mol Biotechnol 26:249–261. https://doi.org/10.1385/MB:26:3:249

Collins AR, Oscoz AA, Brunborg G, Gaivão I, Giovannelli L, Kruszewski M, Smith CC, Stetina R (2008) The comet assay: topical issues. Mutagenesis 23:143–151. https://doi.org/10.1093/mutage/gem051

Crawford JA, Rosenbaum PF, Anagnost SE, Hunt A, Abraham JL (2015) Indicators of airborne fungal concentrations in urban homes: understanding the conditions that affect indoor fungal exposures. Sci Total Environ 517:113–124. https://doi.org/10.1016/j.scitotenv.2015.02.060

Criseo G, Bagnara A, Bisignano G (2001) Differentiation of aflatoxin-producing and non-producing strains of Aspergillus favus group. Lett Appl Microbiol 33:291–295

Diaz-Meco MT, Moscat J (2012) The atypical PKCs in inflammation: NF-κB and beyond. Immunol Rev 246:154–167. https://doi.org/10.1111/j.1600-065X.2012.01093.x

Duque GA, Descoteaux A (2014) Macrophage cytokines: involvement in immunity and infectious diseases. Front Microbiol 5:1–12. https://doi.org/10.3389/fimmu.2014.00491

Eder C (2009) Mechanisms of interleukin-1 b release. Immunobiology 214:543–553. https://doi.org/10.1016/j.imbio.2008.11.007

Fazekas B, Tar A, Kovács M (2005) Aflatoxin and ochratoxin A content of spices in Hungary. Food Addit Contam 22:856–863. https://doi.org/10.1080/02652030500198027

Fischer G, Dott W (2003) Relevance of airborne fungi and their secondary metabolites for environmental, occupational and indoor hygiene. Arch Microbiol 179:75–82. https://doi.org/10.1007/s00203-002-0495-2

Fischer G, Müller T, Schwalbe R, Ostrowski R, Dott W (2000a) Exposure to airborne fungi, MVOC and mycotoxins in biowaste-handling facilities. Int J Hyg Environ Health 203:97–104. https://doi.org/10.1078/S1438-4639(04)70014-0

Fischer G, Müller T, Schwalbe R, Ostrowski R, Dott W (2000b) Species-specific profiles of mycotoxins produced in cultures and associated with conidia of airborne fungi derived from biowaste. Int J Hyg Environ Health 203:105–116. https://doi.org/10.1078/S1438-4639(04)70015-2

Frisvad JC, Smedsgaard J, Samson RA, Larsen TO, Thrane U (2007) Fumonisin B production by Aspergillus niger. J Agric Food Chem 55(23):9727–9732

Garcia-Canton C, Minet E, Anadon A, Anadon A, Meredith C (2013) Metabolic characterization of cell systems used in in vitro toxicology testing: lung cell system BEAS-2B as a working example. Toxicol in Vitro 27:1719–1727. https://doi.org/10.1016/j.tiv.2013.05.001

Geisen R (1996) Multiplex polymerase chain reaction for the detection of potential aflatoxin and sterigmatocystin producing fungi. Syst Appl Microbiol 19:388–392. https://doi.org/10.1016/S0723-2020(96)80067-1

Godet M, Munaut F (2010) Molecular strategy for identification in Aspergillus section Flavi. FEMS Microbiol Lett 304:157–168. https://doi.org/10.1111/j.1574-6968.2009.01890.x

Gu C, Wu L, Li X (2013) IL-17 family: cytokines, receptors and signaling. Cytokine 64:389–399. https://doi.org/10.1016/j.asieco.2008.09.006.EAST

Guindon KA, Foley JF, Maronpot RR, Massey TE (2008) Failure of catalase to protect against aflatoxin B₁-induced mouse lung tumorigenicity. Toxicol Appl Pharmacol 227:179–183. https://doi.org/10.1016/j.taap.2007.10.015

Hardin BD, Robbins CA, Fallah P, Kelman BJ (2009) The concentration of no toxicologic concern (CoNTC) and airborne mycotoxins. J Toxicol Environ Heal—Part A Curr Issues 72:585–598. https://doi.org/10.1080/15287390802706389

Hayes RB, van Nieuwenhuize JP, Raatgever JW, ten Kate FJ (1984) Aflatoxin exposures in the industrial setting: an epidemiological study of mortality. Food Chem Toxicol 22:39–43. https://doi.org/10.1016/0278-6915(84)90050-4

Hedayati MT, Pasqualotto AC, Warn PA, Bowyer P, Denning DW (2007) Aspergillus flavus: human pathogen, allergen and mycotoxin producer. Microbiology 153:1677–1692. https://doi.org/10.1099/mic.0.2007/007641-0

Hinton DM, Myers MJ, Raybourne RA, Francke-Carroll S, Sotomayor RE, Shaddock J, Warbritton A, Chou MW (2003) Immunotoxicity of aflatoxin B1 in rats: effects on lymphocytes and the inflammatory response in a chronic intermittent dosing study. Toxicol Sci 73:362–377. https://doi.org/10.1093/toxsci/kfg074

Hong S-B, Go S-J, Shin H-D, Frisvad JC, Samson RA (2005) Polyphasic taxonomy of Aspergillus fumigatus and related species. Mycologia 97:1316–1329. https://doi.org/10.3852/mycologia.97.6.1316

Hulina A, Grdić Rajković M, Jakšić Despot D, Jelić D, Dojder A, Čepelak I, Rumora L (2018) Extracellular Hsp70 induces inflammation and modulates LPS/LTA-stimulated inflammatory response in THP-1 cells. Cell Stress Chaperones 23:373–384. https://doi.org/10.1007/s12192-017-0847-0

Ishikawa AT, Hirooka EY, e Silva PLA, Bracarense APFRL, Flaiban KKMDC, Akagi CY, Kawamura O, Costa MCD, Itano EN (2017) Impact of a single oral acute dose of aflatoxin B₁ on liver function/cytokines and the lymphoproliferative response in C57BL/6 mice. Toxins 9:1–10. doi: https://doi.org/10.3390/toxins9110374

Jakab GJ, Hmieleski RR, Zarba A, Hemenway DR, Groopman JD (1994) Respiratory aflatoxicosis: a suppression of pulmonary and systemic host defenses in rats and mice. Toxicol Appl Pharmacol 125:198–205. https://doi.org/10.1006/TAAP.1994.1065

Jakšić Despot D, Šegvić Klarić M (2014) A year-round investigation of indoor airborne fungi in Croatia. Arh Hig Rada Toksikol 65:209–218. https://doi.org/10.2478/10004-1254-65-2014-2483

Jakšić Despot D, Kocsubé S, Bencsik O, Kecskeméti A, Szekeres A, Jelić D, Kopjar N, Vágvölgyi C, Varga J, Šegvić Klarić M (2016) Species diversity and cytotoxic potency of airborne sterigmatocystin-producing Aspergilli from the section Versicolores. Sci Total Environ 562:296–304. https://doi.org/10.1016/j.scitotenv.2016.03.183

Jakšić D, Puel O, Canlet C, Kopjar N, Kosalec I, Šegvić Klarić M (2012) Cytotoxicity and genotoxicity of versicolorins and 5-methoxysterigmatocystin in A549 cells. Arch Toxicol 86:1583–1591. https://doi.org/10.1007/s00204-012-0871-x

Jakšić D, Kocsubé S, Bencsik O, Kecskeméti A, Szekeres A, Jelić D, Kopjar N, Vágvölgyi C, Varga J, Šegvić Klarić M (2018) Fumonisin production and toxic capacity in airborne black Aspergilli. Toxicol in Vitro 53:160–171. https://doi.org/10.1016/j.tiv.2018.08.006

Kelly JD, Eaton DL, Guengerich FP, Coulombe RA Jr (1997) Aflatoxin B1 activation in human lung. Toxicol Appl Pharmacol 144:88–95. https://doi.org/10.1006/taap.1997.8117

Lee RJ, Workman AD, Carey RM, B1 C, Rosen PL, Doghramji L, Adappa ND, Palmer JN, Kennedy DW, Cohen NA (2016) Fungal aflatoxins reduce respiratory mucosal ciliary function. Sci Rep 6:1–13. https://doi.org/10.1038/srep33221

Liu BH, Yu FY, Chan MH, Yang YL (2002) The effects of mycotoxins, fumonisin B₁ and aflatoxin B₁, on primary swine alveolar macrophages. Toxicol Appl Pharmacol 180:197–204. https://doi.org/10.1006/taap.2002.9406

Luongo D, Russo R, Balestrieri A, Marzocco S, Bergamo P, Severino L (2014) In vitro study of AFB₁ and AFM₁ effects on human lymphoblastoid Jurkat T-cell model. J Immunotoxicol 11:353–358. https://doi.org/10.3109/1547691X.2013.848250

Manikandan P, Varga J, Kocsubé S, Anita R, Revathi R, Németh TM, Narendran V, Vágvölgyi C, Panneer Selvam K, Shobana CS, Babu Singh YR, Kredics L (2013) Epidemiology of Aspergillus keratitis at a tertiary care eye hospital in South India and antifungal

susceptibilities of the causative agents. Mycoses 56:26–33. https://doi.org/10.1111/j.1439-0507.2012.02194.x

Marchese S, Polo A, Ariano A, Velotto S, Costantini S, Severino L (2018) Aflatoxin B$_1$ and M$_1$: biological properties and their involvement in cancer development. Toxins 10:214. https://doi.org/10.3390/toxins10060214

Massey TE, Smith GBJ, Tam AS (2000) Mechanisms of aflatoxin B$_1$ lung tumorogenesis. Exp Lung Res 26:673–683

McKean C, Tang L, Tang M, Billam M, Wang Z, Theodorakis CW, Kendall RJ, Wang JS (2006) Comparative acute and combinative toxicity of aflatoxin B$_1$ and fumonisin B$_1$ in animals and human cells. Food Chem Toxicol 44:868–876. https://doi.org/10.1016/j.fct.2005.11.011

Medina A, Rodriguez A, Magan N (2014) Effect of climate change on *Aspergillus flavus* and aflatoxin B$_1$ production. Front Microbiol 5:348. https://doi.org/10.3389/fmicb.2014.00348

Meissonnier GM, Pinton P, Laffitte J, Cossalter AM, Gong YY, Wild CP, Bertin G, Galtier P, Oswald IP (2008) Immunotoxicity of aflatoxin B$_1$: impairment of the cell-mediated response to vaccine antigen and modulation of cytokine expression. Toxicol Appl Pharmacol 231:142-149–142-149. https://doi.org/10.1016/j.taap.2008.04.004

Murdock BJ, Falkowski NR, Shreiner AB, Sadighi Akha AA, McDonald RA, White ES, Toews GB, Huffnagle GB (2012) Interleukin-17 drives pulmonary eosinophilia following repeated exposure to *Aspergillus fumigatus* conidia. Infect Immun 80:1424–1436. https://doi.org/10.1128/IAI.05529-11

Olsen J, Dragsted L, Autrup H (1988) Cancer risk and occupational exposure to aflatoxins in Denmark. Br J Cancer 58:392–396. https://doi.org/10.1038/bjc.1988.226

Oppliger A, Rusca S, Charrière N, Vu Duc T, Droz PO (2005) Assessment of bioaerosols and inhalable dust exposure in Swiss sawmills. Ann Occup Hyg 49:385–391. https://doi.org/10.1093/annhyg/meh105

Palanee T, Dutton MF, Chuturgoon AA (2001) Cytotoxicity of aflatoxin B$_1$ and its chemically synthesised epoxide derivative on the A549 human epithelioid lung cell line. Mycopathologia 151:155–159. https://doi.org/10.1023/A:1017985924257

Pariza MW, Johnson EA (2001) Evaluating the safety of microbial enzyme preparations used in food processing: update for a new century. Regul Toxicol Pharmacol 33:173–186. https://doi.org/10.1006/rtph.2001.1466

Paterson RRM, Lima N (2010) How will climate change affect mycotoxins in food? Food Res Int 43:1902–1914. https://doi.org/10.1016/j.foodres.2009.07.010

Perrone G, Gallo A, Logrieco AF (2014) Biodiversity of *Aspergillus* section *Flavi* in Europe in relation to the management of aflatoxin risk. Front Microbiol 5:1–5. https://doi.org/10.3389/fmicb.2014.00377

Piecková E, Wilkins K (2004) Airway toxicity of house dust and its fungal composition. Ann Agric Environ Med 11:67–73

Qian G, Tang L, Guo X, Wang F, Massey ME, Su J, Guo TL, Williams JH, Phillips TD, Wang JS (2014) Aflatoxin B$_1$ modulates the expression of phenotypic markers and cytokines by splenic lymphocytes of male F344 rats. J Appl Toxicol 34(3):241–249. https://doi.org/10.1002/jat.2866

Rao CY, Fink RC, Wolfe LB, Liberman DF, Burge HA (2016) A study of Aflatoxin production by growing on wallboard. Journal of the American Biological Safety Association 2(4):36–42

Ren P, Ahearn DG, Crow Jr SA (1999) Comparative study of Aspergillus mycotoxin production on enriched media and construction material. J Ind Microbiol Biotechnol 23(3):209–213

Roilides E, Lamaignere CG, Farmaki E (2002) Cytokines in immunodeficient fungal infections: an emerging patients therapy. Int J Infect Dis 6:154–163

Samson RA, Houbraken J, Thrane U, Frisvad JC, Andersen B (2010) Food and indoor fungi. Fungal Biodiversity Centre Utrecht, The Netherlands

Samson RA, Visagie CM, Houbraken J, Hong S-B, Hubka V, Klaassen CHW, Perrone G, Seifert KA, Susca A, Tanney JB, Varga J, Kocsubé S, Szigeti G, Yaguchi T, Frisvad JC (2014) Phylogeny, identification and nomenclature of the genus Aspergillus. Stud Mycol 78:141–173

Schweer KE, Jakob B, Liss B, H2 C, Fischer G, Vehreschild M, Cornely OA, Vehreschild JJ (2016) Domestic mould exposure and invasive aspergillosis—air sampling of *Aspergillus* spp. spores in homes of hematological patients, a pilot study. Med Mycol 54:576–583. https://doi.org/10.1093/mmy/myw007

Sharma D, Dutta BK, Singh AB (2010) Exposure to indoor fungi in different working environments: a comparative study. Aerobiologia (Bologna) 26:327–337. https://doi.org/10.1007/s10453-010-9168-9

Singh NP, McCoy MT, Tice RR, Schneider EL (1988) A simple technique for quantitation of low levels of DNA damage in individual cells. Exp Cell Res 175:184–191

Soares C, Rodrigues P, Peterson SW, Lima N, Venâncio A (2012) Three new species of *Aspergillus* section *Flavi* isolated from almonds and maize in Portugal. Mycologia 104:682–697. https://doi.org/10.3852/11-088

Suganuma M, Okabe S, Marino MW, Sakai A, Sueoka E, Fujiki H (1999) Essential role of tumor necrosis factor a (TNF-a) in tumor promotion as revealed by TNF-a deficient mice. Cancer Res 59:4516–4518

Turner MD, Nedjai B, Hurst T, Pennington DJ (2014) Cytokines and chemokines: at the crossroads of cell signalling and inflammatory disease. Biochim Biophys Acta—Mol Cell Res 1843:2563–2582. https://doi.org/10.1016/j.bbamcr.2014.05.014

Van Vleet TR, Klein PJ, Coulombe RA (2002) Metabolism and cytotoxicity of aflatoxin B$_1$ in cytochrome P-450 expressin human lung cells. J Toxicol Environ Health A 65:853–867. https://doi.org/10.1080/0098410029007121

Varga J, Frisvad JC, Samson RA (2011) Two new aflatoxin producing species, and an overview of *Aspergillus* section *Flavi*. Stud Mycol 69:57–80. https://doi.org/10.3114/sim.2011.69.05

Viegas S, Veiga L, Malta-Vacas J, Sabino R, Figueredo P, Almeida A, Viegas C, Carolino E (2012) Occupational exposure to aflatoxin (AFB$_1$) in poultry production. J Toxicol Environ Health A 75:1330–1340. https://doi.org/10.1080/15287394.2012.721164

Yu J, Bhatnagar D, Cleveland TE (2004) Completed sequence of a aflatoxin pathway gene cluster in *Aspergillus parasiticus*. FEBS Lett 564:126–130. https://doi.org/10.1016/S0014-5793(04)00327-8

Zhao M, Xia L, Chen GQ (2012) Protein kinase c delta in apoptosis: a brief overview. Arch Immunol Ther Exp 60:361–372. https://doi.org/10.1007/s00005-012-0188-8;10.1007/s00005-012-0188-8