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IDENTIFICATION AND ENUMERATION OF *FUSARIUM* SPECIES IN POULTRY FEED MIXTURES FROM SLOVAKIA

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Abstract: Thirty-three samples of poultry mixed feeds collected in the region of Nitra (Slovakia) from August 2001–April 2002 were assayed for the incidence of *Fusarium* LINK: Fr. species. In tested samples, the total frequency of isolated fusaria was determined to be 48 % (16 samples) and their counts ranged from 0.2×10^2 to 2.4×10^4 CFU per g of the sample on Dichloran Chloramphenicol Peptone Agar (DCPA). Of the total amount of *Fusarium* isolates (609), the highest part (i.e. 584 isolates) was represented by *Fusarium proliferatum* (Matsushima) Nirenberg, being isolated in all the samples tested. *Fusarium subglutinans* (Wollenw. *et* Reinking) Nelson, Tousson *et* Marasas (in total 24 isolates) was found in 3 positive samples (9%) and *Fusarium oxysporum* Schlecht. Fr. (a single isolate) was found in one positive sample only (3 %). Data of these significant mycotoxin producers found in examined samples with particular mycotoxins as well as their habitats were summarised briefly. The results refer to a large incidence of the potentially toxinogenic *Fusarium* species, mainly *F. proliferatum*, in the feeds of Slovakian origin which represent entry components of the food chain. The study also points out a potential risk of feed contamination with hazardous toxic compounds, especially by carcinogenic fumonisins and cardiotoxic moniliformin. From the hygienic point of view, it will be necessary to continue monitoring and evaluating this occurrence.

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INTRODUCTION

There are many companies producing mixed feeds for poultry nutrition in Slovakia. In general, mixed feeds by self-composition, especially under favourable conditions such as high moisture and increased temperature, represent excellent substrates for growth and reproduction of the chemoorganotrophic microscopic fungi.

A number of morphologically related *Fusarium* species, namely: *F. moniliforme, F. proliferatum, F. napiforme, F. anthophilum, F. dlamini, F. thapsinum* and *F. globosum* are occuring world-wide and can produce a group of structurally-related mycotoxins such as fumonisins [15, 26, 36, 37]. According to IARC, fumonisins are potent human carcinogens placed in class 2B and their level should not exceed 100–200 μg/kg in contaminated corn [26]. Fumonisin B_1 is the most common and most toxic and mainly occurs on maize and maize products [33]. Eight structurally-related fumonisins A_1, A_2, B_1-B_4, C_1 and their isoderivates have been isolated from fungal cultures and chemically characterized since the discovery of the most important member of the group, fumonisin B_1, in 1988 by [11]. However, only fumonisin B_1 and, to a lesser extent, fumonisins B_2 and B_3 are frequently found as natural contaminants of foods and feeds [36].

The toxin plays a causal role in the occurrence of equine leukoencephalomalacia [3], pulmonary oedema syndrome in pigs [13] and liver cancer in rats [11]. Acute

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mycotoxicosis caused by the fumonisins or their toxic effects in poultry were studied for instance by other scientific reports [4, 7, 8, 18, 19, 20, 28]. It has been found that in poultry the toxin may evoke an unusual disease outbreak characterised by black sticky diarhoea, severe reduction in food intake, egg production and body weight follow by lameness and death. Post-mortem examinations showed pale yellow coloured livers with peripheral congestion, mild haemorrhage in proventriculus and watery accumulations in the intestine, decreases in liver, spleen and bursa of Fabricius absolute weights. Thymic cortical atrophy, multifocal hepatic necrosis, biliary hyperplasia, intestinal goblet-cell hyperplasia, muscle necrosis and rickets have also been found. Of the haematological parameters, significant decreases were noted in red blood cell counts, haemoglobin, packed cell volume and white blood cell count. Also, abnormality in shapes of red blood cells (poikylocytes) has been observed.

Moniliformin is further toxin produced by several *Fusarium* species (mainly *F. moniliforme*, *F. proliferatum* and *F. subglutinans*) and is usually found on the corn kernel [10, 17, 26]. In poultry, chronicle intoxication with dietary moniliformin may evoke cardiac injury with subsequent alteration in cardiac electric conductance, and the cause sudden death of the animals [29, 30]. Acute cardiotoxicity of the toxin in broiler chicken was described by Marasas [22]. The producers of the toxins, mainly *F. moniliforme* and *F. proliferatum*, have been found frequently in corn and corn-based products, or in barley and wheat [23, 33].

Since the content of high-energetic cereals (corn, wheat) in poultry mixed feeds represents 40–70%, a contamination of the mixed feeds with the fungi or their toxins may be expected.

The main aim of this work was therefore to determine the species incidence of the genus *Fusarium* in poultry mixed feeds from the region of Nitra (Slovak Republic), to give short comments towards morphology of isolated species and to point at potential risk of feed contamination by *Fusarium* mycotoxins.

MATERIAL AND METHODS

Thirty-three samples of commercial poultry mixed feeds (HYD) were obtained from the State Veterinary Institute in Nitra from September 2001–April 2002. HYD 01 (12 samples), HYD 02 (8 samples), HYD 03 (2 samples), HYD 04 (2 samples), HYD 09 (1 sample) - chickens; HYD 10 (2 samples), HYD 11 (2 sample) - laying hens; HYD 12 (2 samples), HYD 16 (1 sample), HYD 17 (1 sample) - turkeys were analysed.

Each sample with a weight of 20 g was added to 180 ml saline with Tween 80 (0.02%, Medika, Bratislava) and then shaken on a horizontal shaker (TE III. Chirana, Piešťany).

One ml of the suspension was applied onto an agar plate in dilution 10^{-2} to 10^{-5} in 3 replications. For isolation and enumeration of the fusaria Dichloran Chloramphenicol Peptone Agar (DCPA) [5] was used. Incubation was carried out at 25ºC, during the period of 5–7 days in darkeness. Then, colonies of the fusaria were counted and expressed as colony forming units, i.e. CFU per g of the sample. The counts of cfu fusaria and total frequency in the samples were recorded.

After isolation, or in the some cases monosporic isolation, individual species were identified on the basis of their macro- and micromorphology (light microscope CN-F1 CN-hF, HERTEL REUSS) in accordance with other scientific reports [12, 25, 27, 31]. *Fusarium* isolates were incubated at 25ºC, from 7–14 days in darkeness. In specific cases (*F. subglutinans*), incubation under UV-light was also used. Potato Dextrose Agar (PDA) [31] and Synthetisher nährstoffärmer agar (SNA) [12] were used to evaluate the macroscopic and microscopic features, respectively. A frequency of the *Fusarium* isolates was determined and expressed according to amounts and species incidence in examined samples.

RESULTS AND DISCUSSION

Total frequency of isolated fusaria was 48% (in 16 samples) in tested samples and their counts ranged from 0.2×10^2–2.4×10^4 CFU per g of sample (Tab. 1). In total, 609 isolates of the genus *Fusarium* Link: Fr. were determined to belong to 3 species. From the infrageneric point of view, 2 species belonged to section *Liseola* (e.g. *Fusarium proliferatum* (Matsushima) Nirenberg and *Fusarium subglutinans* (Wollenw. *et* Reinking) Nelson, Tousson *et* Marasas), and 1 species belonged to section *Elegans* (e.g. *Fusarium oxysporum* Schlecht: Fr.). A frequency and total counts of the taxa found in positive samples are shown in Table 2.

Fusarium proliferatum was the most frequently isolated species in all positive samples, i.e. in 16 samples (46%). This represented a part of 96% (in total 584 isolates) of the total *Fusarium* isolates found in the examinated mixed feeds samples. This taxon was characteristic by mostly clavate micro-conidia with a flattened base and produced in various long chains and in false heads (Fig. 1B). The micro-conidia chains were produced from both monophialides and polyphialides in the aerial mycelium (Fig. 1C). On PDA, variations in mycelium coloration on colony obverse as well as reverse were from pale orange, pinkish to violet (Fig. 1A). Some of the subsequent *F. proliferatum* isolates produced dark violet to black sclerotia that were abundantly occurring mainly in marginal colony areas. In some isolates, the polyphialide formation was very sparse. Therefore, special attention had to be paid to longer observation of the mounts in order to avoid confusion on morphologically related *Fusarium moniliforme* J. Sheld. (=*F. verticillioides* (Sacc.) Nirenberg. This species does not form polyphialides, but like *F.proliferatum* produces the micro-conidia in chains or rarely in false heads as well [27, 31]. In accordance with [25], the micro-conidia are arranged either in chains, or in false heads so that the main distinctive characteristic between the 2 taxa is either presence or absence of the polyphialides. The macro-

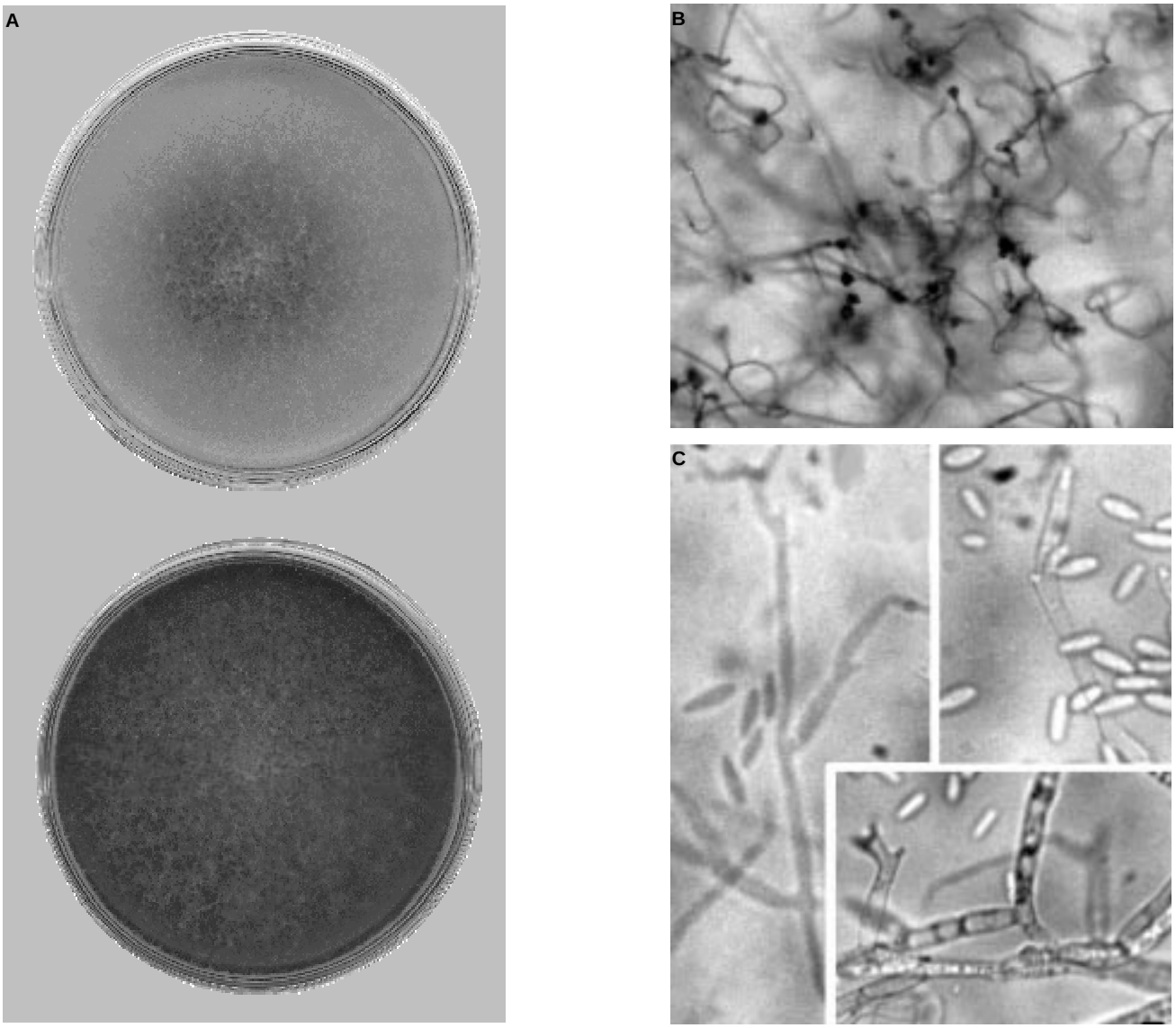

Figure 1. *Fusarium proliferatum*: (A) colonies on PDA after 7 (top) and 14 d (bottom); (B) arrangement of micro-conidia in chains and false heads (×200); (C) polyphialides (×1000).

conidia were not observed, and their formation within this species is rare [24, 30].

Fusarium subglutinans (in total 24 isolates) were found in 3 positive samples (9%). It was characteristic by the production of micro-conidia only in false heads (not in chains) from rich-branched polyphialides, and also from simple phialides (i.e. monophialides). Chlamydospores are missing. On PDA, it forms floccose to centrally powdery-like colonies coloured pale pink in the obverse and pale reverse. On this medium, a quite rich production of small exudate hyaline droplets was observed. Mostly oval or ellipsoidal micro-conidia as well as strong macro-conidia formation were observed on SNA. The macro-conidia were slightly curved to almost straight, with 3–5 septa and with distinct foot-shaped basal cells. The relative length of the apical cell compared with the penultimate cell was approximately equal and showed only a slight bending.

Fusarium oxysporum (a single isolate) was found only in 1 sample (3%) and it was characteristic by formation of kidney to ellipsoidal-shaped microconidia arranged to in false heads from short and stout monophialides. Also, a production of chlamydospores arranged singly or in pairs was observed. The macroconidia were quite similar to those of *F. subglutinans*.

As regards the fumonisin producers, it is elusive how high amount and frequency of *Fusarium proliferatum* isolates were determined in the tested samples during this study. In accordance with the valid state norm 1497/1997-100 that has set up maximum allowable counts of total microscopic fungi in poultry mixed feeds to 30×10^3 CFUper g of the feed, these findings showed that in some examined samples, i.e. HYD 02 (sample No. 6 and 7) and HYD 10 (sample No. 1), the norm was nearly exceeded. In some cases, only cfu of *Fusarium* spp. were so high that they exceeded the norm.

Furthermore, with the exception of *Stachybotrys chartarum*, *Aspergillus fumigatus* and *A. flavus*, the norm does not specify the other problematic groups of fungi which may also represent a health hazard and not only for feeded animals. A potential risk of toxinogenity of the *Fusarium* species found in examined mixed feed samples according to literature data is shown in Table 2. Other mentioned toxins, such as fusaric acid, zearalenon, fusarin C, nivalenol, fusarenon X or sambutoxin produced by the 3 species, are described and discussed by some authors [2, 22, 38]. The ability of *F. proliferatum* (9 isolates) to produce fumonisins was tested by Castella *et al.* [6] and shown that all of the isolates were fumonisin producers. One strain of the species isolated from maize produced approximately 31 µg of fumonisin B_1 and 17 µg of fumonisin B_2 per g of autoclaved corn kernels. According to [35] each of the 12 strains of *F. proliferatum* produced moniliformin (45–16,000 ppm), fumonisin B_1 (27–6140 ppm) and B_2 (5–1,550 ppm), even though the fungi were isolated from dairy cattle feed. Mycotoxin production by *Fusarium proliferatum* isolates from rice with *Fusarium* sheart rot disease was evaluated by [1]. It has been found that all 15 isolates from rough rice samples were the producers of fumonisin B_1, B_2, moniliformin and beauvericin in culture on rice.

All tested strains of *F. moniliforme* (closely related to *F. proliferatum*) tested by [26] in Slovakia produced fumonisin B_1 on sterile corn in amounts detectable by thin-layer chromatography. Beauvericin production by *F. subglutinans* (25 isolates) was also observed [32]. Seven isolates of *F. subglutinans*, isolated from New Zealand maize fields, were found to produce moniliformin at levels ranging from 0.4–64 ppm in maize kernels [16]. Eight isolates of *F. moniliforme* var. *subglutinans* (at present *F. subglutinans*) from swine feed produced 51–540 µg per g on cracked corn, but the toxin was not detected in the feed samples [34]. Eleven of the 15 Canadian isolates of *Fusarium subglutinans* produced moniliformin in corn [9]. All 6 of *F. subglutinans* isolates as well as 14 isolates of *F. oxysporum* (both from Southern Africa) produced moniliformin [29].

A study of [14] in USA has shown a concurrence of fumonisins and moniliformin in food-grade corn (34%) and corn-based food (53%). Sixty-five percent of examinated samples contained fumonisin B_1, ranging in concentration from 28–2.7 µg per kg, and fumonisin B_2 was detected in 29% of the samples with concentration ranging from 31–858 µg per kg of the sample. Fumonisins were also present in 6 of the 18 samples of maize and maize products harvested in small fields and stored by farmers in northern Argentina. The levels of fumonisins ranged from 603–1888 ng per kg of the sample. Czech cereal products from corn harvested in 1995 (71 samples) contained 278 ng of fumonisins B_1–B_3 and from 1996 harvest (76 samples) 131 ng of the same fumonisins per g [26].

All species found in examined mixed feed samples are considered to be pathogens of maize and other cereals, causing stalk rot and cob rot of maize (*F. proliferatum* and

Table 1. Total counts of fusaria found in the samples of poultry mixed feeds on DCPA; cultivation at 25 ˚C, 5 to 7 d in dark; determined species.

Mixed feed	No. of the sample	Counts of the fusaria in CFU.g^{-1}	Isolated species
HYD 01	1	2×10^3	*F. proliferatum*
	6	1×10^3	*F. proliferatum, F. subglutinans, F. oxysporum*
	8	1×10^3	*F. proliferatum*
	9	2×10^2	*F. proliferatum*
	11	2×10^3	*F. proliferatum*
HYD 02	5	2×10^2	*F. proliferatum*
	6	1.6×10^4	*F. proliferatum*
	7	2.4×10^4	*F. proliferatum*
HYD 03	1	2×10^2	*F. proliferatum*
	2	7×10^3	*F. proliferatum*
HYD 04	1	6×10^2	*F. proliferatum, F. subglutinans*
	2	2×10^3	*F. proliferatum*
HYD 10	1	2×10^4	*F. proliferatum, F. subglutinans*
	2	2×10^3	*F. proliferatum*
HYD 11	1	2×10^3	*F. proliferatum*
	2	4×10^3	*F. proliferatum*

Table 2. Counts of isolates and total frequency of *Fusarium* sp. in examined samples of the poultry mixed feeds.

Species	Counts of *Fusarium* isolates	Amount of positive samples	Total frequency in %
Fusarium proliferatum	584	16	48
Fusarium subglutinans	24	3	9
Fusarium oxysporum	1	1	3
In total	609	16	48

Table 3. Potential toxinogenity of *Fusarium* species isolated from mixed feeds [10, 26].

Species	Mycotoxin
Fusarium proliferatum	fumonisins, moniliformin, fuzarin C, fusaric acid, naftoquion pigments
Fusarium subglutinans	fumonisins, moniliformin, fuzarin C, fusaric acid, naftoquion pigments
Fusarium oxysporum	zearalenon, moniliformin, wortmanin, nivalenol, fuzarenon X, sambutoxin

F. subglutinans) and storage rot in maize (*F. oxysporum*) [31]. Both *F. proliferatum* and *F. subglutinans* are well recognised as the natural contaminants of maize in Europe [21]. In accordance with [31], the highest incidence of *F. proliferatum* was determined in 1996, with *F. moniliforme* as prevalent species in the cooler and rainy year of 1998. [26] have also studied a *Fusarium* contamination of the maize kernels in 1995–1998. The authors found that the most frequently determined *Fusarium* species was *F. moniliforme* which predominated in the samples in 1997, being characterised by intensive precipitations and relatively low average monthly temperatures. *F. subglutinans* prefers lower temperatures and therefore its occurrence strongly depends on geographical location and weather conditions in individual years. The highest incidence of the *F. subglutinans* on infected maize kernels has been observed in the cooler year 1998 in southern parts of Slovakia [26]. Whereas, [32] found only a low percentage of positive maize samples contaminated with *F. subglutinans* in the same year and in approximately the same area of Slovakia. *Fusarium subglutinans* was the most prevalent species and 7 isolates produced moniliformin.

In line with [26, 32] findings, we can presume that in Slovakia, the occurrence of *Fusarium* species in mixed feeds is strongly dependent on climatic conditions of a given year, as well as on quality of the storage management of cereals and/or mixed feeds.

It may be concluded that the mixed feeds could be considered as the potential source of *Fusarium* mycotoxins which, through the food chain, can affect both the animal and human health. The study points out the requirement to monitor and evaluate this phenomenon in further works.

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