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ORIGINAL ARTICLE

Mycobiota of berry fruits – levels of filamentous fungi and mycotoxins, composition of fungi, and analysis of potential health risk for consumers

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Abstract

Introduction and Objective. The aim of the study was to determine the presence, concentration and generic composition of filamentous fungi. Selected mycotoxins were also determined: total aflatoxins and deoxynivalenol.

Materials and method. In 2017–2018, 40 samples of strawberry fruits and 40 samples of red raspberry fruits were collected. In 2019–2020, 37 samples of fresh strawberry fruits and 41 samples of fresh red raspberry fruits were collected on conventional farms located in eastern Poland and were subjected to mycological examination. The concentration and species composition of filamentous fungi were determined by the method of plate dilutions on malt agar. The isolated strains were identified using macroscopic and microscopic methods. Samples were also analysed for the presence of aflatoxin B1, total aflatoxin and deoxynivalenol using ELISA tests.

Results. The median concentrations of fungi were moderate or low: 3.079 and 3.491 $\log_{10}$ CFU g^{-1} for strawberries and raspberries, respectively. Fungi of the genus *Cladosporium* prevailed in the mycobiota of berry fruits, accounting for 84.3% of total isolates in raspberries and 34.8% in strawberries. The occurrence of total aflatoxins was detected in the majority of tested samples (73.2% in raspberries and 70.3% in strawberries) but never exceeded the level of 4.0 µg kg^{-1} assumed as safe. Deoxynivalenol has been detected only in raspberries with the prevalence of 58.5%. Its median concentration was 242.0 µg kg^{-1} and in 7 out of 41 samples (17.0%) exceeded the level of 750.0 µg kg^{-1}, assumed as safe.

Conclusions. Filamentous fungi and mycotoxins occurred in the examined berries at levels that mostly do not represent a health risk for immunocompetent people, but might pose such risk for immuno-compromised and/or atopic consumers.

Key words
food safety, strawberries, filamentous fungi, aflatoxins, raspberries, DON

INTRODUCTION

Fresh fruits and vegetables are known to provide health-promoting diet supplements, such as vitamins, minerals, antioxidants and fibres [1]. Their consumption has been encouraged by government health agencies in many countries to protect against a range of illnesses, such as cancers and cardiovascular diseases [2]. The resulting great increase in fruit and vegetable consumption, approximating in the global scale 38.6% between 1990–2015, aroused interest in the microbiological safety of these products [1, 3, 4].

The study presents the issue of the mycological safety of berries (strawberries and raspberries) which is largely unexplored. Nevertheless, this problem needs attention because of the fast consumption of berries in the European Union, for example, in Poland where the yearly consumption of berries exceeds 10.5 kg per person [5]. So far, outbreaks of acute gastrointestinal disease caused by bacteria, viruses or parasites have been linked mostly to the consumption of contaminated vegetables, much less to the consumption of berries [6, 7]. Hitherto reported acute diseases associated with the consumption of strawberries and/or raspberries were caused mostly by noroviruses, hepatitis A viruses and *Cyclospora* protozoans [2], but rarely by bacteria, such as *Escherichia coli* O157:H7 [6], and never by fungi [2].

The lack of acute illnesses caused by fungi associated with berries or other produce contributes to the inaccurate view that fungi occurring in fruits and/or vegetables do not pose any significant hazard for consumers. In fact, although filamentous fungi do not tend to cause any acute disease after the consumption of produce, they do represent a potential cause of chronic diseases, which may be caused mostly by mycotoxins, secondary metabolites able to elicit severe systemic diseases, less often by allergic reactions, or by infections caused by the opportunistic fungal pathogens [8, 9, 10]. Mycotoxins may exhibit nephrotoxic, genotoxic, teratogenic, carcinogenic, and cytotoxic properties and, as a consequence, may cause liver carcinomas, renal dysfunctions, and immunosuppressed states [10]. They include aflatoxins, ochratoxins, patulin, trichothecenes (such as deoxynivalenol or T-2 toxin), fumonisins, zearalenon, and many other compounds which are produced by filamentous fungi, mostly those belonging to the *Aspergillus*, *Penicillium* and *Fusarium* genera, growing on various crops and plant materials [9, 10, 11, 12].

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Until recently, there have been only a few studies on fungi occurring on fruits as potential health hazards, in particular with regard to the species composition. The median concentrations of fungi reported for various fruits sold on the markets of different European and Asian countries ranged within the limits 1.1–4.0 $\log_{10}$ CFU g^{-1}, which is approximately 1.5–2.5 logs lower compared to vegetables [13, 14].

So far, little is known about mycobiota present on berry fruits growing in the field or sold on the markets. The studies to-date have concentrated mostly on plant pathogenic fungi affecting crops of berries, including strawberries and raspberries. The most important fungal pathogens include *Verticillium* spp., causing Verticillium wilt, *Phytophthora* spp., causing crown, leather rot, *Botrytis cinerea* causing grey muld, and *Colletotrichum acutatum* causing anthracnose [15].

To the best of our knowledge, the first research in which the species composition of fungi developing on 'normal' berries (not attacked by pathogens) was determined is an experimental study performed in Washington, D.C. (USA) by Tournas and Katsoudas [8]. The authors incubated the surface-disinfected samples of various supermarket-derived fruit categories (berries, grapes, citrus fruits) for up to two weeks at room temperature, and then determined the percent of samples contaminated by fungi and the species composition of the fungal biota. Berries (mostly raspberries, blackberries, and strawberries) showed the highest prevalence of contamination, ranging from 97–100%. The species *B. cinerea*, a known pathogen of berries, proved to be the most common fungal contaminant, present in 75–78% of berry samples. The other common fungal genera were *Alternaria*, *Cladosporium*, *Fusarium*, *Penicillium*, and *Rhizopus*, followed by *Trichoderma*, *Aureobasidium pullulans* and yeasts. Although the presented study has an experimental character and does not address the primary concentrations of fungi on fruits collected directly from field or markets, it provides the hitherto richest source of the information on fungi that may develop on berries and create a potential health hazard for consumers.

Subsequently, Buyukunal et al. [14] reported the concentrations of fungi ranging from 2.0–2.65 $\log_{10}$ CFU g^{-1} on strawberries sold on Istanbul markets and Hussein et al. [16] found in the samples of strawberries collected from markets in Qena (Egypt) the average concentration of fungi equal tbe 8.66×10^3 CFU g^{-1} (3.938 $\log_{10}$ CFU g^{-1}). The mycobiota comprised 43 species belonging to 15 genera. The prevailing species were *Aspergillus flavus* (26.6% of the total count), *Aspergillus niger* (21.6%) and *Penicillium citrinum* (9.0%), all known as mycotoxin producers.

Other studies on mycobiota of strawberries were oriented towards detection of potentially pathogenic fungal species in various aerial parts of plants: fruits, foliage, and flowers. The prevalence of the individual fungal species, but not the concentration per weight unit of plant, was determined. Rigotti et al. [17] isolated from symptomless field grown strawberries in Switzerland about 40 fungal genera or species, of which more than half were potential plant pathogens. The most common fungi were following: *Alternaria* spp., *B. cinerea*, *Cladosporium* spp., *Epicoccum purpurascens*, *Fusarium* spp. and *Penicillium* spp. Mouden et al. [18] examined the diseased strawberry plants on 7 farms in Morocco and found that *B. cinerea* showed the highest prevalence (90.3%), followed by *Alternaria alternata* (88.1%), *Cladosporium herbarum* (53.1%) and *Cladosporium cladosporioides* (33.3%). Moreover, on some farms, *Colletotrichum acutatum*, *C. gloeosporioides* and *Epicoccum purpurascens* commonly occurred. The results of the above cited authors are in line with those achieved by Abdelfattah et al. [19] who demonstrated with molecular methods that *Botrytis* spp. and *Cladosporium* spp. were the most abundant fungi in strawberries from southern Italy with the percentage of readings ranging from 70% in leaves to 99% in fruit.

Surprisingly, although raspberries are attacked by plant pathogenic fungi, that could also be potentially pathogenic for human consumers to a similar extent as strawberries, in spite of a thorough search of available databases, we were unable to find any study on mycobiota of raspberries. Two of the authors of the current study have already published a part of the results concerning raspberries shown in this study, with respect to the drug resistance of fungal isolates [20]. Overall, a high level of resistance was found which could reduce the treatment efficacy of potential fungal infections by transfer of resistance genes to fruit consumers.

Until recently, only scant information has been available on the prevalence and concentration of mycotoxins in fruits, compared to other foods [11]. Fernández-Cruz et al. in their comprehensive review, [21] indicate aflatoxins (AT), ochratoxin A (OTA), patulin (PAT) and the *Alternaria* toxins (alternariol (AOH), alternariol methyl ether (AME) and altenuene (ATE)) as mycotoxins most commonly found in fruits and their processed products. The authors report that the low concentration of OTA (1.44 μg kg^{-1}) was found in strawberries, whereas low concentrations of AOH (<1.5 μg L^{-1}) were detected in raspberry juice. Similarly, Juan et al. [22] detected low concentrations of AOH ranging from 0.026 – 0.752 μg kg^{-1} in strawberries stored at 22 °C, and low concentrations of AME ranging from 0.011–0.137 μg kg^{-1} in strawberries stored at 6°C. In contrast, very high levels of PAT were found in strawberries (145 μg kg^{-1}) and raspberries (746 μg kg^{-1}) [21]. In the above cited study on fungal contamination of strawberries, Hussein et al. [16] demonstrated the production of aflatoxin and ochratoxin A by selected isolates of *A. flavus* and *A. niger*, which resulted in the concentrations of these mycotoxins approximating 3.5 and 4.1 μg kg^{-1}, respectively.

OBJECTIVE

The aim of the present study, designed as a continuation of earlier studies on the mycological safety of Polish vegetables [23, 24], was to determine the presence, concentration and generic composition of filamentous fungi, as well as the participation of potentially pathogenic species, in 2 species of berry fruits (assuming the common language definition): strawberry (*Fragaria ananassa*) and red raspberry (*Rubus idaeus*).

Considering the significant role of mycotoxins in the pathogenicity of fungal contaminants of vegetables, the scope of the present work was extended by determination of aflatoxins, which are regarded as the greatest health hazard among mycotoxins [9, 10], and previously detected in various fruits [21], including berries [16]. Deoxynivalenol (DON) was selected as the second type of mycotoxins to be determined, and has been identified by the EFSA (European Food Safety Authority) CONTAM Panel [25] as the mycotoxin prevalent in various foods such as cereals, fruits, and legumes. Other reasons for choosing this mycotoxin were: detection of *Fusarium culmorum* and *Fusarium graminearum*, the two most important producers of DON in the course of the present work, as well as the fact that generally the occurrence of this mycotoxin in fruits is largely unexplored.

MATERIALS AND METHOD

Sampling. Samples of fresh, symptomless berry fruits were collected randomly on conventional farms located in the Lublin Province of eastern Poland in two consecutive time periods:

a) 2017–2018, 40 samples of strawberry (*Fragaria ananassa*) fruits and 40 samples of red raspberry (*Rubus idaeus*) fruits were collected from June – September.
b) 2019–2020, 37 samples of fresh strawberry fruits and 41 samples of fresh red raspberry fruits were collected during the same season.

The samples were collected aseptically into clean foil bags and transported to the laboratory.

Preparation of the samples. The samples were analysed immediately after delivery to the laboratory. From each sample, a subsample weighing 20 g was separated, suspended in 180 ml of Ringer solution (Merck KGaA, Germany) with the addition of 10% Tween 80 and homogenized for 5 min using the Bag Mixer 400 SW (Interscience, France). From the homogenates prepared in this way, decimal dilutions were performed which were used for cultures.

Determination of concentration and diversity of filamentous fungi in fruit samples. In order to determine the concentration and species composition of filamentous fungi in the fruit samples, the method of plate dilutions on malt agar (Difco, USA) with chloramphenicol was used, by spreading 0.1 ml of each dilution on the agar surface. The study was conducted in 2 parallel repetitions. Inoculated media were incubated at the temperature of 30 °C for 72 hrs, then at room temperature (22 °C) for 72 hrs, and finally at the temperature of 8 °C for 48 hrs. The grown colonies were counted and differentiated, and the numbers of fungi were expressed as decimal logarithms of the numbers of colony forming units (CFU) in 1 g of the examined material (log$_{10}$ CFU g^{-1}).

For identification, fungal colonies were checked for purity by microscopic and culture methods and subcultured on malt agar slants. The isolated strains were determined using macroscopic and microscopic methods, with the aid of keys and atlases [26, 27, 28]. All isolates were compared to standard strains from the Collection of Fungal Strains at the Institute of Rural Health in Lublin, Poland, which had been determined by phenotypic and genotypic methods. Finally, the species composition of mycobiota was determined for individual samples.

Detection of mycotoxins. Prior to laboratory examination, the reagents and samples were brought to room temperature (20–25 °C). Fruit samples were analysed for the presence of aflatoxin B1 (AFB1), total aflatoxin (the sum of aflatoxins B1, B2, G1, G2 – AFT) and deoxynivalenol (DON). Samples weighing 10 g each were thoroughly mixed with 70% methanol (for aflatoxins) or with distilled water (for deoxynivalenol), and crushed using a Bag Mixer homogenizer. The obtained mixtures were filtered into separate sterile tubes through Whatman No.1 filters and examined by the ELISA method, in accordance with the manufacturer's instructions. Quantitative determinations of mycotoxins in the samples were performed by the immunoenzymatic ELISA method, using commercial sets RIDASCREEN® Aflatoxin B1 30/15, RIDASCREEN® Aflatoxin Total and RIDASCREEN® FAST DON (R-Biopharm, Germany). The content of mycotoxins was calculated according to the Rida® Soft Win programme with reference to prepared standard curve. Standards in 6 concentrations (0, 0.05, 0.15, 0.45, 1.35 and 4.05 μg kg^{-1}) were used for preparing the total aflatoxin standard curve and 6 standards for the aflatoxin B1 curve (0, 1, 5, 10, 20 and 50 μg kg^{-1}). For deoxynivalenol, standards in 5 concentrations were used (0, 222, 666, 2,000 and 6,000 μg kg^{-1}).

Statistical analysis. The results were analysed by the Mann-Whitney non-parametric test and Spearman non-parametric test for correlation, using STATISTICA v. 5.1 package (Statsoft, USA).

RESULTS

Concentrations of filamentous fungi in berry fruits. The concentrations (median, range) of filamentous fungi in logarithmic scale are shown in Table 1, and the mean concentrations of fungi in linear scale and the composition of mycobiota are presented in Table 2. The median concentration of filamentous fungi in raspberries for the total sampling period (2017–2020) was significantly greater than in strawberries (3.491 vs. 3.079 log$_{10}$ CFU g^{-1}), and the difference was highly significant (Mann-Whitney, P<0.00001).

The median concentration of filamentous fungi in berry

Table 1. Concentration of filamentous fungi in the Polish berry fruits

Fruit	Years of sampling	Number of samples (N)	Concentration of filamentous fungi (log$_{10}$ CFU g^{-1}) Median (range)
Strawberry	2017-2018	40	3.041 (2.000 – 3.462)
	2019-2020	37	3.176 (2.778 – 3.556) *
	Total (2017-2020)	77	3.079 (2.000 – 3.556)
Raspberry	2017-2018	40	3.431 (2.602 – 4.114)
	2019-2020	41	3.662 (2.778 – 4.430)
	Total (2017-2020)	81	3.491 (2.602 – 4.430) #
Total berry fruits	2017-2018	80	3.176 (2.000 – 4.114)
	2019-2020	78	3.342 (2.778 – 4.430) **
	Total (2017-2020)	158	3.255 (2.000 – 4.430)

* Concentration of fungi significantly greater in the years 2019-2020 (P=0.0025)
# Concentration of fungi in raspberries significantly greater than in strawberries (P=0.000000)
** Concentration of fungi significantly greater in the years 2019-2020 (P=0.008)

Table 2. Generic composition of filamentous fungi isolated from the Polish berry fruits

Fruit	Mean concentration of commonest genera of filamentous fungi expressed as CFU × $10^2 g^{-1}$ (below percent of the total count)										Concentration of total filamentous fungi, expressed as CFU × $10^2 g^{-1}$, mean ± S.D. (percent of the total count)
	Acremonium spp.	*Alternaria* spp.	*Aureobasidium* spp.	*Botrytis* spp	*Cladosporium* spp.	*Fusarium* spp.	*Mucor* spp.	*Penicillium* spp.	*Rhizoctonia* spp.	Other fungi*	
Strawberry N=77	0.3 (2.1%)	0.7 (4.9%)	0.5 (3.5%)	0.0 (0.0%)	4.9 (34.8%)	1.5 (10.7%)	0.7 (5.0%)	1.2 (8.5%)	0.0 (0.0%)	4.3 (30.5%)	14.1 ± 7.5 (100%)
Raspberry N=81	0.1 (0.3%)	2.0 (4.2%)	0.3 (0.7%)	0.4 (1.0%)	39.1 (84.3%)	0.8 (1.7%)	0.1 (0.2%)	0.2 (0.4%)	0.3 (0.6%)	3.1 (6.6%)	46.4 ± 44.7 (100%)
Total N=158	0.2 (0.7%)	0.7 (2.2%)	0.4 (1.4%)	0.2 (0.7%)	22.5 (73.3%)	1.1 (3.7%)	0.4 (1.4%)	0.7 (2.2%)	0.2 (0.7%)	4.2 (13.7%)	30.6 ± 36.2 (100%)

N = number of examined samples
* Including sterile mycelia

fruits in the sampling period 2019–2020 was significantly greater compared to 2017–2018 (3.342 vs. 3.176 $\log_{10}$ CFU g^{-1}), and the difference proved to be significant (Mann-Whitney, $P<0.01$).

Filamentous fungi were detected in all (100%) samples of examined fruits.

Composition of the mycobiota of filamentous fungi indigenous to berry fruits and potential pathogenicity of isolates. As seen in Table 2 and Figure 1, presenting the generic composition of the fruit mycobiota, the so called 'field fungi' of the genera *Alternaria* and *Cladosporium*, usually observed on live plants, prevailed in the mycobiota of examined fruits, forming together 88.5% of the total fungal population in raspberries and 39.7% in strawberries. *Cladosporium* clearly dominated in the mycobiota of raspberries, forming 84.3% of the total count. In strawberries, its incidence was lower by more than half (34.8%) but still remained the most numerous genus among fungal biota. *Alternaria* accounted for 4.9% and 4.2% in the mycobiota of strawberries and raspberries, respectively. Among other fungal genera, the most common in the mycobiota of strawberries and raspberries were species belonging to the genera: *Fusarium* (10.7% and 1.7% of the total count, respectively), *Penicillium* (8.5% and 0.4%), *Mucor* (5.0% and 0.2%), and *Aureobasidium* (3.5% and 0.7%) (Tab. 2, Fig. 1). The frequency of other genera was distinctly smaller.

The mycobiota of berry fruits showed a marked diversity. Of the 4,385 fungal strains isolated from 158 fruit samples and subjected to the identification procedure, 2,311 could be identified down to the specific level as belonging to 36 species (Tab. 3), and 2,074 were identified down to the generic level as belonging to 17 genera (*Acremonium* spp., *Alternaria* spp., *Aspergillus* spp., *Aureobasidium* spp., *Chaetomium* spp., *Chrysosporium* spp., *Cladosporium* spp., *Fusarium* spp., *Gonatobotrys* spp., *Humicola* spp., *Mucor* spp., *Penicillium* spp., *Rhizoctonia* spp., *Rhizopus* spp., *Stemphylium* spp., *Trichoderma* spp., *Ulocladium* spp.). Altogether, the presence of at least 53 fungal taxons (altogether 36 species and 17 genera) were found in the examined fruit samples.

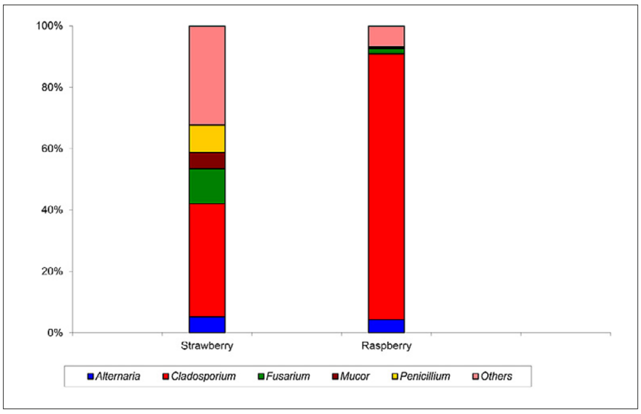

Figure 1. Generic composition of filamentous fungi isolated from two kinds of berry fruits

Table 3 lists all fungal species isolated from berry fruits, reporting the species name and source of isolation, as well as potential pathogenicity and mycotoxins produced with appropriate references. The commonest species were: *Cladosporium sphaerospermum* (present in 67 out of the total of 158 samples), *Alternaria tenuissima* (present in 41 samples), and *Cladosporium cladosporioides* (present in 27 samples). These findings are in accord with the high prevalence of these genera in the total mycobiota shown in Table 2.

As seen in Table 3, of the 36 species of filamentous fungi determined in the examined berry fruits, as many as 34 (94.4%) were reported to be pathogenic for humans and/or animals [9, 11, 12, 26, 29, 30]. The frequency of potential fungal pathogens was very close to the value recorded by us (equal to 95.0%) in the earlier study on leafy and fruit vegetables [24], but much higher compared that found in the study on root vegetables (45.9%) [23]. Most probably, the real number of pathogenic species present in the examined environment was greater, as only strains identified down to the species level were recognized (Tab. 3). However, other fungi which were determined to the generic level, such as *Alternaria* spp., *Aspergillus* spp., *Cladosporium* spp., *Fusarium* spp., *Mucor* spp., or *Penicillium* spp., might also possess pathogenic properties [9, 11, 12, 26, 29].

Concentrations of mycotoxins in berry fruits. The concentrations of aflatoxin B1, total aflatoxin and deoxynivalenol are shown in Table 4. As seen in the Table, aflatoxin B1 (AFB1) was not detected in the examined berry fruits. The concentrations of total aflatoxin (AFT) in the examined berry fruits were low and very similar in strawberries and raspberries (Mann-Whitney, $P>0.05$). Median value, maximal value and prevalence equalled to 1.921 μg kg^{-1}, 3.185 μg kg^{-1}, and 70.3%, respectively, in

Table 3. Species of filamentous fungi isolated from the Polish berry fruits

Species	Source of isolation*	Potential pathogenicity	Most important mycotoxin(s) produced
Acremonium murorum	R(1), S(1)	not reported	
Acremonium strictum	R(4), S(6)	cutaneous mycoses [54]	
Alternaria arborescens	R(2)	mycotoxicoses [41], opportunistic cutaneous mycoses [37]	alternariol, alternariol monomethyl ether, tentoxin, tenuazonic acid, altenuene, altertoxins I, II, III, macrosporin, others
Alternaria infectoria	R(1)	mycotoxicoses [41], opportunistic cutaneous mycoses [53]	as above
Alternaria tenuissima	R(25), S(16)	mycotoxicoses [41], opportunistic cutaneous mycoses [52]	as above
Aspergillus carbonarius	R(1)	mycotoxicoses [11, 12]	ochratoxin A
Aspergillus fumigatus	R(1)	mycotoxicoses [10], mycoses (pulmonary aspergillosis) [55], allergic disease (asthma, HP**) [34]	gliotoxin, fumagilin, verruculogen, viriditoxin
Aspergillus parasiticus	R(1)	mycotoxicoses [10, 11]	aflatoxins
Aspergillus penicillioides	R(2), S(1)	allergic disease (allergic rhinitis, stimulation of the growth of allergenic dust mites), opportunistic mycoses (keratitis) [56, 57]	
Botrytis cinerea	R(14)	allergic disease (HP) [58], pulmonary infection [59]	
Cladosporium cladosporioides	R(16), S(11)	allergic diseases (allergic rhinitis, asthma) [38] opportunistic mycoses [60]	
Cladosporium sphaerospermum	R(30), S(37)	allergic diseases (allergic rhinitis, asthma) [38], opportunistic mycoses [61]	
Curvularia geniculata	S(2)	opportunistic mycoses [51]	
Engyodontium album	R(1)	opportunistic mycoses [62]	
Fusarium culmorum	R(1), S(1)	mycotoxicoses [11, 12]	culmorin, enniatins, fusarins, moniliformin, trichothecenes (DON), zearalenone, others
Fusarium graminearum	R(1)	mycotoxicoses [11, 12]	fusarins, trichothecenes (DON), zearalenone, others
Fusarium oxysporum	R(2), S(6)	mycotoxicoses [55], mycoses (keratitis, onychomycosis, opportunistic invasive fusariosis) [33]	numerous trichothecenes (NT-1, NT-2, others), beauvericin, moniliformin, zearalenon, others
Fusarium poae	R(9), S(7)	mycotoxicoses [63] mycoses (keratitis) [33]	numerous trichothecenes, beauvericin, enniatins, fusarin, others
Fusarium solani	S(1)	mycotoxicoses [12, 55] opportunistic mycoses (keratitis, onychomycosis, mycetoma) [55]	numerous trichothecenes (T-2, DON, others), enniatins
Fusarium verticillioides	R(1), S(1)	mycotoxicoses [9, 12, 55], mycoses (keratitis, onychomycosis, opportunistic invasive fusariosis) [33]	fumonisins B1, B2, B3, beauvericin, fusaric acid, fusarins
Mucor circinelloides	R(1), S(3)	opportunistic infections (mucormycoses) [40]	
Mucor plumbeus	S(9)	not reported	
Mucor racemosus	R(2), S(14)	opportunistic infections (mucormycoses) [40], allergic diseases (asthma, rhinitis) [30]	
Penicillium allii	R(1)	mycotoxicoses [12]	roquefortine C
Penicillium camemberti (P. album)	S(2)	mycotoxicoses [12], allergic diseases (HP, asthma) [34, 64, 65]	cyclopiazonic acid
Penicillium chrysogenum	S(3)	mycotoxicoses [12], allergic disease (HP) [65], opportunistic mycoses [55]	roquefortine C
Penicillium expansum	S(18)	mycotoxicoses [11, 12], allergic disease (HP) [65], opportunistic mycoses [55]	patulin, citrinin, cyclopiazonic acid, peritrem A, chaetoglobosin A
Penicillium tardum	S(1)	mycotoxicoses [66]	rugulosin
Penicillium verrucosum	S(1)	mycotoxicoses [12], allergic diseases (HP) [34]	ochratoxin A, citrinin
Rhizopus oryzae	R(1)	opportunistic mycoses: cutaneous mucormycosis [67]	
Scopulariopsis brevicaulis	S(3)	mycoses (onychomycosis) [36]	
Talaromyces purpurogenus (Penicillium crateriforme)	S(4)	mycotoxicoses [68], opportunistic mycoses: otomycosis [69]	rubratoxins, luteoskyryn, spiculisporic acid, rugulovasins
Trichoderma harzianum	S(1)	mycotoxicoses [70], opportunistic mycoses [71]	harzianum A (trichothecene)
Trichoderma longibrachiatum	R(1)	mycotoxicoses [72] opportunistic mycoses [39]	trilongins (peptaibol class)
Trichothecium (Cephalothecium) roseum	S(2)	mycotoxicoses, allergic diseases (asthma, rhinitis) [73]	trichothecenes
Ulocladium cucurbitae	R(5), S(1)	opportunistic mycoses [74]	

* R = raspberry; S = strawberry. After each letter, the bracketed figure shows the number of samples (from the total of 81 for raspberries and 77 for strawberries), in which the presence of the taxon was detected; ** HP – hypersensitivity pneumonitis.

Table 4. Concentration of mycotoxins in the Polish berry fruits

Fruit Mycotoxin	Aflatoxin B1 (μg kg^{-1}) Median (range)	Prevalence	Total aflatoxin (μg kg^{-1}) Median (range)	Prevalence	Deoxynivalenol (DON) (μg kg^{-1}) Median (range)	Prevalence
Strawberry N=37	<LOD	0.0%	1.921 (<LOD – 3.185)	70.3%	<LOD	0.0%
Raspberry N=41	<LOD	0.0%	2.117 (<LOD – 3.411)	73.2%	242.0 (<LOD – 1060.0)	58.5%
Total samples N=78	<LOD	0.0%	1.981 (<LOD – 3.411)	71.8%	<LOD (<LOD – 1060.0)	30.8%

*LOD = limit of detection

strawberries, and to 2.117 μg kg^{-1}, 3.411 μg kg^{-1}, and 73.2%, respectively, in raspberries (Tab. 4).

Deoxynivalenol (DON) was not detected in strawberries but found to occur at high levels in raspberries with median and maximal values, and a prevalence equal to 242.0 μg kg^{-1}, 1,060.0 μg kg^{-1}, and 58.5%, respectively (Tab. 4). The difference between the concentration of DON in raspberries and strawberries proved to be highly significant (Mann-Whitney, $P<0.00001$).

Correlation between concentrations of filamentous fungi and mycotoxins in berry fruits. The results of the Spearman test for correlation (expressed by 'R' – the Spearman correlation coefficient) between the concentrations of total filamentous fungi and mycotoxins are presented in Table 5. No significant correlation could be found between the concentrations of filamentous fungi and determined mycotoxins (total aflatoxins, DON) in strawberries. A significant correlation was also not found between the concentrations of total aflatoxins and filamentous fungi in raspberries. In contrast, a significant correlation was demonstrated between the concentrations of DON and filamentous fungi in raspberries ($R=0.405$, $P<0.01$). Interestingly, a significant correlation was also found between the concentrations of *Cladosporium* fungi (which distinctly prevailed in the mycobiota of raspberries) and DON ($R=0.382$, $P<0.05$). A highly significant correlation was also found a between the concentrations of filamentous fungi and DON in total berry fruits (raspberries + strawberries) ($R=0.470$, $P<0.00001$).

Table 5. Correlation between concentrations of total filamentous fungi and mycotoxins in the Polish berry fruits

Kind of fruits Tested correlation	Filamentous fungi vs. total aflatoxin (AFT)	Filamentous fungi vs. deoxynivalenol (DON)
Strawberry N=37	R=-0.026 P=0.879 correlation not significant	R=0.000 P=1.000 correlation not significant
Raspberry N=41	R=0.053 P=0.740 correlation not significant	R=0.405 P=0.009 correlation significant
Total fruits N=78	R=0.094 P=0.411 correlation not significant	R=0.470 P=0.00001 correlation highly significant

R = Spearman correlation coefficient

DISCUSSION

Levels of filamentous fungi in berry fruits. The median concentrations of filamentous fungi found in berry fruits amounted to 3.079 $\log_{10}$ CFU g^{-1} in strawberries and 3.491 $\log_{10}$ CFU g^{-1} in raspberries. The concentration found in strawberries was slightly higher, compared to those reported by Buyukunal et al. [14] from strawberries collected in Turkey (2.0–2.65 $\log_{10}$ CFU g^{-1}), and slightly lower compared to mean value (3.938 $\log_{10}$ CFU g^{-1}) found by Hussein et al. [16] in Egypt.

As no quantitative values describing concentration of filamentous fungi in raspberries could be found in the available literature, the median value determined by us for these fruits (3.491 $\log_{10}$ CFU g^{-1}) was regarded as the starting point from which the future results obtained for not diseased raspberries could be compared.

Composition of mycobiota of berry fruits. The prevalence of 'field fungi' in strawberry fruits, mostly those belonging to *Cladosporium*, and to a lesser extent, to *Alternaria* genera, in the current study is in line with the results of field studies by Rigotti et al. [17] and Abdelfattah et al. [19] in Italy, and by Mouden et al. [18] in Morocco, but not with the studies by Tournas and Katsoudis [8] in the USA, who reported a low occurrence of these fungi in strawberry fruits. The occurrence of *Fusarium* spp. and *Penicillium* spp. in the strawberry samples tested in the present study is in accordance with the results of Rigotti et al. [17] and Tournas and Katsoudis [8], who found the presence of these fungi in strawberries. In contrast, the current results differ from those obtained by Hussein et al. [16] in Egypt, who reported the prevalence of the potentially pathogenic *Aspergillus* species in their strawberry samples. The present study differs from the previous studies primarily because of the lack of plant pathogenic species *B. cinerea*, which was reported by Rigotti et al. [17], Tournas and Katsoudis [8], Abdelfattah et al. [19] and Mouden et al. [18] as prevalent or very common species in their strawberry samples.

So far, there are no field studies on the occurrence of filamentous fungi in raspberries. The only data on mycobiota of raspberries are presented in the study by Tournas and Katsoudis [8], who reported the dominance of *B. cinerea*, which was detected in 75% of raspberry samples purchased from supermarkets and incubated at room temperature. By contrast, in the present study, *B. cinerea* was rare – isolated from 17.3% of raspberry samples and formed only 1% of the mycobiota of raspberries, which was distinctly dominated by the genus *Cladosporium* constituting 84.3% of the total count.

Levels of mycotoxins in berry fruits. As no maximal allowable concentrations of mycotoxins in fresh, ready-to-eat, fruits have been proposed to-date, we compared our results concerning aflatoxins concentrations with the proposals of the European Union (EU) for dried fruits intended for direct human consumption [31]. While aflatoxin B1 has not been detected in our samples of berry fruits, the prevalence of total aflatoxin (sum of aflatoxins B1, B2, G1, G2) was relatively high, exceeding 70% in both strawberries and raspberries. Nevertheless, in no case was the maximal safe concentration proposed by EU for dried fruits (equal to 4.0 μg kg^{-1}) exceeded.

In the classification by the European Food Safety Authority (EFSA) experts on the basis of border tests performed in EU countries, the presented risk of exposure to total aflatoxins (AFT) from the consumption of 18 categories of foods, category 'fruit and fruit products', occupies 4th place with mean concentrations ranging from 1.09–1.53 μg kg^{-1} [32], which are lower than the results obtained in this study. The hitherto published data on the AFT concentrations in berry fruits are very scarce. Our results for strawberries are slightly lower compared to value obtained by Hussein et al. [16] for these fruits in Egypt (3.5 μg kg^{-1}). No data concerning the AFT concentration in raspberries could be found, hence our results seem to be the first in this respect. Summarizing, aflatoxins are steadily present in the Polish berries with a high prevalence, but the data are not alarming because the concentrations do not exceed the proposed threshold limit values, and the most hazardous AFB1 is absent.

Regarding the prevalence and concentration of deoxynivalenol (DON) in the examined berries, a distinct difference was noted between strawberries and raspberries. While all the strawberry samples were negative for DON, the prevalence of this trichothecene mycotoxin in raspberries was high (58.5%) with the median concentration equal 242.0 μg kg^{-1}. The DON concentration in 7 out of 41 (17.0%) examined samples exceeded the threshold value (750 μg kg^{-1}) proposed by the experts of EU for cereals intended for direct human consumption [31]. No data could be found on the concentration of DON in berry fruits, except for the results of the border tests for the category of 'berries and small fruits' published by the European Food Safety Authority experts [25]. The results of these tests (mean, range) were equal to 25.0 (0–46.4) μg kg^{-1} for DON and to 45.2 (0–90.4) μg kg^{-1} and 10.0 (0–20.0 μg kg^{-1} for acetylated forms of DON (3-Ac-DON and 15-Ac-DON, respectively).

The results obtained in the current study are circa 10 times greater compared to the above cited data, and seem to indicate that contamination with DON could pose a health hazard for raspberry consumers. At present, the reason for such a high contamination of raspberries with this mycotoxin is unknown. It is noteworthy that the Spearman test showed a significant correlation between the levels of total filamentous fungi and DON in raspberries ($P<0.01$), as well as between the levels of *Cladosporium* (a fungus distinctly dominant in mycobiota of raspberries) and DON ($P<0.05$). Fungal species known as DON producers (*Fusarium culmorum*, *Fusarium graminearum*) were detected in the examined raspberries, but in small quantities (<10^1 CFU g^{-1}).

In this situation, we propose 2 possible explanations for the correlations found:

1) in the tested raspberries there were some time before sampling favourable conditions for development of all (or almost all) filamentous fungi (including *Fusarium*, *Cladosporium* and other genera), which stimulated the growth of all species. As a result of some unknown environmental changes, *Fusarium* species lost viability and/or ability to grow on artificial media, while *Cladosporium* and other species persisted, and hence a positive correlation between the levels of filamentous fungi and DON could be found.
2) trichothecene mycotoxin DON (or hypothetically another substance giving false positive reactions in the test for DON) could be produced by *Cladosporium*. These presumptions, however, need experimental verification.

Incidence of potentially pathogenic species in fruit mycobiota and probable mechanisms of the pathogenicity. Among 36 fungal species isolated from berry fruits, 34 (94.4%) have been described previously as pathogenic or facultatively pathogenic for humans, of which 22, 25, and 14 showed, respectively, mycotoxigenic, infectious and allergenic properties. The most important group of potential pathogens among the berry strains were mycotoxigenic fungi, which constituted 61.1% of the total identified species. It is noteworthy that the ability for mycotoxin production in most cases was connected with allergenic and/or infectious properties, which may potentiate the toxic effect. Altogether, mycotoxic properties were revealed in 22 species, of which only in 6 cases solely, in 7 cases jointly with infectious properties, in 3 jointly with allergenic properties, and in 6 jointly with infectious and allergenic properties [33, 34, 35]. Mycotoxigenic species belonged to the genera: *Fusarium* and *Penicillium* (6 species each), *Alternaria* and *Aspergillus* (3 species each), *Trichoderma* (2 species), *Talaromyces* and *Trichothecium* (1 species each). The pathogenic fungal species classified as 'non-mycotoxigenic' comprised 12 species, of which 7 revealed infectious properties (*Acremonium strictum*, *Curvularia geniculata*, *Engyodontium album*, *Mucor circinelloides*, *Rhizopus oryzae*, *Scopulariopsis brevicaulis* and *Ulocladium cucurbitae*), and 5 joint infectious and allergenic properties (*Aspergillus penicillioides*, *B. cinerea*, *Cladosporium cladosporioides*, *Cladosporium sphaerospermum*, *Mucor racemosus*) [36, 37, 38, 39, 40]. No information was found on the pathogenicity of 2 species isolated from berry fruits (*Acremonium murorum* and *Mucor plumbeus*).

Potential pathogenicity of berry isolates: mycotoxin production. The species isolated from the examined berry fruits are potential producers of more than 41 different mycotoxins that show a wide spectrum of pathogenic effects, targeting various organs in exposed humans and/or animals [9, 10, 11, 12, 41]. The majority revealed hepatotoxic and/or nephrotoxic properties, while some others, e.g. peritrem A, produced by *Penicillium expansum,* are neurotoxic, exhibiting tremorgenic properties [42]. A significant risk is associated with exposure to mycotoxins that possess carcinogenic properties, such as aflatoxins [10]. In the current study, in spite of the rather common prevalence of total aflatoxins in berries, only one strain of *Aspergillus parasiticus*, the known aflatoxin producer, was isolated from a raspberry sample.

The major mycotoxin producers isolated from the examined berries belonged to the genera *Fusarium* and *Penicillium* (6 species each). *Fusarium* species produce a wide range of mycotoxin, including trichothecenes such as deoxynivalenol (DON), detected in this study in large quantities in raspberry samples, fusarins, enniatins, beauvericin and zearalenone [9, 12]. Among the most important mycotoxins produced by *Penicillium* species are patulin, ochratoxin A, citrinin, and cyclopiazonic acid [11, 12], of which low levels of ochratoxin A were reported in strawberries by earlier researchers, while high levels of patulin were detected in both strawberries and raspberries [21].

Fungi belonging to the genus *Alternaria*, represented in the examined berry fruit samples by 3 species, produce more than 70 mycotoxins (alternariol, alternariol methyl ether, tentoxin and others), that show notable toxic properties, such as mutagenicity and carcinogenicity [41, 43]. *Alternaria* toxins have been detected by earlier researchers in raspberry and raspberry juice, but in rather low concentrations [21, 43]. The reason for that could be the ability of red raspberry to produce ellagitannins, bio-active polyphenols that inhibit the growth of *Alternaria alternata* [44]. Juan et al. [22] also detected low levels of *Alternaria* mycotoxins in strawberries.

The species belonging to *Cladosporium*, the dominant fungal genus found in berry fruits in the current study, are widely known because of their allergenic properties, but their mycotoxigenic role remains largely unexplored. According to Alwatban et al. [45], the *Cladosporium* species produce a number of mycotoxins, such as cladosporin, isocladosporin, emodin, epi- and fagi-cladosporic acid, and ergot alkaloids. Thus, it cannot be excluded that some of these compounds could be hazardous for humans. Also, the correlation demonstrated in this study between the levels of *Cladosporium* and DON deserves an experimental elucidation.

The effects of mycotoxins in berry fruits consumers may be aggravated by their strong immunosuppressive properties. Corrier [46] expressed an opinion that consumption of mycotoxins, at levels that do not cause overt clinical mycotoxicosis, suppress immune functions and may decrease resistance to infectious disease. Mycotoxin-induced immunosuppression may be manifested as depressed T or B lymphocyte activity, suppressed immunoglobulin and antibody production, reduced complement or interferon activity, and impaired macrophage-effector cell function. This opinion has been supported by the authors of the EFSA (European Food Safety Authority) report on aflatoxins in food [32] who underlined the immunotoxic effects of these toxins, which are correlated with an increased susceptibility to microbial infections.

Another risk factor highlighted by the results obtained in this study may be the ability of various mycotoxins to synergistic action in low doses [47], which may be enhanced by the diversity of fungal species observed in the study, and the probable diversity of the mycotoxins produced.

Potential pathogenicity of berry isolates: allergenic properties. Out of 14 fungal species revealing allergenic properties that have been isolated from examined berries, 8 are known as causative factors of IgE-dependent asthma and/or rhinoconjuctivitis (3 species of *Alternaria*, 2 species of *Cladosporium*, *Aspergillus penicillioides*, *Mucor racemosus* and *Trichothecium roseum*), 4 were reported as causative factors of hypersensitivity pneumonitis (*B. cinerea, Penicillium chrysogenum*, *Penicillium expansum*, *Penicillium verrucosum*), and 2 as causative factors of both hypersensitivity pneumonitis and asthma (*Aspergillus fumigatus* and *Penicillium camemberti*) (Tab. 3).

Among the allergenic moulds detected in berry fruits, the greatest risk is posed by the *Cladosporium* species, which distinctly dominated in the berry mycobiota, mostly in raspberries. In the second place, *Alternaria* species should be considered because of their common occurrence in the examined samples. The species belonging to both genera produce a wide spectrum of protein allergens causing IgE-dependent asthma and/or rhinoconjuctivitis. They include proteases, ribosomal proteins, enolases, dehydrogenases, heat shock protein, isomerase, manganese superoxide dismutase (MnSOD), flavodoxins and glycoprotein without a known function [29].

An additional risk is associated with similar mechanisms of the respiratory and food allergy mediated by IgE antibodies. According to Popescu [48], up to 80% of all cases of food allergy in adult patients are preceded by IgE-mediated sensitisation (clinical or subclinical) to aeroallergens. In these patients, food allergic symptoms are caused by cross-reactions between ingested food and inhaled allergens sharing the same antigenic determinants. This view is in line with earlier experiments by Luccioli et al. [49] who demonstrated that oral challenge with mould extract elicited allergic symptoms in individuals sensitive to aeroallergenic moulds, and expressed a view that consumption of foods contaminated with fungi may trigger respiratory symptoms in people with allergy to airborne moulds.

As *Cladosporium* and *Alternaria* species detected abundantly in the present study in berry fruits are known factors causing respiratory allergy, it is probable that ingestion of conidia or mycelium fragments of these fungi by berry consumers with pre-existing respiratory sensitization to these allergens, may elicit symptoms of food allergy which may be triggered even by low doses of adverse allergen [48].

Schütze et al. [50] demonstrated in a murine asthma model that exposure to mycotoxins (gliotoxin and patulin) increases the Th2-driven, Ig-E dependent immune response causing asthma and rhinoconjunctivitis. Based on the results of this study, it is presumed that aflatoxins present in berries may enhance such allergic reactions caused by the strains of *Cladosporium* and *Alternaria* dominant in this environment. However, this hypothesis needs an experimental and/or epidemiological confirmation.

Potential pathogenicity of berry isolates: infectious properties. Out of 34 potentially pathogenic species of fungi isolated from the examined berry fruits, as many as 25 species have been reported as causing infections (mycoses) in humans (Tab. 3). In most species, the ability to cause infections was associated with other pathogenic property: causing allergic reaction(s) (in 5 species), ability to produce mycotoxin(s) (in 7 species), or both together (in 6 species). Most of the analysed species (at least 18 out of 25) caused only so-called opportunistic infections, which affect only immunocompromised individuals with lowered immunity, mostly by concomitant disease(s) or by immunosuppressive drugs. Opportunistic fungal infections are mostly superficial (appearing as dermatitis, onychomycosis or keratitis), but may affect also internal organs.

Among fungal species that may cause mycoses in healthy, immunocompetent individuals, the most hazardous among berry isolates appears to be the species *Aspergillus fumigatus,* causing infectious and/or allergic pulmonary diseases [35], isolated in the current study from raspberry. Among other fungal pathogens isolated from berries, noteworthy are *Fusarium* species causing keratitits or onychomycosis in immunocompetent hosts, or invasive fusariosis in those who are immunocompromised [33], as well as *Trichoderma longibrachiatum*, indicated by Hatvani et al. [39] as an emerging human pathogen penetrating into the human environment from agricultural habitats.

The high prevalence is noteworthy in the examined berries of so-called 'melanized' fungi (species containing melanin, also called 'phaeoid' 'dematiaceous' or 'dark' fungi), mainly from the genera *Alternaria*, *Cladosporium* and *Curvularia*), which cause infections described as phaeohyphomycoses, aggravated by melanin, a known virulence factor [51, 52, 53].

The co-existence of mycotoxigenic and potentially infectious fungal species in the examined berries might be associated with adverse effects on consumers, as the co-action of even small doses of mycotoxins may contribute to the initiation and/or exacerbation of the mycotic infection as a result of an abov-mentioned immunosuppressive action [32, 46].

CONCLUSIONS

Filamentous fungi occurred in raspberries and strawberries cultivated in eastern Poland at levels that may be classified as low or moderate. The concentrations were significantly greater in raspberries than in strawberries.

Fungi of the genus *Cladosporium* prevailed in the mycobiota of berry fruits. They were distinctly dominant in raspberries and formed also the most numerous fraction in strawberries. Despite this prevalence, mycobiota of examined berries revealed a high biodiversity and a distinct prevalence of potentially pathogenic species, of which 22, 25, and 14 showed mycotoxigenic, infectious, and allergenic properties, respectively.

A potential risk of foodborne exposure to mycotoxins was confirmed by the common occurrence of total aflatoxins (AFT), which were detected in the majority of tested samples (71.8%), but never exceeded the level of 4.0 $\mu g\ kg^{-1}$ which was assumed as safe. Deoxynivalenol (DON) was detected only in raspberries with the prevalence of 58.5%. Its median concentration was 242.0 $\mu g\ kg^{-1}$ and in 7 out of 41 samples (17.0%) exceeded the level of 750.0 $\mu g\ kg^{-1}$ assumed as safe. A significant correlation was found between the levels of total filamentous fungi and DON.

Taken together, both filamentous fungi and mycotoxins occurred at levels that, in most cases, may be classified as low or moderate, and most probably do not represent a risk of pathogenic effects in healthy, immunocompetent consumers. Nevertheless, considering the ability of mycotoxins to immunosuppression and synergistic action in low doses, a health risk for immunocompromised individuals cannot be excluded, in particular to those subjected to immunosuppression treatment in health care units, or to those with an immune system affected by various diseases, such as recently COVID-19. Another endangered category are people with atopy, exhibiting respiratory hypersensitivity to airborne moulds (mostly to *Cladosporium*, which prevailed in the examined fruits), in which ingested fungi may elicit a pathogenic cross-reaction.

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