Contamination of barley grain with microscopic fungi and their metabolites in Poland in the years 2015–2016

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Abstract

Barley is one of the oldest and most-grown cereals in the world. It owes its health-promoting properties to the presence of nutrients and non-nutrients with strong antioxidant properties. Barley grain is used in the agri-food industry for the production of, among others, feed, beer as well as in the cosmetics industry for care and medicinal purposes. Barley grain during vegetation, storage and processing, is exposed to various stress factors that contribute to the deterioration of grain quality, e.g. by the presence of microscopic fungi.

The purpose of this study was to determine the level of contamination with microscopic fungi and mycotoxins from the group of trichothecenes in barley grain from Poland in a 2-year cycle (2015–2016). A total of 44 grain samples were tested. The analyzed barley samples had a similar content of tested fungal metabolites, as well as levels of microscopic fungi. The ERG concentration averaged 6.82 mg/kg, while in colony forming units this value ranged from 1.35 log CFU/g to over 2.87 log CFU/g. Total trichothecene concentrations were also low and during the two years of the study not exceeding 0,059 mg/kg. DON concentration did not exceed 1250 µg/kg in any of the tested samples. Very significant correlations between the content of trichothecenes and the concentration of ERG indicate that the level of this metabolite is closely related to the content of mycotoxins in the grain.

Key words:

CFU, ergosterol, trichothecenes, barley

Introduction

Barley is one of the oldest and most-grown cereals in the world. Barley accounts for 10% of the cereal crop structure around the world, 13% in the USA and 11% in Poland. Spring barley cultivars grown mainly in Poland are the basic ingredients of cereal mixtures, whose role in cultivation is significant (17%). Nutrition and healing properties have been known since antiquity. Barley contains antioxidants, i.e. vitamins B1, B2, B6, B12, C, E, and other bioactive compounds (phenolic acids, flavonoids). Compared to other cereal grains, barley contains lower amounts of fat. Barley grain is characterized by a high fiber content, which helps primarily in digestion processes, and also supports glucose metabolism. Inside the cell walls of barley is a type of soluble fiber called beta-glucan. Betaglucan is a sticky polysaccharide that the body is unable to absorb. Thanks to this, it can move freely in the digestive tract, absorbing some unnecessary compounds. Despite the fact that beta-glucan is also found in other grains, however, barley grain has a relatively high content. In addition, the mineral salts of potassium, calcium and magnesium in barley naturally lower blood pressure. The presence of elements such as: iron, phosphorus, calcium, magnesium, manganese and zinc ensure adequate mineralization and skeletal resistance. Manganese, iron and zinc contained in barley are ingredients that support the production of collagen. The grain of this cereal is also a rich source of selenium, which plays an important role in the functioning of liver enzymes and helps in the detoxification of some carcinogens in the body [1].

It is thanks to health-promoting properties that barley has found its place in cosmetics. Barley grain is used to make creams and lotions. It has a high content of beta-carotene, thus improving the overall skin tone. Barley grains can also be used as a mild exfoliating, soothing and smoothing agent for dry skin.

Barley cosmetics are also suitable for people with acne skin. This cereal is a source of zinc, which helps in the treatment of acne and in problems with excessively greasy skin. Barley also contains large amounts of lysine and phenolic compounds that have antioxidant, antibacterial and antifungal properties.

Barley grain is used mainly as fodder and to produce malt. Moreover, it is used for consumption purposes in the form of groats, bran and flakes, cereal germ and to a limited extent as an additive to pasta, baby formula and bakery products [2].

In view of the growing importance of barley grain in the agri-food industry it seems justified to require thorough quality control for raw materials and cereal products. The degree of their exposure to microbial contamination is high and possible at any moment starting from the plant vegetation period to each stage of grain processing.

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Microorganisms commonly found in cereal products, microscopic fungi and cereal pathogens colonising kernels are responsible e.g. for the deterioration of technological quality of grain. The greatest threat is posed by mycotoxins produced by microscopic fungi. They are secondary metabolites exhibiting toxic activity both in humans and animals. The problem of mycotoxin contamination of raw materials and products is connected with documented poisoning cases in humans and animals caused by consumption of moulded food or feed. Thus control of their contents in cereals and their milling products is crucial in the context of restrictive legal regulations in the field of health care and safeguarding consumer interests.

Mycotoxin content in grain is inseparably connected with its content of fungal biomass and its presence may indicate the occurrence of a fungus and indirectly – also products of its metabolism.

The basic methods to determine fungal biomass are direct microbiological methods. Advantages of these methods include the possibility to identify individual fungal species, while they reflect only viable fungal biomass. In contrast, increasingly often applied chemical methods, identifying concentrations of specific fungal markers such as ergosterol (ERG) in order to determine contamination levels with microscopic fungi in the tested material, reflect the

contents of both living and dead fungal biomass [3, 4]. Thus when determining the level of mycoflora as ERG concentration we may with high probability infer on grain contamination with mycotoxins or even make an attempt at the estimation of their concentrations, as it is indicated by the significant correlation coefficients specified in literature, determined between deoxynivalenol (DON), one of the most frequently found toxin in cereal grain, total contents of trichothecenes [3, 4], concentration of ochratoxin A [5], the number of colony forming units of microscopic fungi [6] and the concentration of ergosterol. In view of the above, intensive research has been conducted worldwide on the determination of barley grain contamination both in terms of contents of fungal microflora and fusarium toxins [3, 4].

The aim of this paper was to analyse contents of fusarium toxins from the group of trichothecenes, as well as provide quantitative and qualitative assessment of contamination with microscopic fungi using conventional microbiological methods, while also applying a chemical method to determine ergosterol concentration in samples of barley grain analysed in a two-year cycle (2015 – 2016). An additional aspect of this study was to identify the correlation between concentration of trichothecenes in the tested grain, and CFU number and ERG concentration.

Material and methods

Barley grain was collected from cereal silos located at grain purchasing stations and mills throughout Poland in the successive years of the 2015 – 2016 period in the season from October to November in each analysed year applying identical methodology. A total of 44 samples were collected (in individual year it was as follows: 2015 – 19 samples, 2016 – 25 samples), each of 5000 g in accordance with the Regulation on the Methods of sampling of selected foodstuffs to control levels Fusarium toxins and criteria for analytical methods used to determine contents of Fusarium toxins [7, 8].

Analysis of trichothecenes

Grain samples were analyzed for the presence of trichothecenes according to Perkowski et al. (2003) [9]. Sub-samples (10 g) were extracted with acetonitrile/water (82:18) and cleaned up on a charcoal column (Celite 545/charcoal Draco G/60/activated alumina neutral 4:9:5 (w/w/w). Trichothecenes of group A (H-2 toxin, T-2 toxin, T-2 tetraol) were analysed as TFAA derivatives. Trichothecenes of group B (DON, NIV, 3-AcDON, 15-AcDON) were analysed as TMS (trimethylsilylsilyl ether) derivatives. The volume of TMSI/TMCS (trimethylsilyl imidazole/trimethylchlorosilane, v/v 100/1) mixture was added to the dried extract. Next of isooctane were added and the reaction was quenched with of water. The isooctane layer was used for the analysis and of the sample was injected on a GC/MS system. The analyses were run on a gas chromatograph (Hewlett Packard GC 6890) hyphenated to a mass spectrometer (Hewlett Packard 5972 A, Waldbronn, Germany), using an HP-5MS 0.25 mm x 30 m capillary column. The injection port temperature was 280°C, the transfer line temperature was 280°C and the analyses were performed with programmed temperatures, separately for group A and B trichothecenes. Quantitative analysis was performed in a single ion monitored mode (SIM). Qualitative analysis was performed in the SCAN mode (100–700 amu). The limit of detection was 0.001 mg/kg.

Chemical analysis of ergosterol

Ergosterol was determined by HPLC as described by Young (1995) with modifications [3, 4]. Samples of ground grains were placed into culture tubes, suspended in of methanol, treated with of 2M aqueous sodium hydroxide, and tightly sealed. The culture tubes were then placed within plastic bottles, tightly sealed and placed inside a microwave oven (Model AVM 401/1WH, Whirlpool, Sweden) operating at 2450 MHz and 900 W maximum output. Next contents of culture tubes were neutralized with 1M aqueous hydrochloric acid, MeOH were added and extraction with pentane was carried out within the culture tubes. The combined pentane extracts were evaporated to dryness in a nitrogen stream. The sample extract was dissolved in MeOH and were analyzed

by HPLC. The presence of ERG was confirmed by a comparison of retention times and by co-injection of every tenth sample with an ergosterol standard.

Plate flooding method using decimal dilutions

The amounts of microscopic fungi per 1 g grain were assessed using the standard plate method in accordance with the procedure PN-ISO 21527-2: 2009. Microbiology of food and feeds. Horizontal method to determine counts of yeasts and moulds. Part 2: Colony counting method in products with water activity of max. 0.95. The amount of 10g ground tested material (in three replications) was suspended in 90 ml of 0.1% peptone water. After 30 min. samples were shaken for 2.5 min. Next decimal dilutions were prepared from the suspension using 0.1% peptone water solution. For this purpose 1 ml suspension was transferred with a sterile pipette from three produced dilutions onto sterile Petri dishes (with two per each dilution). In each stage plates were flooded with agar medium with rose bengal and chloramphenicol (15 ml) at 45°C. Plates were incubated under aerobic conditions and placed flat in an incubator at 25±1°C for 5–7 days. After incubation colonies were counted on selected plates (adequate for the production of 15 to 150 colonies per plate) and based on the number of counted colonies the number of units forming colonies of microscopic fungi (CFU) was detemrined per 1g tested material (cfu/g). The result was a mean from two replications and it was expressed in log cfu/g.

2.4. The analysis of fungi occurrence in barley

The fungal species composition found in barley grain samples was analysed. The dilution method was used: 1 g of ground grains was put in 10 ml of sterile distilled water and mixed with a magnetic stirrer for 2 min. Next 1 ml of the suspension was transferred onto the PDA medium (Potato-Dextrose Agar) in Petri dishes and spread with the aid of a sterile glass rod on the medium surface. The Petri dishes were incubated at 25°C for 7 days. Growing mycelia were isolated on the PDA and SNA (Synthetic Nutrient – Poor Agar) mediums to identify fungal species. The identification was carried out on the basis of colony and spore morphology.

2.5. Statistical analysis

Recorded results were analysed statistically using the STATISTICA ver. 8.0 programme. In order to compare contents of individual metabolites in samples the multiple comparison procedure was used applying Tukey's method. Moreover, values of Pearson's linear correlation coefficients were also calculated for the significance levels α=0.05, α=0.01 and α=0.001 (*, **, ***) between concentrations of ERG, trichothecenes and the number of CFU. In order to determine the effect of weather conditions on the level of barley grain contamination with mycoflora and mycotoxins multiple regression was applied and Pearson's linear correlation coefficient was determined at the significance level α=0.05 between analysed factors.

Results and discussion

The level of barley grain contamination with microscopic fungi and mycotoxins is one of the basic indicators of its quality. Analyses conducted within this study in a two-year cycle aimed at the determination of the contamination level in barley grain produced in western Poland with microscopic fungi and mycotoxins from the group of trichothecenes. A total of 44 barley grain samples coming from cereal silos were analysed using two methods, i.e. the conventional microbiological method determining colony forming units of microscopic fungi and the chemical method analysing ergosterol concentration. Moreover, the genera of microscopic fungi were identified based on observations of morphology of colonies and hyphae. In view of the fact that among three most frequently identified genera of microscopic fungi there were fungi from the genus Fusarium, mycotoxins from the group of trichothecenes, produced most frequently by them, were determined qualitatively and quantitatively.

Content of microscopic fungi measured in terms of colony forming units in all the barley grain samples was very low, ranging from 1.81 logcfu/g to 2.19 logcfu/g (Table 1). Statistical analysis showed

no significant differences in the number of CFU between samples analysed in individual years.

Chemical analysis of ERG concentration as a specific marker of fungal biomass showed, similarly as in the case of the number of CFU, low contamination of barley grain with microscopic fungi. Mean concentration of this metabolite was 6.82 mg/kg (Table 1).

Apart from the contents of mycoflora the concentration of mycotoxins was also investigated in the cereal samples. The level of A and B trichothecenes in the four years of the study was also low, while DON concentration did not exceed the level of 1250 μg/kg, considered safe for grain for human consumption in any of the tested samples. However, similarly as in the case of ERG concentration, the content of these toxic metabolites was significantly higher in 2008 than in the other years of the study. Mean total concentration of trichothecenes was 0.083 mg/kg (Table 1). Among A trichothecenes STO, T-2 tetraol, T-2 triol, DAS and HT-2 were identified in the analysed barley grain samples. Apart from A trichothecenes also B trichothecenes were detected in the samples (Table 2). The following toxins from this group were identified: DON, Fus-X, 3-AcDON, 15-AcDON and NIV. The most frequently detected toxins were DON (identified in 95% all samples) and NIV (on average in 62% all samples), followed by toxins from group A found in a considerable number of samples (41%).

The conducted qualitative analysis of the fungal microflora composition based on colony morphology and observations of live specimens showed that the most frequently found genera of microscopic fungi in samples of barley grain tested in the years 2015–2016 were Aspergillus, Penicillium and Fusarium (Fig. 1).

Recently a considerably body of literature on the subject concerns the determination of specific weather conditions characteristic of individual years of experiments, which are the necessary prerequisite for the collection of data needed to show the relationship between weather conditions with the conditions

Table 1.
Ranges and mean total toxin concentrations as well as values of log cfu/g in barley grain harvested in the years 2015–2016

Year	Number of samples	Mean ERG concentration (mg/kg)		Mean total trichothecenes concentration (mg/kg)		Log cfu/g	
		Range	Mean	Range	Mean	Range	Mean
2015	19	4.95–9.77	7.33a	0.023–0.091	0.083a	1.68–2.59	2.11a
2016	25	3.48–8.44	6.53b	0.011–0.071	0.066a	1.72–2.83	2.11a

Identical letters in rows – no significant differences at significance level α=0.05

Table 2.
Mean concentrations of A and B trichothecenes in barley grain harvested in the years 2015–2016

Years	Number of samples	Concentration of A trichothecenes (mg/kg)					Concentration of B trichothecenes (mg/kg)				
		STO	T-2 Tetraol	T-2 Triol	DAS	HT-2	DON	Fus-X	3-AcDON	15-AcDON	NIV
2015	19	<LOD	0.004	0.003	0.002	0.002	0.041	0.001	0.001	0.004	0.001
2016	25	0.001	0.003	0.001	<LOD	0.004	0.052	<LOD	0.001	0.001	0.003

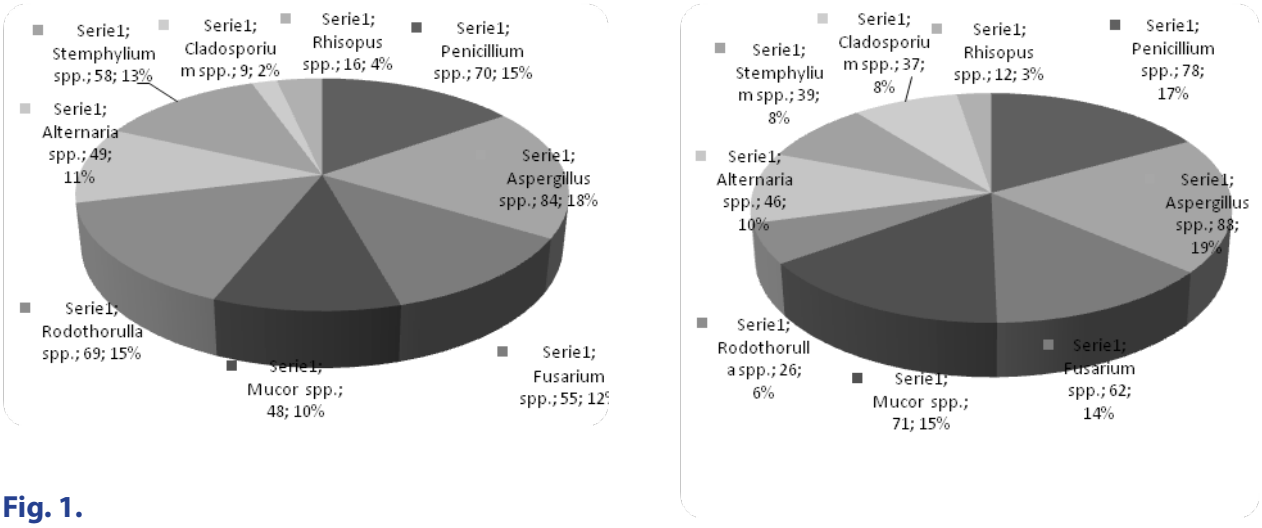

Fig. 1.
Genera of microscopic fungi identified in barley samples collected in Poland in 2015 (A) and 2016 (B)

required for the development of ear blight and increased toxin accumulation in grain. It is reported that the most important conditions in this aspect include precipitation during flowering and elevated humidity during the plant vegetation period. A significant, although lesser effect on these factors was also observed for temperature. When analysing weather conditions in the four years of the study based on data collected from the Institute of Meteorology and Water Management (Fig. 2, 3) it may be stated that there are no significant correlations between precipitation total and temperature in a given month in each year of the study.

Based on the results it was found that barley grain in Poland has low contents of mycotoxins from the group of trichothecenes and mycoflora contamination. When comparing the analysed characteristics with the results recorded for other cereal species, investigated in the years 2015 – 2016, it was found that in terms of grain contamination barley ranks

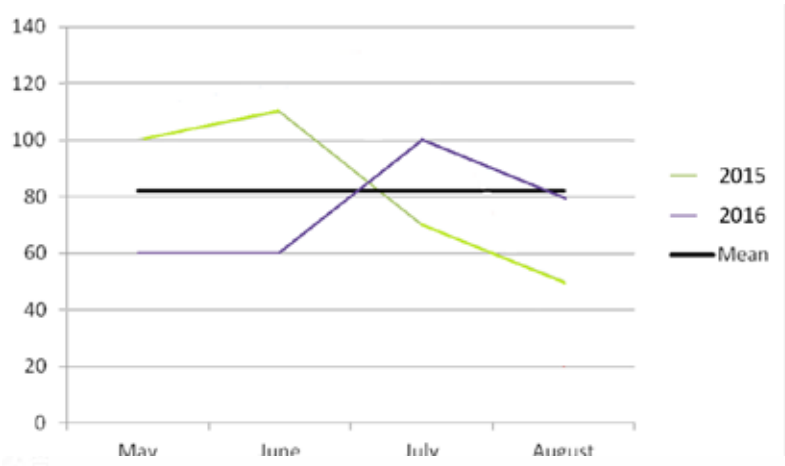

Fig. 2.
Means for temperature in the seasons of 2015–2016 in Poland and their percentage relationships to the means for the last two decades

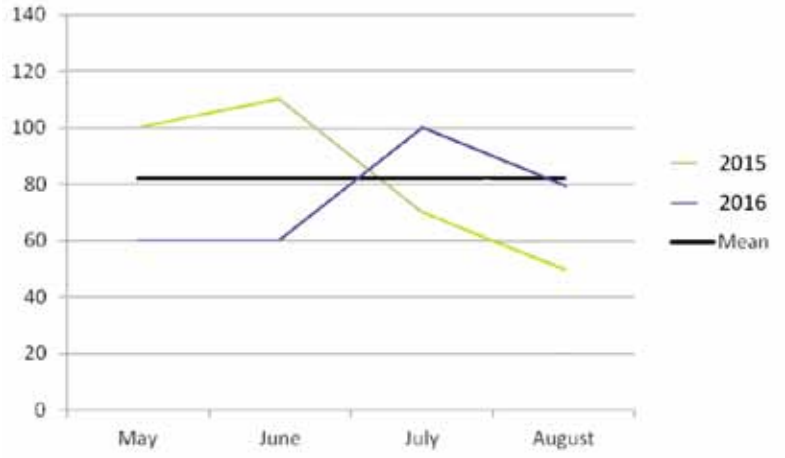

Fig. 3.
Means for rainfall in the seasons of 2015–2016 in Poland and their percentage relationships to the means for the last two decades

4th among cereals grown in Poland. On the basis of ERG concentration, significantly correlated with the total level of toxins, and with the calculated correlation coefficient being considerably higher than that calculated for the relationship of mean total toxin concentration/log CFU, the 5 cereal species grown in Poland may be ranked as follows, starting from the cereal with the lowest ERG content: wheat<triticale<rye<barley<oat. Similar dependencies for ERG concentration were recorded by Perkowski et al. [3, 4]. They determined ERG concentration in samples of barley and wheat grain to be 7.49 mg/kg and 2.39 mg/kg. In studies conducted by other authors mean ERG concentration in comparison to its content in wheat was up to 3 times higher [5]. Concentration of ERG amounting to 3 mg/kg [10] was adopted as the safe content of mycoflora in healthy grain, while Schnörer and Johnson [11] proposed the range of concentrations for this metabolite amounting to 1 – 9 mg/kg as the boundary value for grain for human consumption. Recorded mean ERG concentrations for all analysed samples did not exceed the boundary value specified in literature.

Next to ERG concentration samples of barley grain were also analysed microbiologically. The number of units forming colonies of microscopic fungi was determined, genera of microscopic fungi were identified and the percentage shares of individual genera in the whole population in a given year were calculated. It was found that fungi from the genus Fusarium, next to Alternaria and Penicllium, were identified most commonly. Similarly, Conkova et al. [12] when analysing samples of barley found that the most frequently observed fungi in the population isolated from grain coming from Poland and Slovakia were fungi from the genera Aspergillus, Fusarium, Penicillium and Cladosporium. The content of microscopic fungi measured in terms of colony forming units was low in all samples of barley grain. Mycological contamination of cereals at 103 – 104 cfu/g is assumed to be natural infestation [11]. Similar results to those presented in this paper for CFU in barley grain were reported by Gawrysiak-Witulska et al. [13]. In grain directly after harvest they detected approx. 104 cfu/g. In turn, Baliukoniene et al. [14] found higher numbers of CFU in tested barley grain (4.3 log cfu/g). Samples came from family farms located in Lithuania. Neagu et al. [15] tested samples of barley coming from central Romania. They found a low content of microscopic fungi amounting to 4cfu/g. In comparison to wheat grain the number of CFU in barley was on average by 40% higher [9].

Apart from contents of mycoflora in samples of barley the concentration of mycotoxins was also investigated. Levels of A and B trichothecenes in the course of the four years of the study remained low, while DON concentration did not exceed 1250 μg/kg (established as safe for grain for human consumption) in any analysed sample. However, similarly as in the case of ERG concentration and the number of CFU the content of these toxic metabolites varied in individual years. Others authors investigated DON content in samples of naturally infested barley grain. Similar mean DON concentrations amounting to 138 μg/kg were obtained for Garaleviciene et al. [16]. Mankevičiene et al. [17] in their study detected 198 μg/kg DON in barley grain, while Perkowski et al. [4] on the basis of results recorded by Polish researchers [9, 18, 19] in the years 1997-2003 compared contents of DON, NIV T-2 and HT-2 in samples of barley grain. Among analysed samples showing no FDK presence barley contained 91 – 100 μg/kg. Concentration of NIV was comparable in all tested samples and it amounted to a mean 61 μg/kg in barley grain. Investigations conducted at the time of this study by the American Malting Barley Association (AMBA) on contents of DON and NIV in samples of malting barley showed that presently sown cultivars exhibit high resistance to fungal diseases and their mycotoxin contamination is low. However, AMBA indicated resistance of fodder cultivars, which were also covered in 2009 by the Federation supervision, as in 19% analysed samples the limits for DON contents were significantly exceeded in samples coming from Europe and Asia. Fels-Klerx et al. [20] analysed trichothecene contamination of cereals in north-central Europe in view of climate change. They stated that in the years 2013 – 2016 among the tested 717 samples of barley and 842 samples of wheat the mean concentration of DON was significantly varied, as barley grain contained 3 times more DON than wheat grain, i.e. 797 μg/kg vs. 223 μg/kg, respectively.

For the results recorded in the years 2015–2016 and presented in this study correlations were calculated between the amount of mycoflora and investigated metabolites. In almost 90% investigated cases correlations were significant. In turn, when testing all cereal samples tested in a four-year cycle and applying the three possible combinations for the calculation of correlations at the significance level $P = 0.001$, for ERG/total toxin concentration it was found to be 0.6816, for ERG/log cfu/g it was 0.5501, while for the total toxin concentration/log cfu/g it was 0,5685. Similar or even correlation coefficients for the presented traits were reported by other authors [21]. Highly significant correlations between the content of trichothecenes and ERG concentration, being higher than in the case of the correlations of total toxin concentration/log cfu/g, indicate that the level of this metabolite is inseparably connected with the content of mycotoxins in grain.

Based on the studies presented above, concerning variability in the four-year cycle observed in fungal metabolites and differences between their contents in different cereals it may be stated that the process of accumulation of trichothecenes and ERG is influenced by several factors, e.g. the structure of plants [22], their resistance and weather conditions in the periods of flowering and kernel ripening [23].

In recent years much attention has been devoted in the literature on the subject to the determination of specific conditions required for the development of ear blight and enhanced accumulation of toxins in grain. It is reported that rainfall during flowering and increased humidity during the plant vegetation period are the most significant. A significant effect on these factors is also found for temperature. Analysed weather conditions in the two years of the study based on the data collected from the Institute of Meteorology and Water Management is show Fig 3. Analysed the results concerning both ERG concentration and the contents of trichothecenes in tested grain (Table 1). It was reflected in the presented weather data (Tables 2, 3).

Presented results of studies conducted in a two-year cycle on contents of mycoflora and trichothecenes in barley grain clearly show a low level of contamination with microscopic fungi and toxins in cereals for human consumption in Poland, while the statistically determined slight differences between contents of analysed metabolites between the years of the study are influenced by weather conditions in terms of the development of mycoflora and production of mycotoxins.

Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflict of interest.

Compliance with ethics requirement

This article does not contain any studies with human or animal subjects.

Table 3.
Results of internal correlation analyses

Years	Correlation coefficient				
	ERG/ Σc_{trich}	ERG/DON	ERG/NIV	ERG/log cfu/g	Σc_{trich} /log cfu/g
2015	0.6720**	0.4021*	0.4228*	0.5271**	0.5997**
2016	0.6922**	0.3854*	0.5334*	0.3251	0.6022**

*, **, *** – Pearson's linear correlation coefficients taking into consideration the following significance levels α=0.05, α=0.01, α=0.001

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