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Levels of filamentous fungi and selected mycotoxins in leafy and fruit vegetables and analysis of their 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. Considering the significant role of mycotoxins in the pathogenicity of fungal contaminants of vegetables, the scope of the study was extended by determination of aflatoxins and deoxynivalenol.
Materials and method. In the years 2019–2020, samples of vegetables (lettuce, spinach, tomato, red pepper) collected on conventional farms located in eastern Poland 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 with the use of macroscopic and microscopic methods. Samples were also analyzed for the presence of aflatoxin B1 (AFB1), total aflatoxin (AFT) and deoxynivalenol (DON) using the immunoenzymatic ELISA method.
Results. The median concentrations of filamentous fungi ranged from 2.778–3.204 $\log_{10}$ CFU g^{-1}. Overall, 40 fungal species were identified in the examined vegetables, of which 38 are classified as potentially pathogenic for humans. The mean prevalence values for AFB1 and AFT were moderate or high (16.0–60.0% and 57.8–75.6%, respectively) and very low for DON (0–2.2%). The median concentrations of filamentous fungi, AFB1 and AFT were distinctly greater in leafy vegetables than on non-leafy tomato and pepper fruits, and the differences were highly significant (P<0.001).
Conclusions. The levels of filamentous fungi and mycotoxins in Polish vegetables could be classified as moderate or low. The abundant presence of species with various pathogenic abilities may pose a risk for some categories of people consuming raw vegetables, mostly for immuno-compromised persons or atopics susceptible to food allergy caused by ingested moulds.

Key words
filamentous fungi, vegetables, aflatoxins, deoxynivalenol, food safety, health hazard

INTRODUCTION

The great increase in consumption of fruit and vegetables that has been observed globally in the past 30 years is largely due to the fact that they provide health-promoting diet supplements, such as vitamins, minerals, antioxidants and fibres [1]. This growth, approximating on the global scale 38.6% between 1990–2015, aroused the interest in the microbiological safety of these products [1, 2]. So far, outbreaks of acute gastrointestinal disease after the consumption of vegetables have been reported from many countries worldwide. Bacteria, viruses and parasites of different origin, but never fungi, have been identified as causative agents of these diseases [3]. This fact contributes to the inaccurate view that fungi occurring in vegetables do not pose any significant hazard for consumers. Although filamentous fungi rather do not cause any acute disease after the consumption of vegetables, they do represent a potential cause of chronic diseases. Most of them may be caused by mycotoxins, less often by allergenic reactions, or by infections caused by opportunistic fungal pathogens [4, 5, 6, 7, 8, 9]. Mycotoxins may exhibit nephrotoxic, genotoxic, teratogenic, carcinogenic, and cytotoxic properties and, as a consequence, may cause liver carcinomas, renal dysfunctions, and immunosuppressed states [9]. 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 belonging to the *Aspergillus*, *Penicillium* and *Fusarium* genera, growing on various crops and plant materials [8, 9, 10, 11, 12, 13].

Until recently, there were only a few studies on fungi occurring on vegetables as potential health hazards, in particular with regard to the species composition. The authors most often determined only a total concentrations of fungi defined as 'moulds and yeasts', in most cases ranging within the limits 3.0–6.0 $\log_{10}$ CFU g^{-1}, much lower compared to bacteria [14, 15, 16, 17, 18, 19, 20, 21]. Also until recently, fungi occurring in vegetables were identified down to the generic or specific level only in a small number of studies performed in the USA [6], Venezuela [22], Italy [7], Iran [23], Korea [24] and Poland [25]. The most common were isolates belonging to the *Penicillium*, *Aspergillus*, *Fusarium*,

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Cladosporium, *Alternaria*, and *Geotrichum* genera, of which many were reported as potential producers of hazardous mycotoxins and/or allergens. The greatest mean concentrations of moulds (up to 6.2 $\log_{10}$ CFU g^{-1}) and yeasts (up to 7.4 $\log_{10}$ CFU g^{-1}) were reported by Marinelli et al. [7] from Italy, while concentrations of moulds found in other countries ranged between 2.0–4.5 $\log_{10}$ CFU g^{-1} [6, 22, 23, 24].

So far, there is only scant information on the prevalence and concentration of mycotoxins in vegetables compared to other foods [13]. The hitherto performed studies focused mainly on aflatoxins, the toxins produced by *Aspergillus flavus*, which are classified by the International Agency for Research on Cancer (IARC) as carcinogenic to humans, i.e., as GROUP 1 carcinogens. Of these, the greatest hazard in this respect is posed by aflatoxin B1 (AFB1) [9]. Until recently, the highest levels of AFB1 were detected in the samples of red pepper from Africa and America [26, 27], while in the studies performed on the samples of this vegetable from Asia and Europe either lack of AFB1 [24, 28] or much lower levels [29] were found. By contrast, high levels of total aflatoxins were reported by Hariprasad et al. [30] from India.

In the previous study on mycobiota of root vegetables (carrot and beetroot) in Poland published in 2018, the concentrations of filamentous fungi (moulds) detected ranged from 3.81–5.09 $\log_{10}$ CFU g^{-1} [25]. In the generic composition of the isolated mycobiota (to the best of our knowledge analysed for the first time with regard to vegetables), the *Penicillium* strains were found to be the most numerous (25.3–52.9% of the total count), followed by *Fusarium* strains (5.9–22.6% of the total). Of the 61 species of filamentous fungi determined in the examined root vegetables and adjacent soil, 28 (45.9%), mostly belonging to the prevalent *Penicillium* and *Fusarium* genera, have been reported by earlier authors as pathogenic for humans and/or animals. The potential pathogenicity of the isolates was due mostly to mycotoxin production, less to the ability to cause opportunistic infections and to allergenic properties [25].

OBJECTIVE

The aim of the study was to determine the presence, concentration and generic composition of filamentous fungi, as well as the participation of potentially pathogenic species in four species of leafy and fruit vegetables (lettuce, spinach, tomato and red pepper). The authors believe that the obtained results will be useful in the assessment of the potential health risk for consumers. 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 [8, 9], and were hitherto tested in vegetables as the most common of all mycotoxins and in some cases reaching extremely high values [27, 30]. Deoxynivalenol (DON) was selected as the second type of mycotoxins to be determined. It was identified by Carballo et al. [31] as the most prevalent mycotoxin in food prepared with cereals, vegetables, and legumes. Other reasons for choosing this mycotoxin were: the detection of *Fusarium culmorum*, one of the two most important producers of DON in the earlier study on mycobiota of root vegetables [25], as well as the fact that generally the occurrence of this mycotoxin in vegetables is largely unexplored.

MATERIALS AND METHOD

Collecting and preparation of samples. In the years 2019–2020, samples of four species of fresh vegetable – lettuce (*Lactuca sativa*), spinach (*Spinacia oleracea*), tomato (*Solanum lycopersicum*) and red pepper (*Capsicum annuum*) were subjected to mycological examination. Vegetables were collected on conventional farms located in the Lublin Province of eastern Poland. Samples consisted of 10 vegetable specimens, each weighing approximately 0.5 kg. They were collected at random from various sites of the field. A total number of 180 vegetable samples were collected (100 in the year 2019 and 80 in 2020), 45 (25+20) of each species.

Vegetable samples were separately packed into clean foil bags, and then transported to the laboratory where a mean sample was prepared by mixing the collected specimens. Subsequently, the mean samples were cut into smaller pieces using a sterile scalpel. 20 g samples of fragmented vegetables were homogenized with Universal Laboratory AID type 309 homogenizer for 5 min in 180 ml of a diluent (Ringer's solution), with the addition of 10% Tween 80. From the homogenates prepared in this way, decimal dilutions were performed which were used for cultures.

Determining of concentration and species composition of filamentous fungi in vegetable samples. In order to determine the concentration and species composition of filamentous fungi in the vegetable samples, the method of plate dilutions on malt agar (Difco, USA) with chloramphenicol was used. The study was conducted in two 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 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 [32, 33, 34]. A total number of 546 fungal colonies belonging to 58 fungal taxons (40 species and 18 genera) were isolated and identified. All isolates were compared to standard strains from the Collection of Fungal Strains at the Institute of Rural Health in Lublin, Poland, and had been determined with the 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). Vegetable samples were analyzed for the presence of: aflatoxin B1, total aflatoxin (the sum of aflatoxins B1, B2, G1, G2) 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 BagMixer homogenizer. The obtained mixtures were filtered into separate sterile tubes through Whatman No.1 filters, and examined by the immunoenzymatic 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 a prepared standard curve.The standard in six concentrations (0, 0.05, 0.15, 0.45, 1.35 and 4.05 µg kg^{-1}) was used for preparing the total aflatoxin standard curve, while for preparing the aflatoxin B1 curve the standard was used in the concentrations of 0, 1, 5, 10, 20 and 50 µg kg^{-1}. For deoxynivalenol, standards in five concentrations were used (0, 222, 666, 2,000 and 6,000 µg kg^{-1}).

Statistical Analysis. The results were analyzed by Mann-Whitney non-parametric test and Spearman non-parametric test for correlation, using STATISTICA v. 5.1 package (Statsoft, Tulsa, OK, USA).

RESULTS

Concentrations of filamentous fungi in leafy and fruit vegetables. The concentrations (median, range) of filamentous fungi (in logarithmic scale) are shown in Table 1 and the mean concentrations of fungi in linear scale are presented in Table 2. The average concentration of filamentous fungi in leafy vegetables (lettuce and spinach) were equal to 36.1 × 10^2 CFU g^{-1}, which was 3.9 times greater than in fruit vegetables (tomato and red pepper) equal to 9.2 × 10^2 CFU g^{-1}, and the difference was highly significant (Mann-Whitney, P<0.001).

In singular comparisons, the concentrations of fungi in lettuce and spinach were significantly greater compared to tomato (P<0.001) and to red pepper (P<0.001). No significant differences were observed between the concentrations of fungi in lettuce versus spinach (P>0.05), nor between those in red pepper versus tomato (P>0.05).

Table 1. Concentration of filamentous fungi in the Polish leafy and fruit vegetables

Vegetable	Filamentous fungi (log_{10} CFU g^{-1}) Median (range)	Prevalence[a]
Lettuce N=45	3.204 (0.000 – 4.462)	97.8%
Spinach N=45	3.176 (2.301 – 4.400)	100%
Tomato N=45	2.778 (0.000 – 3.778)	95.6%
Red pepper N=45	2.845 (2.000 – 3.431)	100%
Total samples N=180	3.041 (0.000 – 4.462)	98.3%

N = number of examined samples; [a]Prevalence shows percent of positive samples from which fungi were recovered.

Composition of the mycobiota of filamentous fungi indigenous to vegetables and potential pathogenicity of individual species. As seen in Table 2 and Figure 1, presenting the generic composition of the vegetable mycobiota, the so called 'field fungi' (allergenic fungi that invade the crop in the field before harvest, in contrast to allergenic 'storage fungi' that develop on stored products) of the genera *Alternaria* and *Cladosporium*, usually observed on live plants, dominated in the mycobiota of examined vegetables, forming together 45.4 -66.5% (on average 50.9%) of the total fungal population. Among other genera usually developing on grain and other stored products, the most common were species belonging to the genera *Fusarium* and *Penicillium*, forming together 5.6–19.2% (on average 12.0%) of the total count (Tab. 2, Fig. 1). The frequency of other genera was distinctly smaller with the exception only of *Phoma* on tomato fruits, which constituted 26.0% of the total isolated fungi.

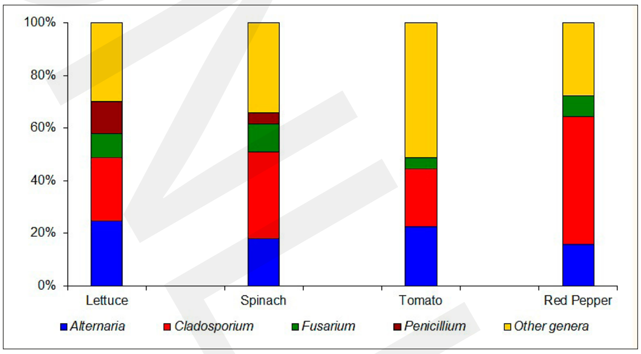

Figure 1. Generic composition of filamentous fungi isolated from four kinds of leafy and fruit vegetables

The vegetable mycobiota showed a marked diversity. Of the 546 fungal strains isolated from 180 vegetable samples and subjected to identification procedure, 389 could be identified down to the specific level as belonging to 40 species (Tab. 3), and 157 were identified down to the generic level as belonging to 18 genera (*Acremonium* spp., *Alternaria* spp., *Aureobasidium* spp., *Chaetomium* spp., *Cladosporium* spp., *Fusarium* spp., *Monascus* spp., *Monilia* spp., *Mucor* spp., *Paecilomyces* spp., *Penicillium* spp., *Phoma* spp., *Stemphylium* spp., *Talaromyces* spp., *Trichoderma* spp., *Trichophyton* spp., *Ulocladium* spp, *Verticillium* spp.). Altogether, the presence of at least 58 fungal taxons (altogether 40 species and 18 genera) were found in the examined vegetable samples.

All fungal species isolated from vegetables are listed in Table 3, giving the species name and source of isolation, as well as potential pathogenicity and mycotoxins produced, with appropriate references. The commonest species were: *Cladosporium cladosporioides* (present in 87 out of the total of 180 samples), *Alternaria tenuissima* (present in 73 samples) and *Alternaria arborescens* (present in 32 samples).

Concentrations of mycotoxins in leafy and fruit vegetables. The concentrations of aflatoxin B1, total aflatoxin and deoxynivalenol are shown in Table 4. The average concentration of aflatoxin B1 (AFB1) in leafy vegetables (0.258 µg kg^{-1}) was 5.6 times greater than in non-leafy vegetables (0.046 µg kg^{-1}), and the difference was highly significant (Mann-Whitney; P<0.001). In singular comparisons, the concentrations of AFB1 in lettuce and spinach were significantly greater compared to tomato (P<0.001) and to red pepper (P<0.001). The concentration of AFB1 in spinach was significantly greater than in lettuce (P<0.05), while no significant difference was observed between the concentrations of AFB1 in red pepper versus tomato (P>0.05). The prevalence of AFB1 (percent of positive samples) in lettuce, spinach, tomato and red pepper was 60.0%, 57.8%, 20.0% and 16.0%, respectively.

The average concentration of total aflatoxin (AFT) in leafy vegetables (1.47 µg kg^{-1}) was 2.1 times greater than in non-leafy vegetables (0.71 µg kg^{-1}), and the difference was

Table 2. Generic composition of filamentous fungi isolated from the Polish leafy and fruit vegetables

Vegetable	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.	*Aspergillus* spp.	*Cladosporium* spp.	*Fusarium* spp.	*Mucor* spp.	*Penicillium* spp.	*Phoma* spp.	*Trichoderma* spp.	Other genera	
Lettuce N=45	0 (0%)	11.5 (27.4%)	0.7 (1.7%)	7.7 (18.4%)	4.1 (9.9%)	1.0 (2.7%)	3.9 (9.3%)	0 (0%)	0.1 (0.2%)	12.8 (30.4%)	$41.8^{a,b} \pm 66.5$ (100%)
Spinach N=45	0.6 (2.1%)	6.4 (21.1%)	0.3 (1.0%)	7.5 (24.7%)	3.3 (10.9%)	1.5 (4.9%)	1.1 (3.6%)	0.2 (0.8%)	0.5 (1.8%)	8.9 (29.1%)	$30.3^{a,b} \pm 45.7$ (100%)
Tomato N=45	0.3 (3.6%)	2.0 (20.9%)	0.1 (0.5%)	2.4 (24.5%)	0.6 (5.6%)	0.2 (1.5%)	0 (0%)	2.5 (26.0%)	0 (0%)	1.7 (17.4%)	9.8 ±14.8 (100%)
Red pepper N=45	0.5 (5.5%)	1.1 (12.8%)	0.1 (0.6%)	4.4 (53.7%)	0.6 (7.3%)	0.1 (0.6%)	0.2 (1.2%)	0 (0%)	0.1 (0.6%)	1.5 (17.7%)	8.6 ±6.7 (100%)
Total N=180	0.3 (1.5%)	5.2 (23.0%)	0.3 (1.3%)	5.5 (24.3%)	2.1 (9.3%)	0.7 (3.1%)	1.3 (5.7%)	0.7 (3.1%)	0.2 (0.8%)	6.3 (27.9)	22.6 ±43.7 (100%)

N = number of examined samples; [a] mean value significantly greater compared to tomato (P<0.001); [b] mean value significantly greater compared to red pepper (P<0.001).

Table 3. Species of filamentous fungi isolated from the Polish leafy and fruit vegetables and their potential pathogenicity for humans

Species	Source of isolation*	Potential pathogenicity	Most important mycotoxin(s) produced
Acremonium murorum	S(5)	not reported	
Acremonium strictum	S(1), T (1)	cutaneous mycoses [35]	
Alternaria alternata	L (3)	mycotoxicoses [36, 37], allergic diseases [38], opportunistic cutaneous mycoses [39]	alternariol, alternariol monomethyl ether, tentoxin, tenuazonic acid, altenuene, altertoxins I, II, III, macrosporin, others
Alternaria arborescens	L(9), S (11), T(5), R (7)	mycotoxicoses [36, 37], allergic diseases [38], opportunistic cutaneous mycoses[40]	as above
Alternaria infectoria	L (2), S (1), T(4), S (1)	mycotoxicoses [36, 37], allergic diseases [38], opportunistic cutaneous mycoses [41]	as above
Alternaria tenuissima	L (22), S (19), T (19), R (13)	mycotoxicoses [36, 37], allergic diseases [38], opportunistic cutaneous mycoses [39]	as above
Aspergillus clavatus	S (2), R (1)	mycotoxicoses [9, 12], allergic diseases (HP**) [42, 43]	cytochalasin E, patulin
Aspergillus flavus	L(1), S(1)	mycotoxicoses [8, 9, 13], mycoses (rhinosinusitis, keratitis, cutaneous infections) [44], allergic diseases (asthma, HP) [42, 44]	aflatoxins
Aspergillus fumigatus	S(3), T(1)	mycotoxicoses [9, 12], mycoses (pulmonary aspergillosis) [45], allergic disease (asthma, HP) [42, 43]	gliotoxin, fumagilin, verruculogen, viriditoxin
Aspergillus niger	L(4), S(2)	mycotoxicoses [13, 46, 9, 12], opportunistic mycoses (mostly otomycosis) [47, 48, 49]	fumonisins B2, B4, B6, ochratoxin A, oxalic acid
Aspergillus westerdijkiae	R(1)	mycotoxicoses [50]	ochratoxin A, penicillic acid
Aureobasidium pullulans	L(1)	allergic disease (HP) [43]	
Cladosporium cladosporoides	L(13), S(16), T(22), R(36)	allergic diseases (allergic rhinitis, asthma) [51], opportunistic mycoses [52]	
Cladosporium macrocarpum	T(1)	allergic diseases (allergic rhinitis, asthma) [51]	
Cladosporium sphaerospermum	L(3), S(2), T(2), R(4)	allergic diseases (allergic rhinitis, asthma) [51, 53], opportunistic mycoses [54]	
Fusarium oxysporum	S(1), T(2), R(1)	mycotoxicoses [11, 12], mycoses (keratitis, onychomycosis, opportunistic invasive fusariosis) [55, 56]	numerous trichothecenes (NT-1, NT-2, others), beauvericin, moniliformin, zearalenon, others
Fusarium poae	L(8), S(5), T(1)	mycotoxicoses [57], mycoses (keratitis) [56]	numerous trichothecenes, beauvericin, enniatins, fusarin, others

Table 3. Species of filamentous fungi isolated from the Polish leafy and fruit vegetables and their potential pathogenicity for humans (continuation)

Species	Source of isolation*	Potential pathogenicity	Most important mycotoxin(s) produced
Fusarium proliferatum	R(1)	mycotoxicoses [8, 11, 58, 59, 12], mycoses (keratitis, onychomycosis, opportunistic invasive fusariosis) [55, 56]	fusaproliferin, fumonisin B1, B2, B3, moniliformin, beauvericin, others
Fusarium solani	L(4)	mycotoxicoses [60, 61]	numerous trichothecenes (T-2, DON, others)
Fusarium verticillioides	T(2)	mycotoxicoses [8, 58, 12], mycoses (keratitis, onychomycosis, opportunistic invasive fusariosis) [55, 56]	fumonisin B1, B2, B3
Geotrichum candidum	S(1)	opportunistic mycoses [62]	
Mucor circinelloides	L(3), S(3), T(1)	opportunistic infections (mucormycoses) [63, 64]	
Mucor plumbeus	L(2), S(3), R(1)	not reported	
Mucor racemosus	L(9), S(17), T(4), R(1)	opportunistic infections (mucormycoses) [63, 64], allergic diseases (asthma, rhinitis) [65]	
Paecilomyces niveus (Byssochlamys nivea)	S(1)	mycotoxicoses [66], allergic diseases (HP) [67]	patulin
Paecilomyces variotii	T(1)	mycotoxicoses [68], allergic diseases (HP) [43, 67], mycoses (mostly opportunistic) [69, 70]	viriditoxin
Penicillium citreonigrum	S(1)	mycotoxicoses [71]	citreoviridin
Penicillium citrinum	L(1)	mycotoxicoses [12] allergic disease (HP) [43]	citrinin
Penicillium discolor	R(1)	mycotoxicoses [72]	chaetoglobosins A, B, C, palitantin, cyclopenin, cyclopenol, cyclopeptin, dehydrocyclopeptin, viridicatin, viridicatol
Penicillium expansum	L(15), S(8), T(1)	mycotoxicoses [11, 12, 13]	patulin, citrinin, cyclopiazonic acid, peritrem A, chaetoglobosin A
Penicillium glabrum	L(4), S(2), T(1)	mycotoxicoses [11], allergic diseases (HP) [43]	ochratoxin A, patulin, penitrem A
Penicillium griseofulvum (urticae)	S(1)	mycotoxicoses [11, 12]	patulin, cyclopiazonic acid
Penicillium olsoni	R(1)	mycotoxicoses [73]	ochratoxin A
Penicillium tardum	R(1)	mycotoxicoses [74]	rugulosin
Scopulariopsis brevicaulis	L(1), S(2)	mycoses (onychomycosis) [75]	
Scopulariopsis fusca	T(1)	mycoses (onychomycosis) [75]	
Talaromyces funiculosus (Penicillium funiculosum)	L(1)	mycotoxicoses [59]	patulin
Trichoderma harzianum	S(2)	mycotoxicoses [76] opportunistic mycoses [77, 78]	harzianum A (trichothecene)
Trichoderma longibrachiatum	R(1)	mycotoxicoses [79] opportunistic mycoses [77, 80, 81]	trilongins (peptaibol class)
Trichoderma viride	L(1), S(1)	mycotoxicoses [82] opportunistic mycoses [77]	trichotoxin A, trichodermin (trichothecenes)

* L – lettuce; S – spinach; T – tomato; R – red pepper. After each letter, the bracketed figure shows the number of samples (out of the total of 45) in which the presence of the taxon was detected;
** HP – hypersensitivity pneumonitis.

highly significant (Mann-Whitney, $P<0.001$). In singular comparisons, the concentration of AFT in lettuce was significantly greater compared to tomato and red pepper ($P<0.001$), while no significant differences were found on comparison of spinach versus tomato and red pepper ($P>0.05$). Concentration of AFT in lettuce was significantly greater than in spinach ($P<0.05$), while no significant difference was observed between the concentrations of AFT in red pepper versus tomato ($P>0.05$). The prevalence of AFT in lettuce, spinach, tomato and red pepper was distinctly greater compared to AFB1 and amounted to 75.6%, 64.4%, 60.0% and 57.8%, respectively.

Deoxynivalenol (DON) has been detected in only one of the 45 tested samples of lettuce and spinach, respectively (prevalence in both cases was equal to 2.2%), and in none of the samples of tomatoes and red pepper.

Correlation between numbers of fungi and mycotoxins concentrations in leafy and fruit vegetables. The results of the Spearman test for correlation between the numbers of total filamentous fungi and the concentrations of aflatoxins are presented in Table 5. The relation between the numbers of fungi and the concentrations of DON was not tested because of the very low incidence of positive results.

Table 4. Concentration of mycotoxins in Polish leafy and fruit vegetables

Vegetable	Aflatoxin B1 (μg kg^{-1}) Median (range)	Prevalence	Total aflatoxin (μg kg^{-1}) Median (range)	Prevalence	Deoxynivalenol (DON) (μg kg^{-1}) Median (range)	Prevalence
Lettuce N=45	0.220 [a,b] (<LOD* – 0.442)	60.0%	1.925 [a,b,d] (<LOD – 7.848)	75.6%	<LOD (<LOD – 627.0)	2.2%
Spinach N=45	0.339 [a,b,c] (<LOD – 0.778)	57.8%	0.518 (<LOD – 4.220)	64.4%	<LOD (<LOD – 296.0)	2.2%
Tomato N=45	<LOD (<LOD – 0.245)	20.0%	0.404 (<LOD – 2.665)	60.0%	<LOD	0.0%
Red pepper N=45	<LOD <LOD – 0.329	16.0%	0.382 (<LOD – 3.295)	57.8%	<LOD	0.0%
Total samples N=180	<LOD (<LOD – 0.778)	38.5%	0.655 (<LOD – 7.848)	64.4%	<LOD (<LOD – 627.0)	1.1%

N - number of examined samples; *LOD - limit of detection; [a] mean value significantly greater compared to tomato (P<0.001); [b] mean value significantly greater compared to red pepper (P<0.001); [c] mean value significantly greater compared to lettuce (P<0.05); [d] mean value significantly greater compared to spinach (P<0.05).

Table 5. Correlation between numbers of total filamentous fungi and concentrations of aflatoxins in Polish leafy and fruit vegetables

Vegetables tested correlation	Filamentous fungi vs. aflatoxin B1 (AFB1)	Filamentous fungi vs. total aflatoxin (AFT)
Leafy vegetables (lettuce + spinach) N=90	R=0.129 P>0.05 correlation not significant	R=0.316 P<0.05 correlation significant
Fruit vegetables (tomato + red pepper) N=90	R= - 0.009 P>0.05 correlation not significant	R=0.105 P>0.05 correlation not significant
Total vegetables (leafy + fruit) N=180	R=0.092 P>0.05 correlation not significant	R=0.303 P<0.001 correlation highly significant

N - Number of examined samples; R - Spearman correlation coefficient

A significant correlation was found between the numbers of fungi and total aflatoxin (AFT) in leafy and total vegetables, but not in fruit vegetables. No correlation was recorded between the numbers of fungi and concentrations of aflatoxin B1 (AFB1).

DISCUSSION

Levels of filamentous fungi in leafy and fruit vegetables. The median concentrations of filamentous fungi in examined vegetables were 2.78–3.20 $\log_{10}$ CFU g^{-1}, which is very close to those reported by Buyukunal et al. [19] from the same kinds of fresh vegetables collected in Turkey (2.5–3.15 $\log_{10}$ CFU g^{-1}). The results obtained in the current study were also similar to those obtained from various vegetables by Tournas [6] in the USA and by Jeddi et al. [23] in Iran (2.0–4.0 $\log_{10}$ CFU g^{-1}), but lower compared to the results obtained by Acevedo et al. [22] in Venezuela and by Marinelli et al. [7] in Italy (4.7–6.8 $\log_{10}$ CFU g^{-1}). The current study did not confirm the prevalence of yeasts, which according to some authors [6, 14, 23] dominate over filamentous fungi (moulds), and therefore heighten the total counts of fungi if expressed as 'Yeast & Mould' up to 5.0–8.0 $\log_{10}$ CFU g^{-1} levels [6, 7, 14, 15, 16, 18, 20, 21, 23], hardly comparable with the present results. The differences in the counts between presented results and other reports may be due to different media used for isolation of the fungi.

Some of the previous authors obtained the highest fungal scores on lettuce and other leafy vegetables [7, 18, 19, 21, 23] which is in line with the above presented results. This seems to indicate an elevated health risk associated with the consumption of vegetables. Nevertheless, their microbial contamination appeared to be lower compared to that found in root vegetables (3.81–5.09 $\log_{10}$ CFU g^{-1}) examined with the same methodology [25].

Composition of mycobiota of leafy and fruit vegetables. Results obtained in the current study showing a distinct prevalence of 'field fungi' from the *Alternaria* and *Cladosporium* genera, are in line with those reported by Tournas [6] from the USA and Jeddi et al. [23] from Iran, who reported *Cladosporium*, *Alternaria*, *Penicillium* and *Geotrichum* as the most common genera among filamentous fungi isolated from leafy and/or fruit vegetables. The results of the current study differ from the data by Acevedo et al. [22] from Venezuela who indicated the genera *Penicillium*, *Aspergillus* and *Fusarium* as the prevalent moulds in vegetable samples, and from those by Costa et al. [85] who reported the *Aspergillus* species as the most prevalent moulds on samples of fresh red pepper from various countries.

The composition of mycobiota found in the present study was significantly different from the earlier study on mycobiota of root vegetables (carrot and beetroot) [25] in which a distinct prevalence of *Penicillium* and *Fusarium* strains was evidenced, while the frequency of *Alternaria* and *Cladosporium* strains was much lower compared to the current study. This could be explained by the immediate contact of root vegetables with the soil containing *Penicillium* and *Fusarium*, whereas leafy and fruit vegetables growing above the soil surface are exposed mostly to airborne fungi, such as *Alternaria* and *Cladosporium*.

Incidence of potentially pathogenic species in vegetable mycobiota and probable mechanisms of pathogenicity. Of 40 species of filamentous fungi determined in the examined leafy and fruit vegetables, as many as 38 (95.0%) were reported to be pathogenic for humans and/or animals (Tab. 3) [8, 11, 12, 32, 65, 83]. The frequency of potential pathogens was much higher compared to earlier study in root vegetables which amounted to 45.9% [25]. 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), whereas species determined to the generic level were not, in spite of the fact that the latter, such as *Alternaria* spp., *Cladosporium* spp., *Fusarium* spp., *Mucor* spp., or *Phoma* spp., might also possess pathogenic properties [8, 12, 32, 83, 84].

The main group of potential pathogens among the strains of fungi isolated from the examined vegetable samples were species reported as mycotoxigenic, which accounted for 70.0% of all identified species. It is noteworthy that the potential ability for mycotoxin production, in most cases, was connected with reported allergenic and/or infectious properties which may potentiate the toxic effect. Altogether, 28 species of fungi from vegetable samples were reported to exhibit mycotoxic properties, of which in only in nine cases solely, in eight cases jointly with infectious properties, in 4 jointly with allergenic properties, and in 7 jointly with infectious and allergenic properties [42, 43, 49, 55].

The species with potential mycotoxic properties included: *Penicillium* (8 species), *Aspergillus* and *Fusarium* (5 each), *Alternaria* (4), *Trichoderma* (three), *Paecilomyces* (2) and *Talaromyces* (one). The pathogenic fungal species classified as 'non-mycotoxigenic' comprised 10 species with reported infectious properties (*Acremonium strictum*, *Geotrichum candidum*, *Mucor circinelloides*, *Scopulariopsis brevicaulis*, *Scopulariopsis fusca*), allergenic properties (*Aureobasidium pullulans*, *Cladosporium macrocarpum*), and joint infectious and allergenic properties (*Cladosporium cladosporoides*, *Cladosporium sphaerospermum*, and *Mucor racemosus*) [40, 51, 63, 64, 75, 81]. The authors did not find any information on the pathogenicity of 2 species isolated from vegetables – *Acremonium murorum* and *Mucor plumbeus*.

POTENTIAL PATHOGENICITY

Mycotoxin production. The species isolated from examined vegetables are the potential producers of more than 45 different mycotoxins that show a wide spectrum of pathogenic effects for humans and animals [5, 8, 9, 11, 13, 36]. The majority revealed hepatotoxic, nephrotoxic, neurotoxic and/or carcinogenic properties [9, 86]. In the current study, the strains of A. flavus, the known producer of carcinogenic aflatoxins, were isolated from the samples of lettuce and spinach, where the highest levels of AFB1 were detected by the above presented analysis (Tab. 3 and 4). However, the obtained results must be interpreted with caution because of the low recovery of A. flavus strains from the tested vegetables.

Mycotoxins produced by *Alternaria* are of major concern in the vegetables studied in the present work, because of the common occurrence of these fungi in the examined samples. *Alternaria* species produce more than 70 mycotoxins that show notable toxic properties, such as mutagenicity, carcinogenicity, induction of DNA strand break, sphingolipid metabolism disruption, or inhibition of enzymes activity and photophosphorylation [36, 37]. *Alternaria* toxins have been detected in many vegetable species, mostly in tomatoes [36, 87, 88], but also in red pepper [89] and carrot [90].

The mycotoxic role of common allergenic species of *Cladosporium*, the most abundant in the vegetables investigated in this study, is largely unexplored. According to Alwatban et al. [91], 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. Corrier [92] 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. This opinion has been supported recently by the authors of the EFSA (European Food Safety Authority) report on aflatoxins in food [93] 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 of the current study may be the ability of various mycotoxins to synergistic action in low doses [94], which may be enhanced by the diversity of fungal species observed in the presented study, and the probable diversity of mycotoxins produced.

Allergenic properties. Out of 16 fungal species revealing allergenic properties that were isolated from examined vegetables, 8 are known as causative factors of IgE-dependent asthma and rhinoconjuctivitis (4 species of *Alternaria*, 3 species of *Cladosporium*, and *Mucor racemosus*), 6 were reported as causative factors of hypersensitivity pneumonitis (*Aspergillus clavatus*, *Aureobasidium pullulans*, *Paecilomyces niveus*, *Paecilomyces variotii*, *Penicillium citrinum*, *Penicillium glabrum*) and 2 as causative factors of both hypersensitivity pneumonitis and asthma (*Aspergillus flavus* and *Aspergillus fumigatus*) (Tab. 3).

Among the allergenic moulds detected in vegetables, the greatest risk was posed by the *Alternaria* and *Cladoporium* species because of their abundant occurrence in the examined samples. They produce a wide spectrum of protein allergens causing IgE-dependent asthma and/or rhinoconjuctivitis.

An additional risk is associated with similar mechanisms of the respiratory and food allergy mediated by IgE antibodies. According to Popescu [95], up to 80% of all cases of food allergy in adult patients are preceded by IgE-mediated sensitisation (clinical or subclinical) to aeroallergens. This view is in line with earlier experiments by Luccioli et al. [96] 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 the *Alternaria* and *Cladosporium* species detected abundantly in all vegetables in the present study are known factors causing respiratory allergy, it is probable that ingestion of spores or mycelium fragments of these fungi by vegetable 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 [95]. Schütze et al. [97] demonstrated in a murine asthma model that exposure to mycotoxins (gliotoxin and patulin) increases the Th2-driven, IgE-dependent immune response causing asthma and rhinoconjunctivitis. Based on the results of this study, it is presumed that aflatoxins present in vegetables may enhance such allergic reactions caused by the strains of *Alternaria* and *Cladosporium*, dominant in this environment. However, this hypothesis needs experimental and/or epidemiological confirmation.

Infectious Properties. Out of 38 potentially pathogenic species of fungi isolated from the examined leafy and fruit vegetables, as many as 23 species have been reported as causing infections (mycoses) in humans (Tab. 3). The ability to cause infections in most species was associated with another pathogenic property: causing allergic reaction(s) (in 3 species), ability to produce mycotoxin(s) (in 8 species), or both of them (in 7 species). Most of the analysed species (at least 13 out of 23) cause only so called 'opportunistic' infections which affect only immunocompromised individuals.

Among fungal species that may cause mycoses in healthy, immunocompetent individuals, the most hazardous are species belonging to the *Aspergillus* genus causing infectious and/or allergic pulmonary diseases: *A. fumigatus* [45, 49] and *A. flavus* [44], isolated in the current study from lettuce and tomato, and from lettuce and spinach, respectively. Among other fungal pathogens isolated from vegetables, noteworthy are the *Fusarium* species causing keratitits or onychomycosis in immunocompetent hosts, or invasive fusariosis in those immunocompromised [55, 56], as well as *Trichoderma longibrachiatum*, indicated by Hatvani et al. [81] as an emerging human pathogen, penetrating into the human environment from agricultural habitats.

The co-existence of mycotoxigenic and potentially infectious fungal species in the examined vegetables might be associated with adverse effects to consumers, as the co-action of even small doses of mycotoxins may contribute to the initiation and/or exacerbation of the mycotic infection as the result of an above-mentioned immunosuppressive action [92, 93].

Levels of mycotoxins in leafy and fruit vegetables. To-date, no maximal allowable concentrations of mycotoxins in fresh, ready-to-eat, vegetables have been proposed. The authors of this study therefore compared their results concerning aflatoxins concentrations with the proposals of the European Union (EU) for dried fruits intended for direct human consumption [98]. The concentrations of aflatoxin B1 measured by the authors in no case exceeded the proposed by EU maximal value equal to 2.0 μg kg^{-1}. In contrast, the EU maximal concentration proposed for total aflatoxin (sum of aflatoxins B1, B2, G1, G2) equal to 4.0 μg kg^{-1} was exceeded in 6 of the 45 samples of lettuce (13.3%) and in 2 of the 45 samples of spinach (4.4%). In none of the tomato and red pepper samples the proposed EU threshold value for total aflatoxin was exceeded.

Until recently, there are only a few studies available on the content of mycotoxins in fresh vegetables, with most of research concerning the contamination of red pepper. Costa et al. [85] expressed an opinion that aflatoxins and ochratoxin A are the most important mycotoxin contaminants of pepper and pepper derivatives. Singh and Cotty [27] found the presence of aflatoxin B1 in 63% of red pepper samples from the markets in the USA and in 93% from Nigerian markets, with the highest concentrations reaching 94.9 μg kg^{-1} in the USA and 156.0 μg kg^{-1} in Nigeria. Asai et al. [26] found the presence of aflatoxin B1 in samples of red pepper from Bolivia and Peru, and suggested that consumption of aflatoxin-contaminated pepper may be related to the development of gallbladder cancer in South America. The contamination of red pepper with aflatoxins seems to be lower in Europe and Asia, as aflatoxins were not detected by Ham et al. [24] in samples of ground red pepper from Korea, nor by Casquete et al. [28] in samples of 'La Vera' smoked paprika, a traditional Spanish food. The results of the current study seem to confirm this lower risk because of the low prevalence (16.0%) and low maximal concentration (0.329 μg kg^{-1}) of aflatoxin B1 in the Polish samples of red pepper.

Hariprasad et al. [30] found the presence of aflatoxins in 69.2% of the samples of green leafy vegetables in India, ranging from 0–88 μg kg^{-1}. In the present study, a slightly greater prevalence in this category of vegetables (70%) was found, but with a much lower maximal value (7.8 μg kg^{-1}). The concentrations of total aflatoxins stated in this study are close to those reported for dried vegetables by Hacıbekiroğlu and Kolak [29] from Istanbul, Turkey (mean equal to 1.7 μg kg^{-1}), while the concentrations of aflatoxin B1 stated in the current study were markedly lower compared to those found by the cited authors (mean equal to 1.0 μg kg^{-1}). Taken together, the current results seem to indicate that in contrast to the concentrations of total aflatoxins, the concentrations and prevalence of the most hazardous aflatoxin B1 are low or very low, in particular in fruit vegetables.

In the current study, only a very low prevalence of deoxynivalenol (DON) was detected in the examined samples of vegetable (1.1%). Because so far no maximal allowable concentrations of DON in vegetables have been proposed, the authors of this study compared their concentrations (296–627 μg kg^{-1}) with the proposals of European Union (EU) for cereals intended for direct human consumption (750 μg kg^{-1}) [63]. Result: the threshold value was not exceeded.

To-date, the prevalence of DON in vegetables is largely unexplored. Carballo et al. [30] found that the prevalence of DON was high (equal to 36%) in food prepared with cereals, vegetables and legumes, but low (5.9%) in tested vegetable samples from Spain. This is similar to the data in the current study which suggest that this trichothecene mycotoxin commonly occurring in grains probably does not pose a health hazard in vegetables.

CONCLUSIONS

Mycobiota of filamentous fungi occurring on vegetables cultivated in eastern Poland reveal a high biodiversity and a distinct prevalence of potentially pathogenic species, mostly mycotoxigenic, followed by potentially infectious and allergenic. A potential risk of foodborne exposure to mycotoxins was confirmed by the common occurrence of aflatoxins which were detected in the majority of tested samples. The risk of exposure to filamentous fungi and aflatoxins is significantly greater in the case of leafy vegetables (lettuce, spinach) than in fruit vegetables (tomato, red pepper).

Both filamentous fungi and mycotoxins occurred at levels that 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, they might pose a health risk for immunocompromised individuals, in particular those subjected to immuno-suppression treatment in health care units, or those with the immune system affected by various diseases, such as the recent COVID-19. Another endangered category are people with atopy, exhibiting respiratory hypersensitivity to airborne moulds (such as *Cladosporium* and *Alternaria* which prevailed in the examined vegetables), in which ingested fungi may elicit a pathogenic cross-reaction.

The obtained results seem to indicate that in contrast to the concentrations of total aflatoxins which are steadily present in Polish vegetables, and even exceed the proposed threshold values (for dried fruits) in leafy vegetables, the concentrations and prevalence of the most hazardous aflatoxin B1 are low or very low, in particular in the fruit vegetables, such as tomatoes or red pepper.

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