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ORIGINAL PAPER

Evaluation of fungal diversity and mycotoxin content in pig feed containing clusterbean (*Cyamopsis tetragonoloba*) meal*

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Abstract

The safety and quality of animal feed are often unknown concerning the content of fungi and mycotoxins. Animal feed may be infested with fungi, leading to contamination with mycotoxins, which pose a potential risk to human and animal health. Currently, the feeding of pigs is mainly based on cereals and soy as protein components. In order to reduce the cost of feed production, other protein sources are used in addition to soy. To assess the health of pig feeds containing guar meal in their composition, a study was conducted to evaluate the presence of toxigenic fungi (culture and qPCR method) and mycotoxins (HPLC). Eight complete feeds produced on the farm containing locally produced feed materials including soybean meal and guar meal as protein components were investigated. The results of the mycobiome analysis showed that the tested feed mixtures were characterised by a high diversity of fungi, with saprophytic species clearly dominating. The qPCR analysis confirmed the presence of *Fusarium* and *P. verrucosum* fungi in the mixtures. Chemical tests of mycotoxin content confirmed the presence of *Fusarium* mycotoxins: DON, 3-AcDON, ZEA, T2 and OTA. Barley grain and guar meal were responsible for the contamination of the feed with the toxins DON, 3-AcDON, ZEA and OTA, while in the case of the T2 toxins, wheat grain and to a lesser extent the premix were responsible. The above correlations were confirmed by a correlation analysis that took into account the different levels of the feed components. Overall, it should be emphasised that the feeds were contaminated with mycotoxins to a low degree, which confirms their high quality. The storage conditions favoured the development of saprotrophic mycoflora and prevented the development of toxigenic species.

Keywords: DON, OTA, T2, qPCR, HPLC

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INTRODUCTION

The quality control and safety of animal feeds is important for the welfare and health of animals and consequently for consumers. The feeding of monogastric animals is based on cereals, which are often home-grown, and on protein components, hence their contamination with mycotoxins and the safety of their use are not known. The most important protein feed in pig and poultry nutrition at present is genetically modified soybean meal (GM-SBM). The changing legal environment and increasing public pressure may eventually lead to a law banning the use of GM feed in animal nutrition. The ban has been postponed until 1st January 2025. In addition, GMOs are banned in organic livestock production, which is gaining more and more followers. Large fluctuations in the price and supply of GM-SBM and the need for affordable protein in animal feed have led to a search for alternative, cheaper, and often local sources of plant protein (Karpiesiuk et al. 2018, Sońta et al. 2020, Świątkiewicz et al. 2021). In Poland, valuable protein sources in pig feed can be rapeseed meal 00 (00-RSM), faba bean (*Vicia faba* L.) seeds from new low-alkaloid varieties (FB), and yellow lupin (*Lupinus luteus* L.) seeds from new low-alkaloid varieties (YL). By-products of food processing, such as canola meal, DDGS (Distiller's Dried Grains with Solubles) or guar meal can be an alternative to the above feeds (Karpiesiuk et al. 2018). Guar with the botanical name *Cyamopsis tetragonoloba* (L.) Taub. is an annual legume belonging to the Fabaceae family, genus Cyanopsis. Due to its origin, it is relatively unknown in Poland and underutilised as a feed component. As a legume, guar is grown in warm climates (Garcia et al. 2023). It is characterised by a deep and well-developed root system that makes it drought-resistant (Mahdipour-Afra et al. 2021), and is grown mainly in India, Pakistan, and Africa (Saeed et al. 2017). In its composition, guar meal contains up to 48-52% protein and 10% dietary fiber. Protein from guar meal can be a supplement or alternative to protein from other protein components (including soybean meal) in pig diets (Biel, Jaroszewska 2018). In addition to the pure availability of protein components, the health and safety of the feed used are also important for animals and humans (Schwarz et al. 2021, Okorski et al. 2022).

However, the use of legume seeds in pig nutrition is limited because of their low availability and, in some cases, the presence of nutritionally unfavourable factors. One factor that often limits or precludes the possibility of using certain feed components is their fungal infestation and the resulting presence of mycotoxins, which negatively affect pig health (Doboszyńska et al. 2005, Doboszyńska et al. 2006, Zielonka et al. 2007). Mycotoxins found in feed are secondary metabolites of molds that can contaminate many types of food and feed throughout the food chain. Although hundreds of fungal toxins are known, only a limited number are generally thought to play an important role in food safety. The most worrisome fungal toxins are produced

by species in the genera Aspergillus. Fusarium and Penicillium, which are commonly found in the field in important food crops and also contaminate them during storage (Okorski et al. 2022). Animal feed is already exposed to mycotoxin contamination during the growth and development phase in the field, depending on weather conditions during vegetation and or during storage. Some factors such as improper processing, packaging, drying techniques, and transportation operations can also affect fungal growth and increase the risk of mycotoxin formation. Unfortunately, mycotoxins are resistant to treatments aiming to neutralize them, which means that they may be present in the final product fed to animals. The negative significance of mycotoxin contamination of animal feed is increasing, and recent improvements in analytical methods for simultaneous determination and confirmation of multiple toxins in animal feed provide the evidence (Okorski et al. 2022). The most widespread toxin-producing fungal species belonging to the genera: *Aspergillus*, *Fusarium* and *Penicillium*, produce mycotoxins that are highly pathogenic to swine, for example ochratoxin A (OTA), deoxynivalenol (DON), and zearalenone (ZEA) – Bennett and Klich (2003).

Quality control of livestock feed for mycotoxin contamination is becoming increasingly important to ensure reliable test results. Studies on the effects of mycotoxins on pigs usually deal with single substances. Meanwhile, feeds may be contaminated with several mycotoxins simultaneously, exacerbating their toxic effects. One can cite studies conducted on young pigs fed diets containing DON, ZEA, and/or fumonisins, which showed that the greatest histopathological changes in the liver and immune system dysfunction occurred in animals fed diets contaminated with multiple mycotoxins simultaneously (Grenier et al. 2011). At high doses, the toxins have direct cytotoxic effects and can be lethal with long-term exposure. The effects of mycotoxins present in individual feedstuffs have been described several times (Zielonka et al. 2007, Savard et al. 2014, Gajęcka et al. 2023). In the available literature, we did not find any information on toxigenic fungi and mycotoxins that could cause both. In the available literature, we did not find any information on toxigenic fungi and mycotoxins colonising both guar meal and pig feed based on this protein component.

Therefore, this study aimed to determine the level of contamination with toxin-producing fungi and toxins in pig mixtures containing cereals and different amounts of guar meal using classical culture, qPCR and HPLC methods.

MATERIALS AND METHODS

Sampling and mycological analysis

Complete feed for fattening pigs during two-phase fattening (stage 1 – 30 to 70 kg body weight (BW), stage 2 – > 70 kg (BW) was used for analyses.

All diets were based on cereals grown in the Region of Warmia and Mazury (NE Poland). Imported soybean and guar meal were used as the main source of protein, at different inclusion levels corresponding to the nutrient requirements of pigs (Table 1). The composition of experimental diets was as follows:

Table 1

Composition of experimental diets (%)

Diet no.	Pig diet	Soybean meal	Guar meal	Barley	Wheat	Premix
1	1st stage of fattening*	21.5	-	45.5	30.0	3.0
2	1st stage of fattening*	16.2	4.9	45.9	30.0	3.0
3	1st stage of fattening*	10.9	9.9	46.2	30.0	3.0
4	1st stage of fattening*	5.5	14.6	46.9	30.0	3.0
5	2nd stage of fattening**	15.0	-	57.5	25.0	2.5
6	2nd stage of fattening**	11.25	3.4	57.85	25.0	2.5
7	2nd stage of fattening**	7.5	6.8	58.2	25.0	2.5
8	2nd stage of fattening**	3.75	10.3	58.45	25.0	2.5

* 30-70 kg BW
** 70-110 kg (BW)

Feed samples were collected randomly, at different time intervals, and combined into bulk composite samples that were analysed in three replications. The samples were frozen and stored at -80°C until used in qPCR analysis and analyses of mycotoxin contamination. Approximately 10 g of feed samples with particle sizes ranging from 0.5 mm to 1 mm (90%) as well as smaller (8%) and larger (2%) particles were employed for the *in vitro* assessment of fungal diversity. Randomly prepared feed samples representing each type of diet were disinfected in 70% ethanol for 3 min and in 1% NaOCl for 3 min in 100-ml glass beakers, and then rinsed for 3 min in sterile water. The feed samples were dried on sterile filter paper before fungal culture isolation. Samples were inoculated onto 9-cm sterile Petri dishes containing potato dextrose agar (PDA) – Leslie and Summerell (2006), at a rate of 10 spots per plate (10 plates per treatment). Approximately 20 mg of feed was transferred each time using a metal spatula. After 14 days, fungal cultures were transferred to sterile Petri dishes filled with PDA medium, and after another 14 days, they were identified based on their morphological and microscopic characteristics (Ellis 1971, Gerlach, Nirenberg 1982, Kwasna et al. 1991, Leslie, Watanabe 2002, Summerell 2006). Biodiversity indices were used to quantify the relationships of mycobiome in studied pig diets in accordance with the formulas in presented in Table 2 (Du et al. 2020).

Using Maxwell 16 equipment and kits suggested for plant material (Promega GMBH, Madison, USA), genomic DNA was extracted from each

Table 2

Biodiversity indices of fungal diversity in pig diets with different dose of guar meal

Index	Mathematical formula
Relative frequency	$RF(\%) = (n_i/N_i) \times 100\%$
Dominance	$Y = (n_i/N_i)f_i$
Species richness	S = no. of species in each variant
Marglef index	$D' = (S - 1)/\ln N_t$
Shanon-Wiener index	$D' = -\sum P_i \ln P_i$
Dominance index	$H' = -\sum p_i$

DNA extraction and qPCR identification of mycotoxin-producing fungi from pig diets

of the investigated pig diets (stored before analysis at 80°C). The amount of DNA was measured spectrophotometrically (Nanodrop ND 2000C, Thermo Fisher Scientific) and fluorimetrically (Qubit™ fluorometer, Thermo Fisher Scientific). The qPCR analysis was carried out using 10 ng of high-quality gDNA (A260/280 ratio ranging from 1.8 to 2.0) from feedstuffs.

The following primers and fluorescently labelled probes were used for qPCR detection of fungal DNA contamination in feed:

F. avenaceum/F. tricinctum: Avetric F: GGCYTTCCTGCGAACTTG; Avetric R: AGCAGTCGAGTTCGTCAACAGA; Avgetric Probe: Fam-CCGTCGAGTCCTCT-MGB (Kulik et al. 2011);

F. poae: Poae_F: GCGGCCGCTTTTGTCA; Poae_R:GCCTTTCCA GCAAGAGATGGT; Poae_probe FAM-AAAGCGGTCGAGTCTG-MGB (Kulik et al. 2011);

F. culmorum /F. graminearum: Tri5 forward: TCTTAACACTA GCGTGCGCCTTC; Tri5 reverse: CATGCCAACGATTGTTTGGAGGGA; FGTri5 probe: Fam-AACAAGGCTGCCCACCACTTTGCT CAGC CT – Tamra (Vegi, Wolf-Hall 2013);

F. sporotrichioides;: FspoA18 fwd: GCAAGTCGACCACTGTGAGTACA; FspoA85 rev: CTGTCAAAGCATGTCAGTAAAAATGAT (Nicolaisen et al. 2008);

P. verrucosum: rRNA forward: TAAGGTGCCGGAATACACGCTCAT; rRNA reverse: TAGTTCATTCGGCCCGTGAGTTGT; rRNA probe: Fam-TCTAGACAGCCCGACGGTGGCCATGGAA GT–Tamra (Vegi and Wolf-Hall 2013).

A qPCR assay was carried out in the ABI 7500 Fast System (Thermo Fisher Scientific). All qPCR reactions were performed in a total volume of 20 µL containing 3 µl of genomic DNA solutions. 1× TaqMan Universal PCR Master Mix with ROX (Thermo Fisher Scientific). 4 µl of primer mix (160-300 nM) and 2 µl of probe (80-150 nM). QPCR amplification was programmed with one cycle of denaturation at 95°C for 3 min and 40 cycles at 95°C for 15 s, 60°C for 15 s and 72°C for 60 s. Each sample was loaded

in triplicate onto a single qPCR plate. Calculations of the amount of gDNA were performed using the method described in previous studies (Okorski et al. 2017, 2022).

Isolation of mycotoxins from feedstuffs and HPLC analysis

Mycotoxin standards were purchased from Biopure® Referenzsubstanzen GmbH, Austria. Water for HPLC, organic solvents for HPLC, salts and other chemicals were purchased from Sigma Aldrich (Saint Louis, Missouri, USA). Water for the mobile phase HPLC was purified using the Milli-Q system (Millipore, Bedford, MA, USA). In this study, we used sample purification procedures to determine the levels of trichothecenes, zearalenone (ZEA) and ochratoxin A (OTA) in the samples according to the methodology described by Okorski et al. (2017).

HPLC analyses were performed using a Nexera UHPLC instrument from Shimadzu (Kyoto, Japan). Chromatography of zearalenone was performed using a Hypersil ODS column (2.1 mm×100 mm, 5 μm) using an isocratic mobile phase of water and acetonitrile (60/40) at a column temp. of 30°C. The detector parameters were: 274 nm (excitation) and 418 nm (emission). Chromatographic separation of the trichothecenes of group A was achieved with a Zorbax® Bonus-RP, (2.1 mm×150 mm; 5 μm) at 30°C. The HPLC was operated with a gradient mobile phase system consisting of ammonium formate buffer (10 mmol, formic acid and ammonium formate, pH 3.6), i.e. phase A, and acetonitrile (phase B) at a flow rate of 0.3 ml min^{-1}. Chromatographic separation of the trichothecenes of group B was performed using a Hypersil® ODS column (2 mm×125 mm, 5 μm) at 45°C. The gradient mobile phase system consisted of a mixture of water/acetonitrile/methanol (mobile phase A: 96/2/2) and acetonitrile (mobile phase B) at a flow rate of 0.5 ml min^{-1}. Detection was carried out using a detector set to a wavelength of 220 nm. Analytical determination of ochratoxin A was carried out after chromatographic separation using an HP Hypersil® ODS column (2.1 mm×100 mm, 3 μm) at 40°C. The method used an isocratic mobile phase of acetonitrile/water/acetic acid with a flow rate of 0.4 ml min^{-1}. The fluorescence detector was set to an excitation wavelength of 330 nm and emission to 460 nm.

Statistical analysis

The results were processed in the Statistica vs. 13.0 application (data analysis software system, IBCO Software Inc.) (2017), with the use of the *Tukey's HSD* tests $\alpha<0.01$ for all comparisons. Standard deviation and homogenous groups (A, B, C) identifying significant differences between means were determined (means denoted by the same letter are not significantly different at $\alpha=0.01$). Correlation coefficients were calculated to determine the strength of relationships between variables. Relationships between amounts of DNA mycotoxin-producing fungi and mycotoxin concentration in the stu-

died pig diet are presented in the form of regression charts using GraphPad Prism vs 10.0.0 (GraphPad Software, Boston, Massachusetts USA, www.graphpad.com).

RESULTS AND DISCUSSION

Mycological analysis

A total of 1131 fungal cultures were isolated from the tested feeds, which contained varying proportions of guar meal (Table 3). Toxigenic species were represented by fungi of the genus Fusarium: *F. avenaceum, F. culmorum,*

Table 3

Fungal cultures isolated from pig diet with different dose of guar meal

No	Species/Genus of Fungus	Diet no.								Total
		1*	2*	3*	4*	5*	6*	7*	8*	
1	*Alternaria alternata* (Fr.) Keissl	21	32	34	41	18	27	33	55	261
2	*Alternaria tenuissima* (Kunze) Wiltshire		1		2		1		3	7
3	*Aspergillus* sp.	1			3		1		4	9
4	*Aspergillus niger* Van Tieghem.	3		1	1		1		2	8
5	*Aureobasiudium bolleyi* (R. Sprague) Arx.	5				7				12
6	*Aureobasidium pullulans* (de Bary & Löwenthal)	1			1		2	1		5
7	*Botrytis cinerea* Pers.	2			1	2	2			7
8	*Cladosporium cladosporioides* (Fresen.) de Vries	13	15	19	11	21	12	18	19	128
9	*Cladosporium herbarum* (Pers.) Link	1					7			8
10	*Colletotrichum* sp.				3				4	7
11	*Curvularia* spp.		2	2	2		2	2		10
12	*Drechslera* spp.			2	3		4	3		12
13	*Epicoccum nigrum* Link	1	23	1	2			1		28
14	*Fusarium avenaceum* (Fr.) Sacc.	18	2	2	1	13	4	5		45
15	*F. equiseti* (Corda) Sacc.	7								7
16	*F. culmorum* (Wm.G. Sm.) Sacc.	1		2		1	1	2		7
17	*F. graminearum* Schwabe.	2	3		12	5	2	1	11	36
18	*F. oxysporum* Schlecht.	1	1	1	3	1	1	1	4	13
19	*F. poae* (Peck) Wollenw.	3	1	1	2	4	3	1	1	16
20	*F. solani* (Mart.) Sacc		1		2		2	1	2	8

cont. Table 3

No	Species/Genus of Fungus	Diet no.								Total
		1*	2*	3*	4*	5*	6*	7*	8*	
21	*F. sporotrichioide*s Sherb.	4	1	3		6	3	3	1	21
22	*F. tricinctum* (Corda) Sacc.	3		6			2	1		12
23	*Graphium* spp.		1				2	1		4
24	*Helminthosporium* sp.		2	3	2	5	8	9	4	33
25	*Mortierella alpina* Peyronel	1					4			5
26	*Mortierella clonocystis* W. Gams			1			1	5		7
27	*Mucor circinelloides* Tiegh.	1				2	1	1		5
28	*Mucor niveus* Leys.	1		4				3		8
29	*Mucor hiemalis* Wehmer	1		2	4			1		8
30	P*aecilomyces varioti* Bainier				2		3			5
31	*Penicillium* sp.	12	21	18	34	18	26	25	27	181
32	*Phoma* sp.					1				1
33	*Rhizoctonia solani* (Kuhn)		2	1	4		2	2	5	16
34	*Rhizopus nigricans* Ehrenb.	12	13	12	7	15	8	9	14	90
35	*Stachybotrys chartarum* (Ehrenb.) S. Hughes				2				1	3
36	*Trichoderma* sp.	1	3				3			7
37	*Trichoderma viride* Pers. ex S. F. Gray				2			2		4
38	*Trichothecium roseum*	5	9	10	14	12	3	5	1	59
39	*Ulocladium* sp.			1						1
40	*Zygorhynchus circinelloides* Pišpek	1					1			2
41	*Zygorhynchus exponens* Burgeff	5		2						7
42	Mycelia sterilla	2		4		3	4	5	2	20
Total		129	133	131	161	134	143	141	160	1131

1*, 2*, 3*... the composition of the feed is presented in Table 1.

F. equiseti, F. graminearum, F. oxysporum, F. poae, F. solani, F. sporotrichioides, F. tricinctum and Trichothecium roseum. A few unidentified isolates of the genera *Aspergillus* and *Penicillium* were also isolated, some species of which have the ability to synthesise mycotoxins. The species present in all the feed mixtures analysed were: *Alternaria alternata, Cladosporium cladosporioides, Rhizopus nigricans*.

In a study by Nagerabi and Elshafie (2001), representatives of 59 species, including *Aspergillus, Rhizopus* and *Curvularia*, were isolated from guar (cluster bean), with fungi of the genera *Alternaria* and *Fusarium* domi-

nating the mycobiome of the seeds. Another study showed that seeds from India were colonised by eight fungal species, including *Alternaria cyamopsidis*, *Aspergillus* sp., *Chaetomium* sp., *Curvularia lunata*, *Memnoniella echinata*, *Penicillium vinaceum*, *Rhizopus oryzae* and *Strachybotrys* sp. (Singh et al. 2005). The important role of *Alternaria* fungi as pathogens in Africa was confirmed by the work of Westhuizen and Van Der Beack (1987).

In a study by El-Rayes et al. (2022), the following fungal species were isolated and identified from *Cyamopsis tetragonoloba* seeds: *Alternaria alternata, Fusarium solani, Fusarium oxysporum, Macrophomina phaseolina, Rhizoctonia solani, Sclerotium rolfsii*, and other unknown fungi. Our own study showed the presence of *A. alteranta* and both *F. oxysporum* and *F. solani* in the material examined. The mentioned species are also typical microflora colonising legume seeds, including soybean (Pszczółkowska et al. 2019, 2020). Studies by Pareek and Varma (2015) have shown the important role of *F. solani* as a seed-borne pathogen of cluster beans both on the seed surface and as a result of infestation of their internal structures. The study by Poonam et al. (2020) also shows that *F. solani* and *R. solani* are important pathogens of cluster beans. This demonstrated that they cause yield losses of up to 20%, while at the same time different cultivated varieties have different resistance to the infestation. Seed infestation may be caused by previous infection of cluster bean plants with *Fusarium* fungi (Mathur, Shekhawat 1987). A study by Gautam et al. (2016) showed that *Fusarium* fungi were isolated from roots infected with cluster bean fusariosis, including: *F. acutatum, F. oxysporum*, and *F. solani*. According to Dwivedi and Dubey (1993), guar seeds are mainly colonised by representatives of *Aspergillus*, including *A. flavus*. In our study, this species was not found; however, *A. niger* and *Aspergillus spp.* were isolated. Another disease of major economic importance in India that is transmitted with cluster bean seeds is anthracnose (Shivran et al. 2023). The occurrence of the disease has also been confirmed in Mexico (García-León et al. 2022). In our study, the occurrence of a few cultures of *Colletotrichum spp.* was confirmed in forage varieties contaminated with bean seeds.

Analysis of the biodiversity indices of the fungal communities present in the different feeds showed that the different feed mixtures differed in the proportion of toxin-producing and saprotrophic species (Table 4). All calculated indices characterising the species diversity of the studied fungal communities were higher in saprotrophic species. The most colonized by fungi was forage No. 8, which contained the highest percentage of barley grain and a high percentage of guar meal, while having a low addition of soybean meal. This forage was characterised by the highest relative abundance (Rf) of saprotrophic fungi with a simultaneously low percentage of species capable of synthesising mycotoxins. In addition, mixture No 8 had the highest index of the dominance of saprotrophic fungi (Y), Shannon-Wiener index (H') and dominance index (λ). Analyses of the diversity of seed-borne fungi using

Table 4

Fungal diversity in pig diets with different amount of guar meal

Diversity Index	Group of Fungi	Diet no.							
		1*	2*	3*	4*	5*	6*	7*	8*
Relative frequency (Rf)	mycotoxin producing fungi	3.89	1.59	2.21	3.01	3.71	1.86	1.77	1.77
	saprotrophs	7.52	10.17	9.37	11.23	8.05	10.79	10.70	12.38
Dominance (Y)	pathogens	0.15	0.03	0.05	0.09	0.14	0.03	0.03	0.03
	saprotrophs	0.56	1.03	0.88	1.26	0.65	1.16	1.14	1.53
Species richness (S)	mycotoxin producing fungi	9.00	7.00	7.00	6.00	7.00	9.00	13.0	6.00
	saprotrophs	19.00	11.00	16.00	19.00	10.00	22.00	20.00	13.00
Marglef index (D')	mycotoxin producing fungi	2.11	2.08	1.86	1.42	1.61	2.63	4.01	1.67
	saprotrophs	4.05	2.11	3.00	3.72	1.77	4.37	3.96	2.43
Shannon-Wiener index (H')	mycotoxin producing fungi	0.34	0.13	0.19	0.21	0.32	0.15	0.14	0.12
	saprotrophs	0.66	0.86	0.81	0.79	0.68	0.85	0.86	0.87
Dominance index (λ)	mycotoxin producing fungi	0.04	0.03	0.03	0.03	0.04	0.03	0.03	0.03
	saprotrophs	0.05	0.03	0.05	0.05	0.05	0.04	0.03	0.06

1*,2*, 3*... the composition of the feed is presented in the Table1

the indices employed in our study are common in this type of research (Pszczółkowska et al. 2019, 2020). Previous studies have shown that lupine and pea seeds have a mycobiome with a dominant role of saprotrophic species. This was advantageous because under such conditions the proportion of pathogenic species was low.

The presence of saprotrophic fungi on seeds and in feed mixtures is related to the natural process associated with storage, which is influenced by thermal and moisture conditions and the presence of inoculum capable of reproduction in the tested material.

qPCR identification of mycotoxin-producing fungi from pig diets

The qPCR analyses included detection of genotypes responsible for the synthesis of DON and ZEA (*F. culmorum /F. graminearum*) and, in addition, eniatins (*F. avenaceum/F. tricinctum/ F. poae*), T2 toxin (*F. poae/F. sporotrichioides*) and OTA (*P. verrucosum*) – Table 5.

The highest proportion of *F. avenaceum/F. tricinctum* genotype was recorded in mix 1 - 12.1 pg, and the lowest in mix 5- 2.3 pg DNA. The genotype of *F. culmorum/F. graminearum* was found most frequently in mixture 4 (12.3 pg) and least frequently in mixture 1 (4.1 pg). The genotype of *F. poae* was present in all forage mixtures in relatively low amounts, ranging from

Table 5

Quantification qPCR mycotoxins producing fungi in pig diet

Diet no.	Amount of DNA (pg)				
	F. avenaceum / F. tricinctum	*F. culmorum / F. graminearum*	*F. poae*	*F. sporotrichioides*	*P. verrucosum*
1*	12.1^{A*}±0.6**	4.1^{D}±0.7	4.2^{A}±0.5	1.2^{B}±0.5	10.2^{E}±2.5
2*	7.1^{BC}±0.8	5.4^{CD}±0.8	2.9^{B}±0.5	0.9^{B}±0.4	17.4^{D}±4.6
3*	9.23^{B}±0.7	6.11^{C}±0.6	3.1^{AB}±0.6	0.8^{B}±0.3	19.3^{C}±5.5
4*	8.92^{B}±0.5	12.3^{A}±0.9	2.8^{B}±0.4	0.9^{B}±0.5	23.7^{C}±4.8
5*	2.3^{DE}±0.6	5.2^{CD}±0.5	3.8^{A}±0.5	2.1^{A}±0.4	9.3^{E}±1.2
6*	4.2^{D}±0.7	4.9^{CD}±0.6	3.4^{AB}±0.7	1.1^{B}±0.4	45.2^{B}±7.2
7*	3.6^{DE}±0.6	5.9^{C}±0.7	2.9^{B}±0.5	0.8^{B}±0.6	48.1^{B}±6.7
8*	4.78^{D}±0.8	9.4^{B}±0.8	2.7^{B}±0.6	0.7^{B}±0.2	65.4^{A}±5.1

* Averages marked with the same letters are not statistically different p-0.01,
** standard deviation,
1*, 2*,3*... the composition of the feed is presented in the Table 1

4.2 pg in mixture 1 to 2.7 pg in mixture 8. *F. sporotrichiodes* was also detected in all tested forage mixtures and the amount of DNA found was low (0.7-2.1 pg). DNA of *P. verrucosum* was also detected in all mixtures (Table 5). The highest amount of DNA of this species was found in feed 8 (65.4 pg) and the lowest in mixtures 5 and 1 (9.3 and 10.2 pg, respectively).

The examination technique used in our study, based on classical fungal culture, relies on the knowledge of fungal biology and, in particular, the nature of sporulation. Therefore, other investigation techniques using molecular biology methods or sensors based on the detection of volatile compounds produced by *Fusarium* fungi should be used to verify the extent of infection of plant material (Pszczółkowska et al. 2016, Borowik et al. 2023). Our previous research on the detection of toxigenic fungi in pig feed (Okorski et al. 2017) and in various components such as wheat seeds (Okorski et al. 2022) demonstrated that the qPCR technique is an effective tool for investigating the contamination of biological material with both toxigenic fungi and mycotoxins.

HPLC analysis of mycotoxin contamination of pig diets

Chemical analyses showed the presence of mycotoxins in all tested feed mixtures (Table 6). The content of individual mycotoxins depended on the proportion of each component in the feed mixture. The highest levels of ochratoxin A were present in feeds dedicated to the second fattening period. The lowest level of OTA mycotoxins was found in mixtures without guar meal. A similar ratio was found for the other tested mycotoxins (DON,

Table 6

Mycotoxin content of feeds with different proportion/type of protein component

Diet no.	OTA (μg kg^{-1})	DON (μg kg^{-1})	3-AcDON (μg kg^{-1})	T-2 (μg kg^{-1})	ZEA (μg kg^{-1})
1*	9.36^{F*}±1.63**	19.55^{D}±1.84	5.49^{F}±0.97	2.28^{A}±0.19	14.52^{D}±1.74
2*	22.18^{E}±0.98	20.01^{D}±0.29	4.87^{F}±0.26	1.74^{AB}±0.21	14.39^{D}±0.97
3*	29.33^{D}±4.74	21.47^{D}±3.50	7.52^{E}±1.25	1.96^{AB}±0.19	17.64^{D}±0.25
4*	34.25^{C}±3.78	27.16^{C}±2.36	10.73^{D}±3.34	1.53^{AB}±0.20	33.40^{C}±1.26
5*	7.43^{F}±0.69	14.59^{E}±0.31	4.32^{F}±2.03	1.95^{AB}±0.46	15.23^{D}±0.68
6*	44.38^{B}±1.23	35.3^{B}±2.56	17.62^{C}±1.11	1.72^{AB}±0.41	45.84^{B}±0.97
7*	44.85^{B}±1.17	35.87^{B}±2.20	19.88^{B}±2.38	1.35^{B}±0.20	37.12^{C}±7.46
8*	50.41^{A}±5.43	42.74^{A}±3.90	27.12^{A}±1.44	1.63^{AB}±0.33	61.57^{A}±15.15

* Averages marked with the same letters are not statistically different p-0.01,
** Standard deviation,
1*, 2*, 3*... the composition of the feed is presented in the Table 1.

3-AcDON and ZEA), except T2 toxin, which was found in a higher amount in mixtures without the addition of guar meal as a protein source (2.28 μg kg^{-1}).

In general, it can be concluded that the amounts of mycotoxins found in the present study were low, which could be due to the high diversity of saprotrophic fungi detected by the culture method. Contamination of guar by mycotoxins has been described in a few studies (Zhang et al. 2014, Berthiller et al. 2015). The studies showed that guar was contaminated by aflatoxins, fumonisins, DON, ZEA and OTA. The finding that guar meal was a source of contamination of the tested feed mixtures was confirmed by mathematical analysis included in our study.

The linear correlation analysis between the percentages of each feed ingredient showed that the increasing percentage of guar meal and barley in each feed mixture were responsible for the contamination of the feeds with mycotoxins: OTA, DON, 3-AcDON and ZEA. Indeed, a positive correlation was observed between the percentage of guar and the amount of OTA (R=0.59), DON (R=0.41), 3-AcDON (R=0.38) and ZEA (R=0.42). The above data are consistent with the results obtained by a team led by Zhang et al. (2014).

There was a positive correlation between the content of OTA, DON, 3-AcDON and ZEA in the diet and the percentage of barley in the diet, recording correlation coefficients for OTA: R=0.55, DON: R=0.63, 3-AcDON: R=0.70 and ZEA: R=0.65 (Table 7). It can be concluded that barley was more responsible than guar flour for the contamination of feeds with mycotoxins synthesised by *Fusarium* and OTA fungi. In a previous study, the level of infection in swine feed was shown to depend on the proportion of each

Table 7

Correlation between amount of mycotoxin (μg kg^{-1}) and the percentage of individual components (plant material) in the pig diet

Composition of diets (%)	Amount of mycotoxin (μg kg^{-1})				
	OTA	DON	3-AcDON	T-2	ZEA
Soybean meal	-0.83**	-0.72**	-0.73**	0.58*	-0.73**
Guar meal	0.59*	0.41*	0.38*	-0.46*	0.42*
Wheat	-0.49*	-0.59*	-0.66*	0.27	-0.61*
Barley	0.55*	0.63*	0.70*	-0.33	0.65*
Premix	-0.11	-0.21	-0.32	0.27	-0.16

** significant at p-0.01, * significant at p-0.05

component in the tested feed (Okorski et al. 2017), whereas soybean meal was the source of contamination of feed with DON, and T2 toxin. The level of T2 toxin was positively correlated with the proportion of barley and wheat in the feed. This was partially confirmed in this study. Based on mathematical analysis, it was shown that soybean meal (R=0.58) was mainly responsible for the contamination of the tested feed variants with T2 toxin, and based on the mathematical analysis, it appeared that mainly soybean meal was responsible for the contamination of the tested T2 feed variants and – to a lesser extent (not statistically significant) – wheat with the toxin (R=0.58 and R=0.27, respectively).

To relate the results of the toxigenic fungal DNA content to the results of the HPLC analysis, a linear regression analysis was performed (Figure 1).

The mathematical analysis confirmed the correlation between the DNA amount of *F. culmorum/F. graminearum* genotype (*Tri5*) and the densities of DON (R=0.41) – Figure 1*a*, 3-AcDON (R=0.42) – Figure 1*b* and ZEA (R=0.52) – Figure 1*c*. There was also a positive correlation between the amount of mycotoxin T2 and the amount of DNA of *F. poae* (R=0.62) – Figure 1*d* and *F. sporotrichioides* (R=0.74) – Figure 1*e*. Mathematical analyses confirmed that the species *P. verrucosum* (R=0.82) – Figure 1*f* was responsible for the contamination of OTA feed mixtures. Previous studies have shown that there is a positive correlation between the results of qPCR analyses of the presence of toxigenic fungal gDNA and the content of mycotoxins in plant material (Okorski et al. 2017, 2022).

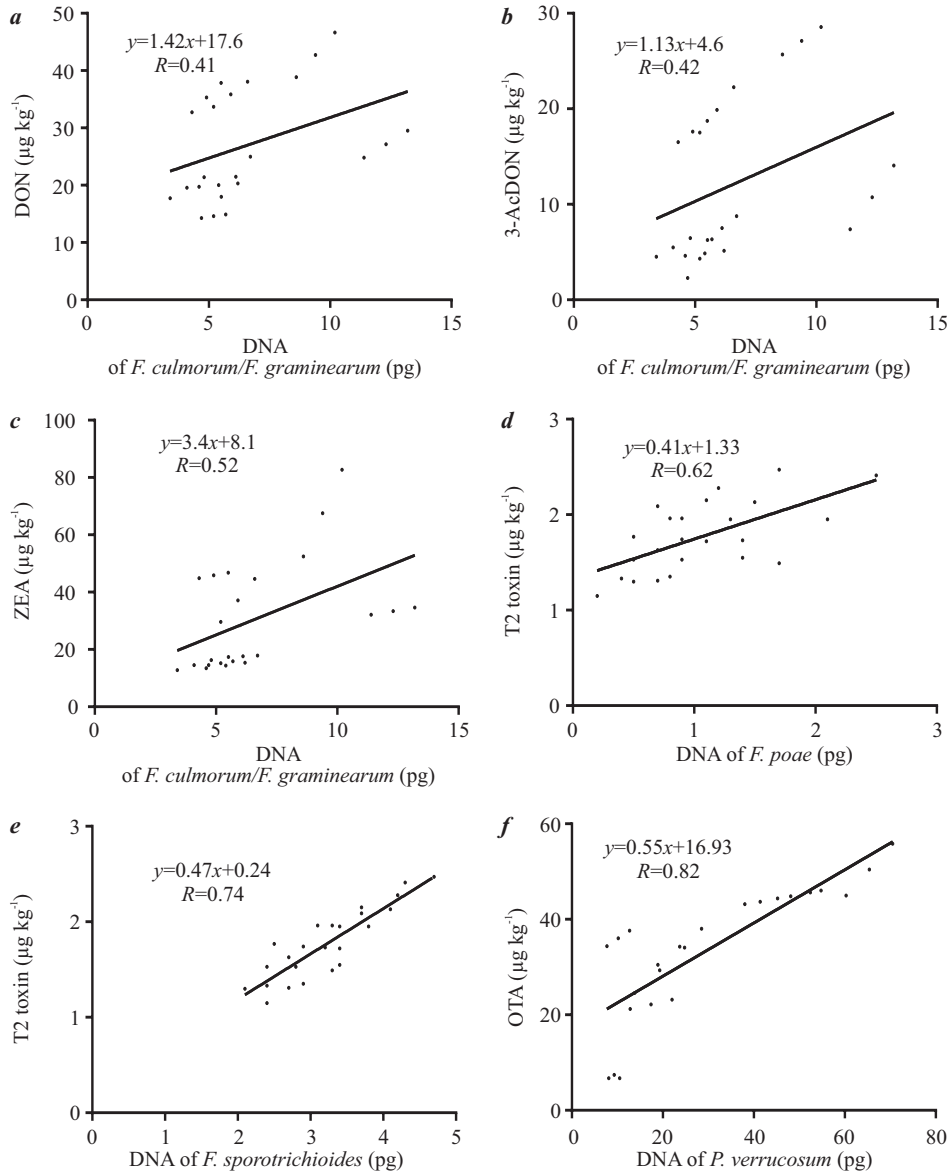

Fig. 1 Correlation between DNA amounts of mycotoxin producing fungi: *F. culmorum*/*F. graminearum* (*a*,*b*,*c*), *F. poae* (*d*), *F. sporotrichioides* (*e*), *P. verrucosum* (*f*) and mycotoxins DON (*a*), 3-AcDON (*b*), ZEA (*c*), T-2 (d,e), OTA (f) detected in pig diets

CONCLUSIONS

1. The mycobiome of the pig feeds tested was differentiated and had a high proportion of saprotrophic species.

2. The species responsible for the synthesis of mycotoxins were mainly propagated by the fungi *Fusarium* and *Trichotecium roseum*.

3. QPCR analyses showed that the forage mixtures were infected with *Fusarium species* (*F. culmorum, F. graminearum, F. avenaceum, F. tricinctum, F. poae, F. sporotrichioides*) and *P. verrucosum*.

4. The levels of *Fusarium* mycotoxins from the trichothecenes and ZEA group and of OTA were low and depended on the composition of the feed mixture.

5. Mathematical analyses confirmed that the addition of barley grain and guar meal were responsible for the contamination of the feeds with the mycotoxins DON, ZEA, and OTA, while T2 toxin contamination was related to the increasing proportion of soybean meal in the feeds and, to a lesser extent, to wheat grain and premix.

6. The content of mycotoxins in all mixtures was below the maximum permissible values. The lowest content of analyzed mycotoxins (OTA, DON, 3AcDON and ZEA) was found in mixtures based on soybean meal, and these mixtures are the safest for pig health.

Author contributions

Conceptualization, K.K., methodology, K.K., M.P.Ś and A.O., validation, K.K. and W.K., formal analysis, K.K., investigation, K.K.,W.K., A.O., M.Ś-P., A.P., J.A.D., U.CZ.; resources, K.K., W.K., data curation, K.K., W.K., A.O., M.Ś-P., writing – original draft preparation, K.K., W.K., A.O., writing – review and editing, K.K., visualization, K.K., W.K., A.O., M.Ś-P., supervision, K.K., project administration, K.K., funding acquisition, K.K. and W.K. All authors have read and agreed to the published version of the manuscript.

Conflicts of interest

The authors declare no conflict of interest.

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