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Three-Year Survey of *Fusarium* Multi-Metabolites/Mycotoxins Contamination in Wheat Samples in Potentially Epidemic FHB Conditions

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Abstract: *Fusarium* head blight (FHB) is a fungal disease of cereals including wheat, which results in significant economic losses and reductions in grain quality. Additionally, the presence of *Fusarium* spp. results in productions of mycotoxins/metabolites, some of which are toxic in low concentrations. The liquid chromatography with tandem mass spectrometry (LC-MS/MS) method was applied to 216 wheat samples from field conditions diseased with FHB. Data obtained show that out of 28 metabolites detected, deoxynivalenol (DON), deoxynivalenol-3-glucoside (D3G), enniatin B (ENN B), enniatin B1 (ENN B1), culmorin, 15-hydroxyculmorin, and aurofusarin were the most prevalent mycotoxins/metabolites over three years (2014–2016). In 2014–2016, 100, 100 and 96% of the samples were contaminated with zearalenone (ZEN). Of the masked mycotoxins, D3G occurred at a high incidence level of 100% in all three investigated years. Among emerging mycotoxins, moniliformin (MON), beauvericin (BEA) and enniatins (ENNs) showed high occurrences ranging from 27 and 100% during three investigated years. Co-occurrence of *Fusarium* mycotoxins/metabolites was high and almost all were highly correlated to each other but their possible synergistic, additive, or antagonistic effects of toxicity, should be taken into consideration. Our results demonstrated that modified and emerging mycotoxins/metabolites contributed substantially to the overall contamination of wheat grains. To avoid disparagement, it is necessary to analyse these forms in future mycotoxin monitoring programs and to set their maximum levels.

Keywords: crop season; emerging mycotoxins; *Fusarium*; LC-MS/MS; mycotoxins; wheat

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1. Introduction

Fusarium spp. occur regularly each year in cereal crops over the globe, and additional concerns have created new insight into the extremely negative effects of mycotoxins on human and animal health. *Fusarium* head blight (FHB), mainly caused by *Fusarium graminearum* and *F. culmorum*, can significantly reduce grain yield and quality of wheat, and produce mycotoxins that affect food safety [1]. Nowadays, the term "food safety" is increasingly mentioned and hence mycotoxins are increasingly attracting attention, thus encouraging plant biologists and breeders to work on solutions to find resistant wheat genotypes to this widespread disease. Screening and identifying FHB resistant genes in wheat germplasm for development of resistant wheat varieties is the most effective way to manage FHB [2]. But however, agronomic practices and fungicides reduce the risk of damage to some extent. The best fungicide applications, considering the timing and dose of application, can partially reduce FHB symptoms [3]. Furthermore, creation of resistant varieties is hampered as FHB resistance has been classified into multiple types [4]. Type I

resistance is attributed to reduction of initial infection, type II resistance prevents spread of infection within the spike and type III confers resistance to mycotoxin accumulation. Type II resistance has been widely used in breeding programs due to its effective performance in reducing the impact of FHB on grain production. There is also evidence that FHB resistant wheat genotypes accumulate far fewer mycotoxins than susceptible ones [5]. FHB continues to threaten susceptible wheat varieties where environmental conditions such as high humidity and temperature persist during flowering. Warm and humid environmental conditions ideally propagate the pathogen which may result in severe disease outbreaks with substantial crop losses [6]. Typical FHB symptoms include water soaked lesions on glumes, followed by discoloration that spreads from the point of infection to the adjacent spikelets. As infection progresses, symptoms of wilting and blight spread over the entire spike, indicating premature senescence of infected spikes [7]. As previously mentioned, *Fusarium* mycotoxins in wheat are the main secondary metabolites that occur at levels of potential concern for human and animal health [8]. Deoxynivalenol (DON), zearalenone (ZEN), nivalenol (NIV), fumonisins (FB), T-2, and HT-2 toxins are the most important *Fusarium* mycotoxins occurring on a worldwide basis in cereal grains [9]. All of the above mentioned mycotoxins belong to the trichothecene's groups A and B, with the exception of FB and ZEN, which are listed as the most toxic [10]. Furthermore, as food and feed contaminants, they may cause alimentary hemorrhage and vomiting, while direct contact causes dermatitis [11]. In addition, the acute symptoms of trichothecenes detriments are gastroenteritis, nausea, anorexia, growth retardation, endocrine damage and immunosuppression [12]. The main biological effect of the non-steroidal estrogenic mycotoxin ZEN and its metabolites (especially α-zearalenol) is reproductive toxicity [13]. Limits for some mycotoxins have been recommended and specified in unprocessed cereals, milling products, and cereal end-use products: 200–1750 μg kg^{-1} for DON, 20–400 μg kg^{-1} for ZEN, 200–4000 μg kg^{-1} for the sum of B1 + B2 fumonisins (FB1 + FB2 combined) [14], and 15–1000 μg kg^{-1} for the sum of HT-2 and T-2 toxins [15]. European Commission has not yet given any legislative for NIV, but the European Food Safety Authority set a tolerated daily intake (TDI) of up to 1.2 μg kg^{-1} body weight per day [16].

The situation with the occurrence of mycotoxins becomes even more dangerous by the presence of modified *Fusarium* mycotoxins and so-called emerging mycotoxins [17]. *Fusarium* mycotoxins can be altered in their chemical structure, with unexpectedly high toxicity in the digestive tract of humans/animals although the metabolic fate is still not very well studied [18]. They may escape detection methods as they are chemically different from parental mycotoxins and there are currently no regulations for these newly developed metabolites/mycotoxins [19,20]. Previous reports have demonstrated the conversion of DON into DON-3-glucoside (D3G) as well as modifications of ZEN to glucoside or sulphate form [21,22]. Moreover, it was reported about glucoside forms of NIV in wheat products [23]. D3G is formed through the glycosylation of DON as the response to the detoxification process, but the greatest danger is hidden in the fact that D3G has been found in wheat lines with low FHB susceptibility [24]. Although D3G was found in wheat grains at concentrations that reached or exceeded the maximal permitted levels for DON, the toxicity of D3G remains still unknown [25]. On the other hand, the total amount of conjugated forms of ZEN exceeded the concentration of the parental mycotoxin [17], so these forms of ZEN should not be underestimated as conjugated ZEN derivatives can be efficiently hydrolysed. It is also interesting to note that NIV usually co-occurs with DON in wheat grains, where its modified form detected was nivalenol-glucoside (NIV-3G) [26].

Unlike some *Fusarium* mycotoxins, such as DON, T-2, HT-2, FB, and ZEN, whose presence in food and feed has been regulated by authorities, no limits have been set for emerging mycotoxins, such as enniatins (ENNs) [27]. Special attention should be paid to ENN B, as its potential toxicity may be enhanced by co-occurrence with other mycotoxins [28]. The role of other emerging mycotoxins, such as beauvericin (BEA), fusaproliferin (FUP) and moniliformin (MON), is not very well understood up today [29]. It has been reported that BEA is inducer of reactive oxygen species (ROS) leading to cell apoptosis but also it has very

efficient effects in the anticancer, antimicrobial, and insecticidal activities [30]. Although an anti-inflammatory activity of FUP was found [31], there is evidence of FUP phytotoxicity causing structural changes in chloroplasts of plants [32]. In the study of Bertuzzi et al. [33], it was reported that MON reduces the activity of glutathione peroxidase and glutathione reductase, thus increasing oxidative stress in plants. Overall, multi-mycotoxin contamination is very common because mycotoxins have additive/synergistic interactions that pose an additional risk to food safety [8]. This is supported by the fact that co-occurrence of mycotoxins in cereals has been previously reported [34,35]. In addition to problem in food safety, *Fusarium* spp. and their co-occurrence have a detrimental effect on the processing and rheological quality of wheat. FHB infection and its mycotoxins may reduce wheat milling performance, with a strong negative effect on end-use quality [36]. Also, FHB epidemic reduces processing quality in susceptible wheat varieties; primarily, sedimentation value and gluten index, and hence, had negative impact on rheological properties [37]. Of even greater concern, however, is the fact that the technological process can produce conjugated forms of mycotoxins in wheat end-use products [38]. Moreover, microorganisms used in fermentation and malting processes may transform mycotoxins into conjugated forms or even increase some parental forms of mycotoxins [1]. Nevertheless, according to some studies increased temperatures or fermentation can reduce the concentration of some *Fusarium* mycotoxins [39,40]. Nevertheless, the masked forms have significant toxicity due to their conversion by plant's metabolism or technological process.

The objectives of the present study were to investigate the occurrence of *Fusarium* mycotoxins/metabolites and their modified forms in wheat grain under potentially epidemic FHB conditions in three consecutive years and to determine the correlations between the levels of different mycotoxins in contaminated wheat samples.

2. Materials and Methods

2.1. Wheat Material and Field Conditions

Eight winter wheat varieties/lines ranging in FHB resistance and 28 of their progeny were included in the experiment (Supplementary Figure S1). These 36 genotypes were planted in October in three consecutive growing seasons (2013/2014, 2014/2015 and 2015/2016) at location Botinec, Croatia (45°45′11.49″ N; 15°56′4.98″ E) following randomized complete block design with two replicates. Each experimental plot was consisted of two rows with a length of 1 m and a row spacing of 0.25 m. The previous crop in all three growing seasons was rapeseed (*Brassica napus* subsp. *napus*). Fertilization was adapted to intensive wheat production. Before sowing, nitrogen (N), phosphorus and potassium (7:20:30), and UREA (carbonyl diamide with 46% N) in the amount of 300 kg ha^{-1} and 150 kg ha^{-1} were added in the soil. Calcium ammonium nitrate (CAN with 27% N) was added at the beginning of vegetation (185 kg ha^{-1}) and in the phase of intensive growth (110 kg ha^{-1}) (GS 31–33). For weed protection, 0.8 g L^{-1} of Axial 50 EC (pinoxaden 50 g L^{-1}) and Starane 250 (fluroxypyr 360 g L^{-1}) were applied (GS 21–23). Foliar protection was performed using the fungicide Amistar Opti (chlorothalonil 480 g L^{-1} plus azoxystrobin 80 g L^{-1}) at a rate of 2.5 L ha^{-1} (GS 37–38). To prevent insect's influence plants were treated with the insecticide Karate Zeon (lambda cyhalothrin 50 g L^{-1}) at a rate of 0.15 L ha^{-1} (GS 59–60).

Weather data were obtained from the Croatian Meteorological and Hydrological Service. At location Botinec weather data were different in May and June for three consecutive years with precipitation of 88 mm in May and 171 mm in June in 2014, 151 mm in May and 60 mm in June in 2015 and 101 mm in May and 133 mm in June in 2016 (Figure 1). Regarding temperature, the monthly mean values for May were 23, 23 and 24 °C in 2014–2016, respectively, while the monthly mean values for June were 28, 26 and 28 °C in the same years (Figure 2).

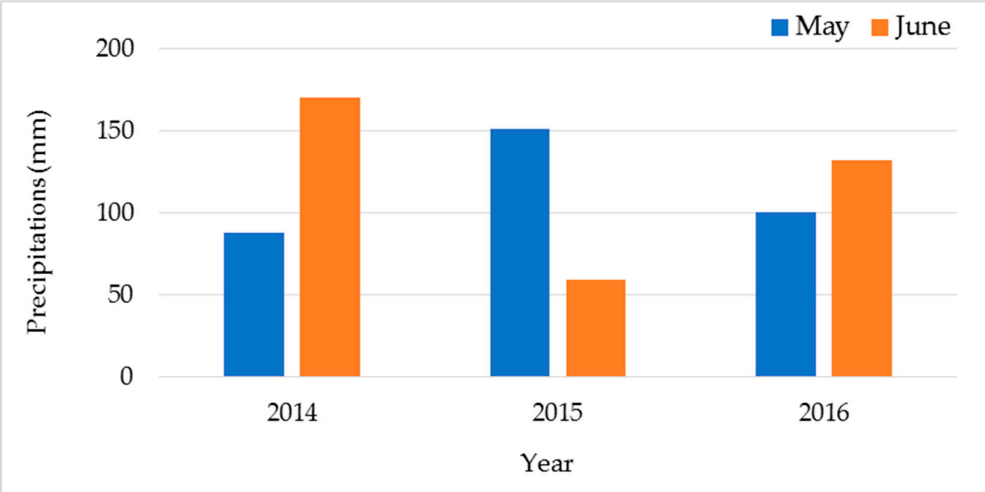

Figure 1. Rainfall in mm in May and June during three consecutive years.

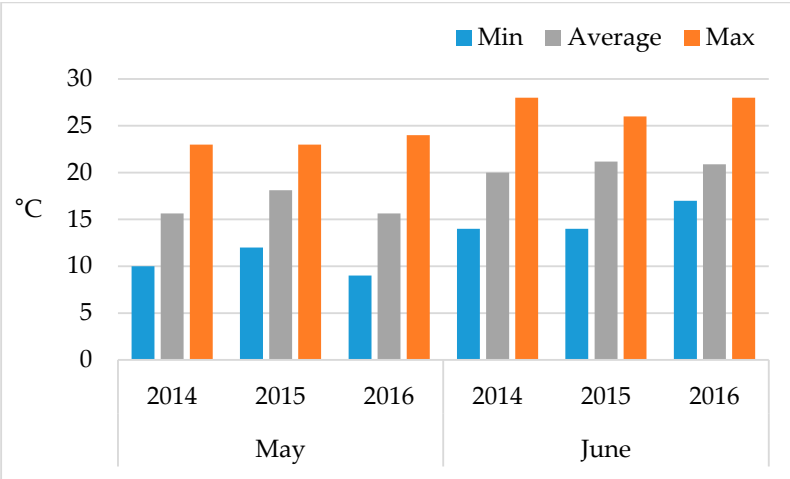

Figure 2. Mean daily temperatures in May and June during three consecutive years.

2.2. Inoculum Production and Inoculation

The four isolates of *F. graminearum* were used for artificial inoculation of plants and were previously collected from wheat in Croatian fields. For inoculum production the bubble-breeding method was used using the medium *Vigna radiata* [L.] R. Wilczek [41]. The concentration of inoculum was set up to 500,000 spores mL^{-1}. Each wheat genotype in the field experiment was inoculated separately at the flowering stage (GS 65–69) in the early morning, and the inoculation procedure was repeated two days later using the back-pack sprayer. Grain samples (total n = 216) of wheat genotypes (n = 36) in two replicates in three years of investigation were harvested when grain moisture was below 13%.

2.3. Disease Assessment

In each plot, the percentage of visually infected spikelets was estimated 18, 22, 26, and 30 days after the first inoculation. The area under the disease progress curve (AUDPC) was calculated for each plot using the following equation:

$$\text{AUDPC} = \Sigma \{[(yi + yi - 1)/2] \times (xi - xi - 1)\}$$

where Σ is the sum over four observations, yi is the score of visually infected spikelets on the ith day, and xi is the day of the ith observation. At harvest maturity (about 13% grain moisture), 10 randomly selected ears were taken from each plot, manually threshed

and the number of *Fusarium* damaged kernels (FDK) was determined and expressed as a percentage of the total number of kernels in the sample.

2.4. Mycotoxin Analysis

Determination of *Fusarium* mycotoxins/metabolites in 216 wheat samples was performed by liquid chromatography with tandem mass spectrometry (LC-MS/MS) at the Department of Agrobiotechnology (IFA-Tulln), Institute of Bioanalytics and Agro-Metabolomics, University of Natural Resources and Life Sciences Vienna (BOKU), Austria, according to the same method previously described in the study by Sunic et al. [6]. Sample values below the LOD (<LOD) were replaced by a constant value of LOD/2 before statistical analysis of the data.

2.5. Statistical Analysis

Combined analysis of variance (ANOVA) was performed across three years and 36 genotypes using the GLM procedure in the statistical program SAS/STAT (SAS Institute Inc., Cary, NC, USA) [42]. Pearson correlation r values were determined using GraphPad Prism 9.4.1 [43].

3. Results

For three consecutive years, Fusarium head blight (FHB) severity was estimated by the area under the disease progress curve (AUDPC) and Fusarium damaged kernels (FDK). In addition, 28 *Fusarium* metabolites/mycotoxins were detected in 216 analysed wheat samples (36 genotypes × 3 years × 2 replicates). Furthermore, these metabolites/mycotoxins were classified into four different groups (trichothecenes and their derivatives, zearelenone and its derivatives, emerging mycotoxins and other *Fusarium* metabolites (Table 1, Figure 3). Analysis of variance revealed a significant effect of year for FDK as well as for all mycotoxins except HT-2 glucoside, moniliformin and enniatin B3, whereas genotype was significant for AUDPC for general resistance, FDK and all mycotoxins except T-2 toxin, HT-2 glucoside and equisetin (Table 1). On the other hand, the genotype × environment interaction was significant for FDK and only nine of the 28 mycotoxins analysed.

Table 1. Analysis of variance for area under the disease progress curve (AUDPC) for general resistance, *Fusarium* damaged kernels (FDK), and content of 28 mycotoxins in grain of 36 wheat genotypes grown in three vegetation years; F values and their significances (F Sign.) are shown.

		Year (Y)		Genotype (G)		G × Y	
Trait		**F**	**F Sign.**	**F**	**F Sign.**	**F**	**F Sign.**
AUDPC for general resistance		1.5	ns	24.8	**	1.0	ns
Fusarium damaged kernels		53.6	**	17.2	**	1.7	**
Trichothecenes and their derivatives	Deoxynivalenol	66.0	**	16.8	**	1.1	ns
	DON-3-glucoside	96.3	**	13.5	**	1.7	**
	3-acetyldeoxynivalenol	75.5	**	6.3	**	0.9	ns
	15-acetyldeoxynivalenol	72.9	**	7.2	**	1.5	*
	Nivalenol	29.7	**	13.4	**	1.2	ns
	T-2 toxin	10.9	**	1.4	ns	0.9	ns
	HT-2 toxin	9.6	**	3.0	**	0.9	ns
	HT-2 glucoside	1.9	ns	1.3	ns	0.8	ns
Zearelenoneand its derivatives	Zearalenone	14.0	**	10.9	**	1.0	ns
	Zearalenone-sulphate	51.3	**	11.1	**	2.7	**
	α-zearalenol	6.3	**	2.5	**	0.8	ns
	β-zearalenol	4.4	*	2.8	**	0.6	ns

Table 1. *Cont.*

		Year (Y)		Genotype (G)		G × Y	
	Trait	**F**	**F Sign.**	**F**	**F Sign.**	**F**	**F Sign.**
Emerging mycotoxins	Moniliformin	1.0	ns	2.9	**	0.6	ns
	Beauvericin	81.3	**	8.7	**	2.4	**
	Enniatin A	17.6	**	4.9	**	1.6	*
	Enniatin A1	18.9	**	5.4	**	1.8	**
	Enniatin B	7.8	**	4.7	**	1.1	ns
	Enniatin B1	17.4	**	6.0	**	1.6	*
	Enniatin B2	5.3	**	4.1	**	0.9	ns
	Enniatin B3	2.6	ns	2.8	**	0.9	ns
Other *Fusarium* metabolites	Culmorin	134.5	**	15.6	**	4.9	**
	5-hydroxyculmorin	11.9	**	4.1	**	0.7	ns
	15-hydroxyculmorin	12.5	**	4.0	**	0.6	ns
	15-hydroxyculmoron	20.2	**	3.5	**	0.7	ns
	Aurofusarin	154.6	**	16.2	**	5.9	**
	Apicidin	3.0	**	1.8	*	0.7	ns
	Chrysogin	10.8	**	6.3	**	0.8	ns
	Equisetin	3.3	*	1.1	ns	0.9	ns

* and **, F significant at $p < 0.05$ and $p < 0.01$, respectively; ns—F not significant.

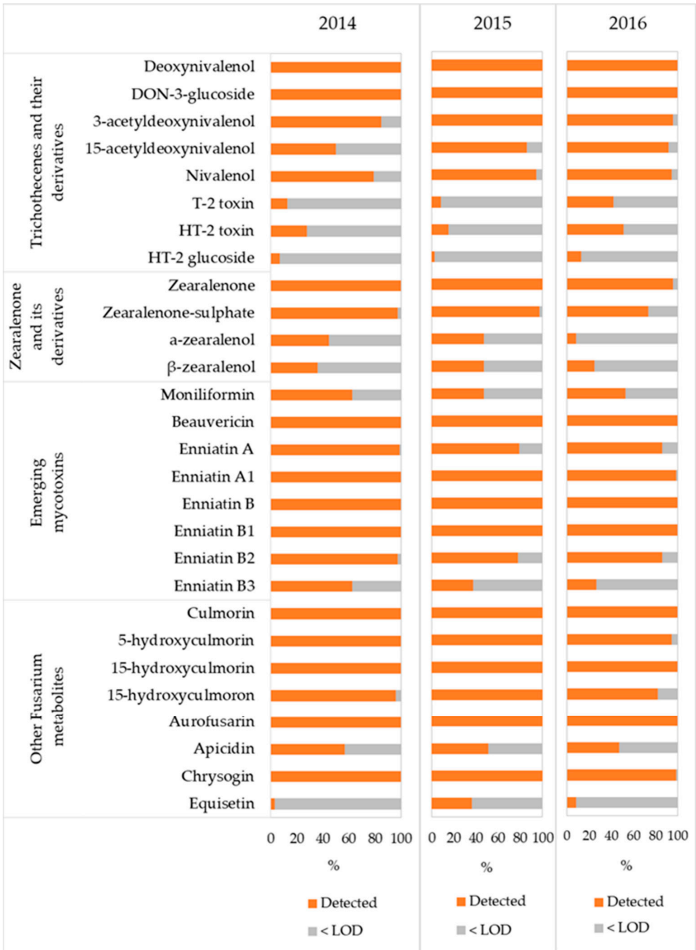

Figure 3. Percentage of detected mycotoxins by class in 72 analysed wheat samples (36 genotypes x 2 replicates) in 2014, 2015 and 2016 growing seasons. The Microsoft Excel Spreadsheet Software was used to create figure.

3.1. Fusarium Head Blight Assessment

Mean disease severity, measured by area under the disease progress curve (AUDPC) for general resistance and Fusarium damaged kernels (FDK) of 36 wheat genotypes varied over a wide range in all three years of the study (Supplementary Table S1). Values of AUDPC for wheat genotypes ranged from 11 to 710, 4 to 860, and 7 to 810 in 2014, 2015, and 2016, respectively, with the corresponding mean values across genotypes of 255, 284, and 276. FDK values also varied widely among genotypes, ranging from 0.6 to 44.8%, 0.3 to 64.3% and 1.3 to 56.1% in 2014, 2015, and 2016, respectively, with mean FDK values across all genotypes of 11.3% in 2014 and approximately twice as high in 2015 and 2016 (21.1% and 22.2%, respectively).

3.2. Trithothecenes and Their Derivatives

Among all trichothecenes detected in analysed wheat samples the greatest concentration in all three investigated years was observed for DON (found in 100% of the wheat samples) with mean values of 3244.7, 5379.8 and 6742.8 μg kg^{-1} in 2014, 2015 and 2016, respectively (Figure 3, Supplementary Table S2). Considering the means of genotypes over three years, 34/36 of means exceeded the maximum permitted level of DON by legislation (1250.0 μg kg^{-1}), with 12,374.6 μg kg^{-1} being the maximal level found. All wheat samples were contaminated with D3G while the means of each genotype in three years varied between 70.4 and 733.9 μg kg^{-1} (Figure 3, Supplementary Table S2). Similar to concentration of DON, the mean concentration of D3G was higher in the last two years of investigation (2014–297.6 μg kg^{-1}, 2015–375.9 μg kg^{-1}, 2016–565.9 μg kg^{-1}). 3-acetyl-deoxynivalenol (3-ADON) was detected in 85, 100 and 96% of wheat samples, in 2014, 2015 and 2016, respectively, with a minimum of only 4.8 μg kg^{-1}, and a maximum of 144.2 μg kg^{-1}. Nevertheless, mean values of 3-ADON in a particular year did not exceed 90.0 μg kg^{-1} (Figure 3, Supplementary Table S2).

Percentage of positive samples with 15-acetyl-deoxynivalenol (15-ADON) were 50, 86 and 92% in 2014, 2015 and 2016, respectively (Figure 3), with the highest observed concentration of 380.7 μg kg^{-1}, but with the highest mean levels in last two years of investigation. Percentage of positive samples contaminated with NIV were 79, 94 and 94% in the three consecutive years with a minimum level of 5.0 μg kg^{-1} and a maximum level of 220.2 μg kg^{-1}, and the highest mean concentration of 66.5 μg kg^{-1} observed in 2016 (Supplementary Table S2).

T-2, HT-2 and HT-2 glucoside toxins had a lesser occurrence because only 13, 8 and 42% of samples contained T-2 toxin, 28, 15 and 51% of samples contained HT-2 toxin, and 7, 3 and 13% of samples contained HT-2 glucoside toxin, in 2014, 2015, and 2016 crop season, respectively. The lowest concentration of 0.4 μg kg^{-1} was detected for T-2 toxin, while the highest concentration of 40.2 μg kg^{-1} was found for HT-2 toxin (Figure 3, Supplementary Table S2).

3.3. Zearelenone, Zearelenone-Sulphate, α- and β-Zearalenol

Zearalenone (ZEN) was present in 100, 100 and 96% of the samples, while ZEN-sulphate was present in 97, 97 and 74% samples, respectively, during the three crop seasons, with mean values of genotypes of 49.3, 49.4 and 23.6 μg kg^{-1} in 2014, 2015 and 2016, respectively (Figure 3, Supplementary Table S3). The minimum mean levels of ZEN and ZEN-sulphate in investigated wheat genotypes were 1.7 and 2.6 μg kg^{-1}, while the maximum mean values were 194.9 and 797.9 μg kg^{-1} in three investigated years. α-zearalenol was present in 44, 47 and 8% of samples, and β-zearalenol in 36, 47 and 25% of samples in 2014, 2015 and 2016, respectively, with the lowest mean value of genotypes detected in 2016 (Figure 3, Supplementary Table S3). The highest mean values of α- and β-zearalenol for genotypes were 13.4 and 10.1 μg kg^{-1}, respectively.

3.4. Moniliformin, Beauvericin and Enniatins

The rates of contamination with MON were 63, 47 and 53% in set of 216 samples, while BEA was observed in 100% of samples during three crop seasons (Figure 3, Supplementary Table S4). The highest mean concentration of MON was 65.2 μg kg^{-1}, while BEA did not exceed 8.0 μg kg^{-1}. ENN A was present in samples in a rate of 99, 79 and 86% during three years. However, all samples were contaminated with ENN A1, ENN B and ENN B1 in a rate of 100%, except in 2016 where ENN A1 was present in 99% samples (Figure 3). The rates of contamination with ENN B2 were 97, 78 and 86%, respectively, while ENN B3 was present in 63, 38 and 26% of samples during three years, respectively (Figure 3). The highest mean value of 242.6 μg kg^{-1} in genotypes was observed for ENN B1, and further 128.3 μg kg^{-1} for ENN B and 120.2 μg kg^{-1} for ENN A1. The rank order of mean values of these mycotoxins during investigated years was as follow: 67.7 > 26.3 > 22.5 μg kg^{-1} for ENN B1, 35.4 > 19.9 > 14.5 μg kg^{-1} for ENN B, 33.3 > 10.0 > 10.3 μg kg^{-1} for ENN A1, in 2014, 2015 and 2016, respectively (Supplementary Table S4).

3.5. Other Fusarium Metabolites

All wheat samples (n = 216) were contaminated with culmorin, 15-hydroxyculmorin, aurofusarin and chrysogin in a rate of 100%, except in 2016 where chrysogin contamination was 99% (Figure 3). Mean concentration in three investigated years of culmorin ranged between 295.5 and 3157.2 μg kg^{-1}; and further 369.4 and 9011.7 μg kg^{-1}, 106.5 and 17,138.0 μg kg^{-1}, 11.3 and 234.9 μg kg^{-1} for 15-hydroxyculmorin, aurofusarin and chrysogin, respectively. In 2014 culmorin had the mean value of 1257.1 μg kg^{-1}, while in 2015 and 2016 mean values increased to 1570.3 and 2451.8 μg kg^{-1} (Supplementary Table S5). 15-hydroxyculmorin and chrysogin showed the highest mean concentration in 2015 (4347.1 μg kg^{-1} and 125.8 μg kg^{-1}), while aurofusarin had the highest concentration of 8214.4 μg kg^{-1} in 2016. 5-hydroxyculmorin was present in 2016 with a rate of 94%, while 15-hydroxyculmoron was present in 2014 and 2016 with the rates of 96 and 82% in 216 samples. The maximum mean values of 5-hydroxyculmorin and 15-hydroxyculmoron were 5550.0 and 1204.1 μg kg^{-1}. Apicidin was observed in 57, 51 and 47% of samples, while equisetin was found in much lower number of samples (3, 36 and 8%) in the three investigated years (Figure 3). The highest mean values of apicidin and equisetin in genotypes were 30.6 and 1.7 μg kg^{-1} (Supplementary Table S5).

3.6. Co-Occurrence and Correlation of Fusarium Mycotoxins/Metabolites

Very high co-occurrence of mycotoxins has been observed in all investigated wheat samples (Supplementary Tables S2–S5). Further, correlation analysis based on 36 genotypic means over two replicates and three years were performed to show relationships among *Fusarium* severity traits (AUDPC for general resistance and FDK) and *Fusarium* mycotoxins/metabolites (Figure 4, Supplementary Table S6). A strong positive correlation (0.93) was found between AUDPC for general resistance and FDK. Significant positive correlations were also observed between the two measures of FHB severity and the levels of all mycotoxins except equisetin, and were in most cases higher for FDK than for AUDPC for general resistance. Both AUDPC for general resistance and FDK showed the strongest correlations (r > 0.90) with DON and its derivatives, zearalenone, and four other *Fusarium* metabolites. Correlations between 28 metabolites/mycotoxins ranged from moderate to very strong in most cases, except in the case of equisetin, the only *Fusarium* metabolite without any significant correlation with other metabolites/mycotoxins. The most distinguished positive correlations were observed between DON and its derivatives, as well as between DON and its derivatives with aurofusarin and culmorin and its derivatives, NIV, T-2 and HT-2 toxins with BEA and ENNs, while chrysogin showed the strongest positive correlations with DON, ZEN and culmorin and their derivatives.

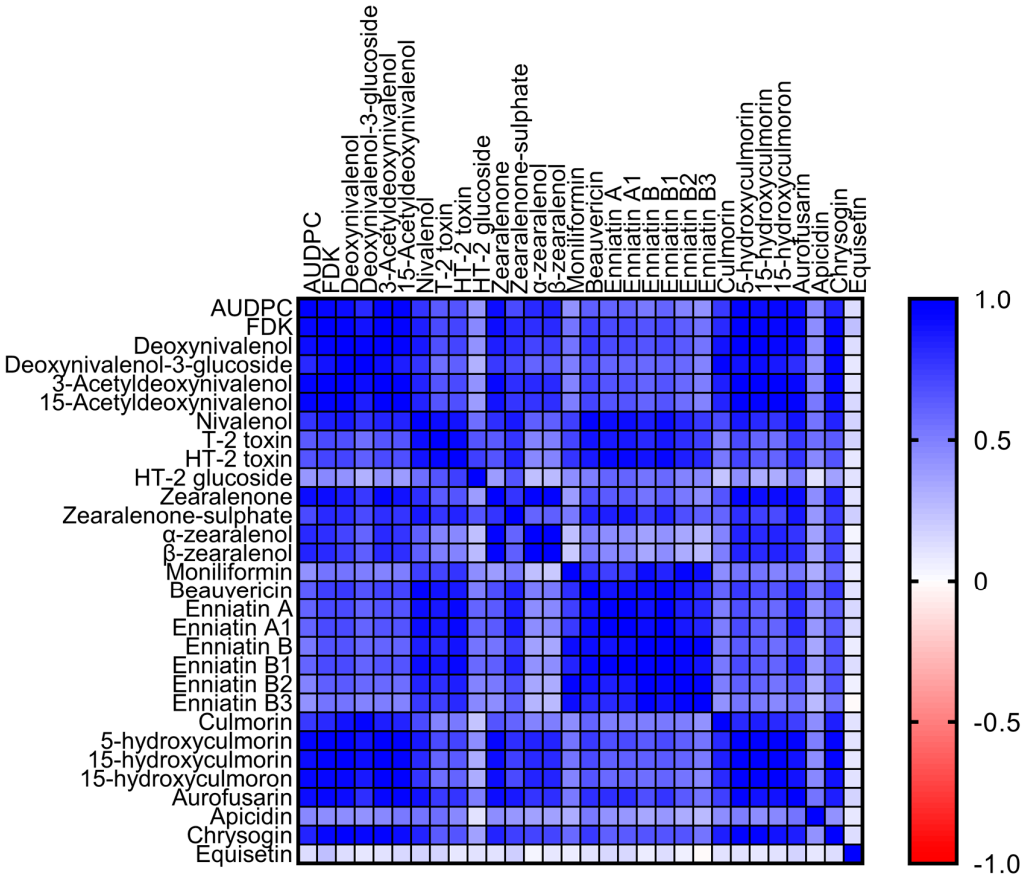

Figure 4. Heatmap presenting Pearson correlation matrix of area under the disease progress curve (AUDPC), *Fusarium* damaged kernels (FDK) and 28 *Fusarium* mycotoxins/metabolites in 36 wheat genotypes. Pearson correlation r values were determined using GraphPad Prism 9.4.1, San Diego, CA, USA. Colors are added for better visualization. The colors span from dark blue to dark red, where dark blue denotes a r value of 1, and dark red indicates a r value of −1.

4. Discussion

Fusarium spp. are fungi that can produce mycotoxins, that pose a potential danger for humans and animals as they can be found in a great variety of food and feed products [44]. The severity of FHB during a given crop season depends on precipitation during wheat flowering, whereas increased levels of *Fusarium* mycotoxins are often observed in seasons with frequent rainfall and high humidity. In the current research the influence of the genotype and year was significant for all *Fusarium* metabolites/mycotoxins, except the influence of genotype for T-2 toxin, HT-2 glucoside, ENN B3 and apicidin, and except the influence of year for HT-2 glucoside, MON and equisetin. Only nine metabolites had significant G × Y interaction. A significant year effect on the concentration of most mycotoxins/metabolites in the current study could be due to observed differences in the precipitation and temperatures, as prevalent factors that have an important effect on *Fusarium* infection, as it has been already observed in previous studies [1,45]. Stanciu et al. [13] suggested that precipitation levels influence fungi and mycotoxin development to a greater extent, compared to temperatures. Previous research showed that FHB susceptible wheat varieties are characterized by a much greater accumulation of DON than the resistant varieties [1]. Further, similarly to results of the current study, previously it was reported that DON significantly correlated with other investigated mycotoxins, and therefore it can be concluded that DON content can be used in the selection of FHB resistant genotypes resulting in lower total toxicity [6,37]. Furthermore, in the current research the level of FHB severity and FDK correlated well with mycotoxins present in the grain, but there was no significant correlation observed for equisetin. It was evident that more FHB infected grains

(with increased % of FDK) were shrivelled and discoloured and thus associated with higher mycotoxin concentrations.

4.1. Trichothecenes and Their Modified Forms

The group with trichothecenes is comprised from large family of structurally related mycotoxins produced by various *Fusarium* species [46]. DON, NIV, 3-ADON, 15-ADON, and fusarenon-X are included in type-B trichothecenes, while type-A group is comprised of T-2 and HT-2 toxin, diacetoxyscirpenol and neosolaniol [47] most of which were detected in wheat samples in the current study. Mycotoxins belonging to type-B trichothecenes group are resistant to milling, processing and heating which results in entrance of these mycotoxins in the food [48]. In the current research, acetylated fungal derivatives 3-ADON and 15-ADON as well as the derived D3G were detected in high occurrence in the most of investigated wheat samples. Similar results were obtained in previous research of Spanic et al. [4]. In the current research, the mixture of four aggressive *F. graminearum* species was used for inoculation, whereas previously it was reported that *F. graminearum* was found to produce three forms of DON (namely DON, 3-ADON and 15-ADON) [49]. Also, this is supported by the fact that DON and its derivatives were significantly positively correlated.

One of the most important type-B trichothecene is DON, being the most prevalent contaminant of cereals and end-use products [50]. In the current research, DON and its derivative forms were one of the most abundant in trichothecenes' group. Similarly, in the study of Nathanail et al. [51] DON and its glucosylated form were found in 93 and 81% of the samples. Concentrations of DON were unusually high in 2015 and 2016, due to increased precipitation in May, thus exceeding the maximum legislative limits for unprocessed wheat grains placed on the market for first-stage processing. Mean concentrations of D3G were not as high as for DON, but still this masked mycotoxin is representing huge concern. On the opposite to the current research, it was reported that D3G concentrations even exceeded those of DON [52]. During some processing, such as malting, DON was successfully converted into D3G [53]. Further problem with D3G is his hydrolysis during mammalian digestion that will return D3G in toxic precursor DON [52]. Thus, the increase in toxicity may occur directly or indirectly by transformation to the parental form of mycotoxin during digestion in the gastrointestinal system [18]. Ovando-Martínez et al. [24] reported that at higher DON concentration, a decrease in the D3G content occurs. On the opposite, in correlation matrix of the current research, D3G and DON were significantly positively correlated. This discrepancy could arise from different sets of genotypes as well as different environmental conditions in the two studies, and in the case of D3G production could be partly explained by the significant genotype × environment interaction observed in the current study. Metabolites such as 3-ADON and D3G were characterised by significantly lower toxicity then DON [54]. On the other hand, 15-ADON is the only derivative of DON whose toxicity is comparable with DON [55]. The levels of 3-ADON and 15-ADON were increased in wheat samples during last two years of investigation (2015 and 2016). In these two years the amount of precipitation during flowering period in May was higher compared to 2014, probably resulting in increased level of 3-ADON and 15-ADON. So, it can be concluded that the DON and its derivatives, dependent on the growing conditions in a particular season, were also significantly affected by the wheat genotype, but only 15-ADON and D3G showed significant G x Y interaction. 15-ADON and D3G should be taken into account in terms of food safety because D3G as "masked" mycotoxin can be converted into DON while 15-ADON is more toxic than DON. Although, another trichothecene B, NIV, is not usually produced in high concentrations, oxidative stress and toxicity induced by NIV contamination are higher than that described for DON [56]. NIV is also very dangerous mycotoxin because it can have damaging effects on mammals through immunotoxicity and hemotoxicity [57]. Further, these authors found similar concentration of NIV in cereals and their products (107.2 μg kg^{-1}), as can be seen in the current research. It was expected to find NIV in samples in increased occurrence in all

samples, because it is an accompanying mycotoxin of DON, as isolates of *Fusarium* spp. that produce DON, also produce NIV [49].

In the current research the occurrence of type-A trichothecenes T-2 and HT-2 toxin and its derivative was lower, compared to other trichothecenes and their modified forms. Similarly, it was concluded for T-2 and HT-2 toxin for maize kernels where they were significantly less common, compared to other toxins produced by *Fusarium* spp. [58]. T-2 toxin can be metabolized into HT-2 toxin and thus the toxicity of T-2 might partly be attributed to HT-2 [59]. Somewhat increased occurrence of T-2 and HT-2 toxin was found in the last year of investigation that could be influenced by more or less equal distribution of rainfall in May and June, before and after flowering. This is in accordance with the results of Hjelkrem et al. [60] who reported that HT-2 + T-2 contamination in oats was influenced by weather conditions both pre- and post-flowering.

4.2. Zearalenone and Its Derivatives

The main characteristic of ZEN is its estrogenic activity causing reproductive disorders in both humans and animals [61]. ZEN was identified in 100% of wheat samples during the first two years of our investigation; whereas its presence was significantly affected by genotype and year. Similar concentrations of ZEN as in the current research were observed by Tan et al. [62] who found that ZEN in maize kernel samples ranged from <LOD to 163.58 μg kg^{-1}. In the current research, the maximum concentration of that toxin in some genotypes exceeded permitted levels while ZEN-sulphate maximum level was 4-fold higher then ZEN's. This could be very hazardous, as for example, ZEN-14-sulphate was produced by *F. graminearum* but yet with unknown toxic effects [63]. Anyway, the sum of ZEN and its modified forms should be taken into account in the health risk management. This is supported by the observation of Gonzalez Pereyra et al. [64] were the presence of highly oestrogenic metabolites, like α-zearalenol and the masked ZEN-4-sulphate, increased the overall toxicity of ZEN contaminated silage. However, glucosylated masked forms of ZEN were not detected in the current research. Therefore, in investigated genotypes they do not represent dangerous, but in other cases they can be very unsafe as they are unstable in the digestive tract of mammals and could formed the main form ZEN [65]. In contrast to DON, ZEN had the highest mean value across genotypes in 2014, and was significantly affected by year but not by G × Y interaction. In opposite to our results, Vogelgsang et al. [66] demonstrated that year had a highly significant effect on both the DON contamination rate and the average content, however no effect of year was observed for ZEN or NIV. Potential risk is also hiding in the fact that ZEN gets synthetized during the malting [53] but also its thermostability is potential danger [67]. Previously, Abid-Essefi et al. [68] reported that toxic effects seem to be relieved by the metabolism of ZEN into α-zearalenol and β-zearalenol. In the current study, the rates and levels of contamination with reduced forms of ZEN, α- and β-zearalenol toxins were low, thus not contributing to toxicity of ZEN.

4.3. Emerging Mycotoxins

In recent years, special attention has been dedicated to so-called "emerging mycotoxins". The huge problem is that there is currently no legislation that would regulate the content of compounds from the group of emerging mycotoxins. Beside MON, BEA, ENNs and FUP, more *Fusarium* metabolites with toxicity falls in the category of emerging mycotoxins. In the current research, MON, BEA and ENNs compounds were found in all investigated wheat samples in all three years. MON was represented with more than 60% in 2014 and somewhat to lesser extent in last two years of investigation, while BEA, ENN B and ENN B1 were present in all investigated years in 100% samples. MON is a mycotoxin that can disrupt the Krebs cycle and cause adenosine triphosphate (ATP) deficiency, causing muscle weakness, heart and respiratory failure in animals [58]. Hietaniemi et al. [69] reported a mean level of 190 μg kg^{-1} and a maximum level of 850 μg kg^{-1} for MON in cereals from Finland. In our study, maximum levels of MON were much lower, where in three years, average value among genotypes did not exceed 66 μg kg^{-1}. Observed differences

among studies could be the consequence of different *Fusarium* spp. present in inoculum, whereas *F. proliferatum* was reported as main producer of MON [70]. BEA is mycotoxin structurally similar to the ENNs, but differs in the nature of the N-methylamino acid, and induces programmed cell death [71]. This toxin is also involved in antimicrobial and antibiotic activities [72]. The same authors described ENNs with cytotoxic activities and genotoxicity, while on the other side exhibiting antifungal and antimicrobial activities. Both, ENNs and BEA, have cytotoxic effects as a result of the induction of oxidative stress [73,74]. In the current research, ENN B1, ENN B and ENN A1 were found in relatively higher concentrations as compared to other "emerging" mycotoxins. Similar results were reported by Reisinger et al. [75] who found that ENN B and ENN B1 were the most abundant with median concentrations of 7 and 6 μg kg^{-1}, respectively, and maximum concentrations of 429 and 555 μg kg^{-1}, respectively. Maximum reported concentrations for BEA in grains and in cereal-based food were 6400.0 and 844.0 μg kg^{-1}, respectively [76]. This was much higher than it was found in the current study, where maximum mean value of BEA in three years was 8.0 μg kg^{-1}. This concentration is much closer to median concentration of 9 μg kg^{-1} found in maize silages [75]. As can be seen from previous studies, all these emerging mycotoxins pose a certain danger for human and animals, and that is why the investigations of their content and occurrence in wheat must not be neglected. Emerging mycotoxins of *Fusarium* spp. with their increased concentrations in possible epidemic conditions should be of concern to official food control authorities and should be incorporated in future legislation.

4.4. Other Fusarium Metabolites

In last few years' metabolite culmorin was also assigned in the group of "emerging mycotoxins" that usually comes with trichothecene mycotoxins thus influencing their toxicity [77]. In the current study mean highest concentrations for culmorin exceeded 2.5 fold the maximal permitted level for DON (250.0 μg kg^{-1}). For 5-hydroxyculmorin and hydroxyculmorin the permitted level was exceeded 4.4 and 7.3 fold, respectively, while for 15-hydroxyculmoron maximal concentration was at the permitted level for DON. Uhlig et al. [78] observed that in natural conditions the concentration of culmorin was about 3-fold higher than concentration of DON. It was previously reported that mixtures of culmorin with DON, 3-ADON, 15-ADON, or NX-3, but not with NIV, inhibited growth of wheat roots in a synergistic manner [79]. It is important to note that culmorin and DON are likely characterised by synergistic toxicity [80]. Culmorin and its derivatives were highly represented in wheat samples in each investigated year in the current study. Results of Streit et al. [81] showed a high occurrence of other *Fusarium* metabolites in natural conditions, where 63, 63, 13, and 7% of feed and feed raw materials (n = 83) was positive for culmorin, 15-hydroxyculmorin, 5-hydroxyculmorin, and 15-hydroxyculmorone. In the study of Spanic et al. [4] aurofusarin was detected in the range of 735.0 to 63,098.0 μg kg^{-1} when significantly fewer samples were investigated than in the current research. In the present study maximum value of aurofusarin was 3.7 fold lower, compared to research of Spanic et al. [4], probably due to different wheat genotypes or *Fusarium* isolates used in these two studies. Recently, it has been reported on the possible induction of oxidative stress by aurofusarin [82]. Sunic et al. [6] reported about connection between production of major *Fusarium* mycotoxins and pigments. However, the available literature data concerning aurofusarin is limited, and further research is needed for better understanding of occurrence and levels of aurofusarin in wheat samples. Chrysogin is *Fusarium* metabolite previously reported in the concentration up to 1320 μg kg^{-1} [6] in contrast to the current study where occurrence was high, but concentrations were 5.6 fold lower. Compared to the concentration of aurofusarin, apicidin had lower mean concentrations in wheat samples in the present study and no significant differences in apicidin concentration among 36 wheat genotypes were found. Although apicidin was detected in low concentration in the current study, we need to be careful with this metabolite as previously Khoshal et al. [83] ranked apicidin as the most toxic as can be seen from their ranking of metabolites acording to order of

toxicity: apicidin > enniatin A1 > DON > beauvericin > enniatin B > enniatin B1 > emodin > aurofusarin. Equisetin was the only metabolite investigated in the present study showing no correlations with other mycotoxins/metabolites and with very low concentrations. Similarly, low occurrence of equisetin was previously found by Spanic et al. [25].

5. Conclusions

The co-occurrence of several mycotoxins/metabolites under potentially FHB epidemic conditions in individual samples confirms the importance of using credible analytical methods for monitoring of *Fusarium* mycotoxins/metabolites in wheat. This is especially important in food risk assessment as most of them are showing synergic or additive effects, and as we observed they are significantly positively correlated. The amount of mycotoxins in wheat grains can be decreased by utilization of FHB resistant genotypes. Also, our results underline the potential of *F. graminearum* to produce multi-mycotoxins simultaneously under the influence of various factors related to the genotype or the environment. In conclusion, both the crop season and genotype significantly affected the levels of mycotoxins/metabolites in wheat grain in response to *Fusarium* infection. Significant differences in the contamination pattern were observed among years for all mycotoxins, except for HT-2 glucoside, MON and equisetin. The differing levels of mycotoxins in three investigated years may be a result of different precipitation patterns among years. In 2016 an equal distribution of precipitations across May and June increased the occurrence of trichothecenes, and decreased the occurrence of ZEN and its derivatives. Special attention needs to be given to masked and emerging mycotoxins, as in the current study they incidence was high in all three investigated years. Mycotoxin content in wheat should be monitored continuously, as the annual levels may vary depending on rainfall and temperature changes, wheat variety type etc.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13030805/s1, Figure S1: Mean values of AUDPC for general resistance and Fusarium damaged kernels (FDK); Table S1: Area under the disease progress curve (AUDPC) for general resistance and Fusarium damaged kernels (FDK) of 25 wheat genotypes in 2014, 2015 and 2016; Table S2: Mean values of deoxynivalenol and its derivatives, nivalenol, T-2 and HT-2 toxin and its derivative in three years; Table S3: Mean values of zearelenone, zearelenone-sulphate, α- and β-zearalenol in three years; Table S4: Mean values of moniliformin, beauvericin and enniatins in three years; Table S5: Mean values of other Fusarium metabolites in three years; Table S6: Pearson's correlation coefficients between AUDPC for general resistance, Fusarium damaged kernels (FDK) and concentrations of mycotoxins/metabolites in wheat samples.

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References

1. Spanic, V.; Marcek, T.; Abicic, I.; Sarkanj, B. Effects of Fusarium head blight on wheat grain and malt infected by *Fusarium culmorum*. *Toxins* **2018**, *10*, 17. [CrossRef]
2. Zhang, W.; Boyle, K.; Brûlé-Babel, A.L.; Fedak, G.; Gao, P.; Robleh Djama, Z.; Polley, B.; Cuthbert, R.D.; Randhawa, H.S.; Jiang, F.; et al. Genetic characterization of multiple components contributing to Fusarium Head Blight Resistance of FL62R1, a canadian bread wheat developed using systemic breeding. *Front. Plant Sci.* **2020**, *26*, 11–580833. [CrossRef] [PubMed]

3. Mesterhazy, A.; Toth, B.; Varga, M.; Bartok, T.; Szabo-Hever, A.; Farady, L.; Lehoczki-Krsjak, S. Role of fungicides, application of nozzle types, and the resistance level of wheat varieties in the control of Fusarium head blight and deoxynivalenol. *Toxins* **2011**, *3*, 1453–1483. [CrossRef] [PubMed]
4. Spanic, V.; Viljevac Vuletic, M.; Horvat, D.; Sarkanj, B.; Drezner, G.; Zdunic, Z. Changes in antioxidant system during grain development of wheat (*Triticum aestivum* L.) and relationship with protein composition under FHB stress. *Pathogens* **2020**, *9*, 17. [CrossRef]
5. Wilde, F.; Miedaner, T. Selection for Fusarium head blight resistance in early generations reduces the deoxynivalenol (DON) content in grain of winter and spring wheat. *Plant Breed* **2006**, *125*, 96–98. [CrossRef]
6. Sunic, K.; Kovac, T.; Loncaric, A.; Babic, J.; Sulyok, M.; Krska, R.; Drezner, G.; Spanic, V. *Fusarium* secondary metabolite content in naturally produced and artificially provoked FHB pressure in winter wheat. *Agronomy* **2021**, *11*, 2239. [CrossRef]
7. Bushnell, W.R.; Hazen, B.E.; Pritsch, C. Histology and physiology of Fusarium head blight. In *Fusarium Head Blight of Wheat and Barley*, 1st ed.; Leonard, K.J., Bushnell, W.R., Eds.; The American Phytopathological Society: St. Paul, MN, USA, 2003; Chapter 3; pp. 44–83.
8. Rodrigues, I.; Naehrer, K. A three-year survey on the worldwide occurrence of mycotoxins in feedstuffs and feed. *Toxins* **2012**, *4*, 663–675. [CrossRef]
9. Cheli, F.; Pinotti, L.; Rossi, L.; Dell'Orto, V. Effect of milling procedures on mycotoxin distribution in wheat fractions: A review. *LWT Food Sci. Technol.* **2013**, *54*, 307–314. [CrossRef]
10. Shank, R.A.; Foroud, N.A.; Hazendonk, P.; Eudes, F.; Blackwell, B.A. Current and future experimental strategies for structural analysis of trichothecene mycotoxins—A prospectus. *Toxins* **2011**, *3*, 1518–1553. [CrossRef]
11. Bennett, J.W.; Klich, M. Mycotoxins. *Clin. Microbiol. Rev.* **2003**, *16*, 497–516. [CrossRef]
12. Lemos, A.C.; de Borba, V.S.; Badiale-Furlong, E. The impact of wheat-based food processing on the level of trichothecenes and their modified forms. *Trends Food Sci. Technol.* **2021**, *111*, 89–99. [CrossRef]
13. Stanciu, O.; Juan, C.; Berrada, H.; Miere, D.; Loghin, F.; Mañes, J. Study on trichothecene and zearalenone presence in Romanian wheat relative to weather conditions. *Toxins* **2019**, *11*, 163. [CrossRef]
14. Commission Regulation (EC) No 1881/2006 of 19 December 2006 on the Setting Maximum Levels for Certain Contaminants in Foodstuffs. Available online: https://eur-lex.europa.eu/legal-content/EN/ALL/?uri=CELEX%3A32006R1881 (accessed on 28 January 2023).
15. Commission Recommendation (ER) No 2013/165/EU of 27 March 2013 on the Presence of T-2 and HT-2 Toxin in Cereals and Cereal Products. Available online: https://eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX%3A32013H0165 (accessed on 28 January 2023).
16. EFSA. Panel on Contaminants in the Food Chain. Scientific Opinion on risks for animal and public health related to the presence of nivalenol in food and feed. *EFSA J* **2013**, *11*, 3262. [CrossRef]
17. Berthiller, F.; Crews, C.; Dall'Asta, C.; De Saeger, S.; Haesaert, G.; Karlovsky, P.; Oswald, I.P.; Seefelder, W.; Speijers, G.; Strokaet, J. Masked mycotoxins: A review. *Mol. Nutr. Food Res.* **2013**, *57*, 165–186. [CrossRef] [PubMed]
18. Ekwomadu, T.I.; Akinola, S.A.; Mwanza, M. *Fusarium* mycotoxins, their metabolites (free, emerging, and masked), food safety concerns, and health impacts. *Int. J. Environ. Res. Public Health* **2021**, *18*, 11741. [CrossRef]
19. Broekaert, N.; Devreese, M.; de Baere, S.; de Backer, P.; Croubels, S. Modified *Fusarium* mycotoxins unmasked: From occurrence in cereals to animal and human excretion. *Food Chem. Toxicol.* **2015**, *80*, 17–31. [CrossRef]
20. Iqbal, S.Z. Mycotoxins in food, recent development in food analysis and future challenges: A review. *Curr. Opin. Food Sci.* **2021**, *42*, 237–247. [CrossRef]
21. Berthiller, F.; Dall'Asta, C.; Schuhmacher, R.; Lemmens, M.; Adam, G.; Krska, R. Masked mycotoxins: Determination of a deoxynivalenolglucoside in artificially and naturally contaminated wheat by liquid chromatography—Tandem mass spectrometry. *J. Agric. Food Chem.* **2005**, *53*, 3421–3425. [CrossRef]
22. Borzekowski, A.; Drewitz, T.; Keller, J.; Pfeifer, D.; Kunte, H.J.; Koch, M.; Rohn, S.; Maul, R. Biosynthesis and characterization of zearalenone-14-sulfate, zearalenone-14-glucoside and zearalenone-16-glucoside Using Common Fungal Strains. *Toxins* **2018**, *10*, 104. [CrossRef]
23. Lee, S.Y.; Woo, S.Y.; Tian, F.; Song, J.; Michlmayr, H.; Kim, J.B.; Chun, H.S. Occurrence of deoxynivalenol, nivalenol, and their glucosides in Korean market foods and estimation of their population exposure through food consumption. *Toxins* **2020**, *12*, 89. [CrossRef]
24. Ovando-Martínez, M.; Ozsisli, B.; Anderson, J.; Whitney, K.; Ohm, J.B.; Simsek, S. Analysis of deoxynivalenol and deoxynivalenol-3-glucoside in hard red spring wheat inoculated with *Fusarium Graminearum*. *Toxins* **2013**, *5*, 2522–2532. [CrossRef] [PubMed]
25. Spanic, V.; Katanic, Z.; Sulyok, M.; Krska, R.; Puskas, K.; Vida, G.; Drezner, G.; Sarkanj, B. Multiple fungal metabolites including mycotoxin formation in naturally infected and Fusarium inoculated wheat samples. *Microorganisms* **2020**, *8*, 578. [CrossRef]
26. Nakagawa, H.; Ohmichi, K.; Sakamoto, S.; Sago, Y.; Kushiro, M.; Nagashima, H.; Yoshida, M.; Nakajima, T. Detection of a new Fusarium masked mycotoxin in wheat grain by high-resolution LC–Orbitrap™ MS. *Food Addit. Contam. A* **2011**, *28*, 1447–1456. [CrossRef] [PubMed]
27. Prosperini, A.; Berrada, H.; Ruiz, M.J.; Caloni, F.; Coccini, T.; Spicer, L.J.; Perego, M.C.; Lafranconi, A. A Review of the mycotoxin enniatin B. *Front. Public Health* **2017**, *5*, 304. [CrossRef] [PubMed]

28. Fernandez-Blanco, C.; Font, G.; Ruiz, M.J. Interaction effects of enniatin B, deoxinivalenol and alternariol in Caco-2 cells. *Toxicol. Lett.* **2016**, *241*, 38–48. [CrossRef]
29. Nazari, F.; Sulyok, M.; Kobarfard, F.; Yazdanpanah, H.; Krska, R. Evaluation of emerging *Fusarium* mycotoxins beauvericin, enniatins, fusaproliferin and moniliformin in domestic rice in Iran. *Iran J. Pharm. Res.* **2015**, *14*, e125357. [CrossRef]
30. Wu, Q.; Patocka, J.; Nepovimova, E.; Kuca, K. A Review on the synthesis and bioactivity aspects of beauvericin, a Fusarium Mycotoxin. *Front. Pharmacol.* **2018**, *9*, 1338. [CrossRef]
31. Kuang, Q.-X.; Lei, L.-R.; Li, Q.-Z.; Peng, W.; Wang, Y.-M.; Dai, Y.-F.; Wang, D.; Gu, Y.-C.; Deng, Y.; Guo, D.-L. Investigation of the anti-inflammatory activity of fusaproliferin analogues guided by transcriptome analysis. *Front. Pharmacol.* **2022**, *13*, 881182. [CrossRef]
32. Ceranic, A.; Svoboda, T.; Berthiller, F.; Sulyok, M.; Samson, J.M.; Güldener, U.; Schuhmacher, R.; Adam, G. Identification and functional characterization of the gene cluster responsible for fusaproliferin biosynthesis in *Fusarium proliferatum*. *Toxins* **2021**, *13*, 468. [CrossRef]
33. Bertuzzi, T.; Giorni, P.; Rastelli, S.; Vaccino, P.; Lanzanova, C.; Locatelli, S. Co-Occurrence of moniliformin and regulated *Fusarium* toxins in maize and wheat grown in Italy. *Molecules* **2020**, *25*, 2440. [CrossRef]
34. Bouafifssa, Y.; Manyes, L.; Rahouti, M.; Mañes, J.; Berrada, H.; Zinedine, A.; Fernández-Franzón, M. Multi-occurrence of twenty mycotoxins in pasta and a risk assessment in the Moroccan population. *Toxins* **2018**, *10*, 432. [CrossRef] [PubMed]
35. Mahdjoubi, C.K.; Arroyo-Manzanares, N.; Hamini-Kadar, N.; García-Campaña, A.M.; Mebrouk, K.; Gámiz-Gracia, L. Multi-mycotoxin occurrence and exposure assessment approach in foodstuffs from Algeria. *Toxins* **2020**, *12*, 194. [CrossRef] [PubMed]
36. Siuda, R.; Grabowski, A.; Lenc, L.; Ralcewicz, M.; Spychaj-Fabisiak, E. Influence of the degree of fusariosis on technological traits of wheat grain. *Int. J. Food Sci. Technol.* **2010**, *45*, 2596–2604. [CrossRef]
37. Spanic, V.; Dvojkovic, K.; Babic, J.; Drezner, G.; Zdunic, Z. Fusarium head blight infestation in relation to winter wheat end-use quality—A three-year study. *Agronomy* **2021**, *11*, 1648. [CrossRef]
38. Malachova, A.; Dzuman, Z.; Veprikova, Z.; Vaclavikova, M.; Zachariasova, M.; Hajslova, J. Deoxynivalenol, deoxynivalenol-3-glucoside, and enniatins: The major mycotoxins found in cereal-based products on the Czech market. *J. Agric. Food Chem.* **2011**, *59*, 12990–12997. [CrossRef] [PubMed]
39. Ryu, D.; Hanna, M.A.; Bullerman, L.B. Stability of zearalenone during extrusion of corn grits. *J. Food Prot.* **1999**, *62*, 1482–1484. [CrossRef] [PubMed]
40. Desjardins, A.E.; Manandhar, G.; Plattner, R.D.; Maragos, C.M.; Shrestha, K.; McCormick, S.P. Occurrence of *Fusarium* species and mycotoxins in Nepalese maize and wheat and the effect of traditional processing methods on mycotoxin levels. *J. Agric. Food Chem.* **2000**, *48*, 1377–1383. [CrossRef]
41. Spanic, V.; Lemmens, M.; Drezner, G. Variability of components of fusarium head blight resistance among wheat genotypes. *Cereal. Res. Commun.* **2013**, *41*, 420–430. [CrossRef]
42. SAS Institute Inc. *SAS/STAT 9.2 Users Guide*; SAS Inc.: Cary, NC, USA, 2009.
43. Motulsky, H.J. *Analyzing Data with GraphPad Prism*; GraphPad Software Inc.: San Diego, CA, USA, 1999.
44. Awuchi, C.G.; Ondari, E.N.; Ogbonna, C.U.; Upadhyay, A.K.; Baran, K.; Okpala, C.O.R.; Korzeniowska, M.; Guiné, R.P.F. Mycotoxins affecting animals, foods, humans, and plants: Types, occurrence, toxicities, action mechanisms, prevention, and detoxification strategies—A Revisit. *Foods* **2021**, *10*, 1279. [CrossRef]
45. Wegulo, S. Factors influencing deoxinivalenol accumulation in small grain cereals. *Toxins* **2012**, *4*, 1157–1180. [CrossRef]
46. Alizadeh, A.; Braber, S.; Akbari, P.; Kraneveld, A.; Garssen, J.; Fink-Gremmels, J. Deoxynivalenol and its modified forms: Are there major differences? *Toxins* **2016**, *8*, 334. [CrossRef] [PubMed]
47. Toxicology and Occurrence of Nivalenol, Fusarenon X, Diacetoxyscirpenol, Neosolaniol and 3- and 15-Acetyldeoxynivalenol: A Review of Six Trichothecenes. RIVM Report 388802024/2002. Available online: https://www.rivm.nl/bibliotheek/rapporten/388802024.pdf (accessed on 15 January 2023).
48. Sugita-Konishi, Y.; Park, B.J.; Kobayashi-Hattori, K.; Tanaka, T.; Chonan, T.; Yoshikawa, K.; Kumagai, S. Effect of cooking process on the deoxynivalenol content and its subsequent cytotoxicity in wheat products. *Biosci. Biotechnol. Biochem.* **2006**, *70*, 1764–1768. [CrossRef]
49. Castañares, E.; Albuquerque, D.R.; Dinolfo, M.I.; Pinto, V.F.; Patriarca, A.; Stenglein, S.A. Trichothecene genotypes and production profiles of *Fusarium graminearum* isolates obtained from barley cultivated in Argentina. *Int. J. Food Microbiol.* **2014**, *179*, 57–63. [CrossRef]
50. Turner, P.C. Deoxynivalenol and nivalenol occurrence and exposure assessment. *World Mycotoxin J.* **2010**, *3*, 315–321. [CrossRef]
51. Nathanail, A.V.; Syvähuoko, J.; Malachová, A.; Jestoi, M.; Varga, E.; Michlmayr, H.; Adam, G.; Sieviläinen, E.; Berthiller, F.; Peltonen, K. Simultaneous determination of major type A and B trichothecenes, zearalenone and certain modified metabolites in Finnish cereal grains with a novel liquid chromatography-tandem mass spectrometric method. *Anal. Bioanal. Chem.* **2015**, *407*, 4745–4755. [CrossRef]
52. Berthiller, F.; Krska, R.; Domig, K.J.; Kneifel, W.; Juge, N.; Schuhmacher, R.; Adam, G. Hydrolytic fate of deoxynivalenol-3-glucoside during digestion. *Toxicol. Lett.* **2011**, *206*, 264–267. [CrossRef] [PubMed]
53. Spanic, V.; Zdunic, Z.; Drezner, G.; Sarkanj, B. The pressure of *Fusarium* disease and its relation with mycotoxins in the wheat grain and malt. *Toxins* **2019**, *11*, 198. [CrossRef]

54. He, Y.; Yin, X.; Dong, J.; Yang, Q.; Wu, Y.; Gong, Z. Transcriptome analysis of Caco-2 cells upon the exposure of mycotoxin deoxynivalenol and its acetylated derivatives. *Toxins* **2021**, *13*, 167. [CrossRef] [PubMed]
55. Springler, A.; Hessenberger, S.; Reisinger, N.; Kern, C.; Nagl, V.; Schatzmayr, G.; Mayer, E. Deoxynivalenol and its metabolite deepoxy-deoxynivalenol: Multi-parameter analysis for the evaluation of cytotoxicity and cellular effects. *Mycotoxin. Res.* **2017**, *33*, 25–37. [CrossRef] [PubMed]
56. Nagashima, H. Deoxynivalenol and nivalenol toxicities in cultured cells: A review of comparative studies. *Food Saf.* **2018**, *6*, 51–57. [CrossRef]
57. Zingales, V.; Fernández-Franzón, M.; Ruiz, M.-J. Occurrence, mitigation and in vitro cytotoxicity of nivalenol, a type B trichothecene mycotoxin–Updates from the last decade (2010–2020). *Food Chem. Toxicol.* **2021**, *152*, 112182. [CrossRef] [PubMed]
58. Bryła, M.; Pierzgalski, A.; Zapaśnik, A.; Uwineza, P.A.; Ksieniewicz-Woźniak, E.; Modrzewska, M.; Waśkiewicz, A. Recent research on *Fusarium* mycotoxins in maize-A Review. *Foods* **2022**, *11*, 3465. [CrossRef]
59. EFSA. Panel on Contaminants in the Food Chain. Appropriateness to set a group health based guidance value for T2 and HT2 toxin and its modified forms. *EFSA J.* **2017**, *15*, e04655. [CrossRef]
60. Hjelkrem, A.G.R.; Aamot, H.U.; Brodal, G.; Strand, E.C.; Torp, T.; Edwards, S.G.; Dill-Macky, R.; Hofgaard, I.S. HT-2 and T-2 toxins in Norwegian oat grains related to weather conditions at different growth stages. *Eur. J. Plant Pathol.* **2018**, *151*, 501–514. [CrossRef]
61. Poor, M.; Kunsagi-Mate, S.; Sali, N.; Koszegi, T.; Szente, L.; Peles-Lemli, B. Interactions of zearalenone with native and chemically modified cyclodextrins and their potential utilization. *J. Photochem. Photobiol. B* **2015**, *151*, 63–68. [CrossRef]
62. Tan, H.; Zhou, H.; Guo, T.; Zhang, Y.; Ma, L. Zein-bound zearalenone: A hidden mycotoxin found in maize and maize-products. *Food Control* **2021**, *124*, 107903. [CrossRef]
63. Keller, J.; Borzekowski, A.; Haase, H.; Menzel, R.; Rueß, L.; Koch, M. Toxicity assay for citrinin, zearalenone and zearalenone-14-sulfate using the nematode Caenorhabditis elegans as Model Organism. *Toxins* **2018**, *10*, 284. [CrossRef] [PubMed]
64. Gonzalez Pereyra, M.L.; Sulyok, M.; Baralla, V.; Dalcero, A.M.; Krska, R.; Chulze, S.N.; Cavaglieri, L.R. Evaluation of zearalenone, α-zearalenol, β-zearalenol, zearalenone 4-sulfate and β-zearalenol 4-glucoside levels during the ensiling process. *World Mycotoxin J.* **2014**, *7*, 291–295. [CrossRef]
65. Shkhaliyeva, I.; Teker, T.; Albayrak, G. Masked mycotoxins of deoxynivalenol and zearalenone—Unpredicted toxicity. *Biomed. J. Sci. Tech. Res.* **2020**, *29*, 22288–22293. [CrossRef]
66. Vogelgsang, S.; Musa, T.; Bänziger, I.; Kägi, A.; Bucheli, T.D.; Wettstein, F.E.; Pasquali, M.; Forrer, H.R. *Fusarium* mycotoxins in Swiss wheat: A survey of growers' samples between 2007 and 2014 shows strong year and minor geographic effects. *Toxins* **2017**, *9*, 246. [CrossRef]
67. Ben Salah-Abbès, J.; Belgacem, H.; Ezzdini, K.; Abdel-Wahhab, M.A.; Abbès, S. Zearalenone nephrotoxicity: DNA fragmentation, apoptotic gene expression and oxidative stress protected by *Lactobacillus plantarum* MON03. *Toxicon* **2020**, *175*, 28–35. [CrossRef]
68. Abid-Essefi, S.C.; Bouaziz, E.; El Golli-Bennour, Z.; Ouanes, H.B. Comparative study of toxic effects of zearalenone and its two major metabolites α-zearalenol and β-zearalenol on cultured human Caco-2 cells. *J. Biochem. Mol. Toxicol.* **2009**, *23*, 233–243. [CrossRef]
69. Hietaniemi, V.; Ramo, S.; Yli-Mattila, T.; Jestoi, M.; Peltonen, S.; Kartio, M.; Sievilainen, E.; Koivisto, T.; Parikka, P. Updated survey of *Fusarium* species and toxins in Finnish cereal grains. *Food Addit. Contam. A* **2016**, *33*, 831–848. [CrossRef]
70. Waśkiewicz, A.; Łukasz, S. Mycotoxins biosynthesized by plant-derived *Fusarium* isolates. *Arh. Hig. Rada Toksikol.* **2013**, *63*, 437–446. [CrossRef]
71. Logrieco, A.; Moretti, A.; Castella, G.; Kostecki, M.; Golinski, P.; Ritieni, A.; Chelkowski, J. Beauvericin production by *Fusarium* species. *Appl. Environ. Microbiol.* **1998**, *64*, 3084–3088. [CrossRef] [PubMed]
72. Křížová, L.; Dadáková, K.; Dvořáčková, M.; Kašparovský, T. Feedborne mycotoxins beauvericin and enniatins and livestock animals. *Toxins* **2021**, *13*, 32. [CrossRef] [PubMed]
73. Agahi, F.; Álvarez-Ortega, N.; Font, G.; Juan-García, A.; Juan, C. Oxidative stress, glutathione, and gene expression as key indicators in SH-SY5Y cells exposed to zearalenone metabolites and beauvericin. *Toxicol. Lett.* **2020**, *334*, 44–52. [CrossRef] [PubMed]
74. Cimbalo, A.; Alonso-Garrido, M.; Font, G.; Frangiamone, M.; Manyes, L. Transcriptional changes after enniatins A, A1, B and B1 ingestion in rat stomach, liver, kidney and lower intestine. *Foods* **2021**, *10*, 1630. [CrossRef]
75. Reisinger, N.; Schürer-Waldheim, S.; Mayer, E.; Debevere, S.; Antonissen, G.; Sulyok, M.; Nagl, V. Mycotoxin occurrence in maize silage—A neglected risk for bovine gut health? *Toxins* **2019**, *11*, 577. [CrossRef]
76. EFSA Panel on Contaminants in the Food Chain (CONTAM). Scientific Opinion on the risks to human and animal health related to the presence of beauvericin and enniatins in food and feed. *EFSA J.* **2014**, *12*, 3802. [CrossRef]
77. Weber, J.; Vaclavikova, M.; Wiesenberger, G.; Haider, M.; Hametner, C.; Fröhlich, J.; Berthiller, F.; Adam, G.; Mikula, H.; Fruhmann, P. Chemical synthesis of culmorin metabolites and their biologic role in culmorin and acetyl-culmorin treated wheat cells. *Org. Biomol. Chem.* **2018**, *16*, 2043–2048. [CrossRef] [PubMed]
78. Uhlig, S.; Eriksen, G.S.; Hofgaard, I.S.; Krska, R.; Beltrán, E.; Sulyok, M. Faces of a changing climate: Semi-quantitative multi-mycotoxin analysis of grain grown in exceptional climatic conditions in Norway. *Toxins* **2013**, *5*, 1682–1697. [CrossRef]
79. Wipfler, R.; McCormick, S.P.; Proctor, R.; Teresi, J.; Hao, G.; Ward, T.; Alexander, N.; Vaughan, M.M. Synergistic phytotoxic effects of culmorin and trichothecene mycotoxins. *Toxins* **2019**, *11*, 555. [CrossRef] [PubMed]

80. Woelflingseder, L.; Warth, B.; Vierheilig, I.; Schwartz-Zimmermann, H.; Hametner, C.; Nagl, V.; Novak, B.; Sarkanj, B.; Berthiller, F.; Adam, G. The Fusarium metabolite culmorin suppresses the in vitro glucuronidation of deoxynivalenol. *Arch. Toxicol.* **2019**, *93*, 1729–1743. [CrossRef] [PubMed]
81. Streit, E.; Schwab, C.; Sulyok, M.; Naehrer, K.; Krska, R.; Schatzmayr, G. Multi-mycotoxin screening reveals the occurrence of 139 different secondary metabolites in feed and feed ingredients. *Toxins* **2013**, *5*, 504–523. [CrossRef] [PubMed]
82. Jarolim, K.; Wolters, K.; Woelflingseder, L.; Pahlke, G.; Beisl, J.; Puntscher, H.; Braun, D.; Sulyok, M.; Warth, B.; Marko, D. The secondary *Fusarium* metabolite aurofusarin induces oxidative stress, cytotoxicity and genotoxicity in human colon cells. *Toxicol. Lett.* **2018**, *284*, 170–183. [CrossRef]
83. Khoshal, A.K.; Novak, B.; Martin, P.G.P.; Jenkins, T.; Neves, M.; Schatzmayr, G.; Oswald, I.P.; Pinton, P. Co-occurrence of DON and emerging mycotoxins in worldwide finished pig feed and their combined toxicity in intestinal cells. *Toxins* **2019**, *11*, 727. [CrossRef]