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INCIDENCE OF FUMONISINS, MONILIFORMIN AND *FUSARIUM* SPECIES IN POULTRY FEED MIXTURES FROM SLOVAKIA

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Abstract: A total of 50 samples of poultry feed mixtures of Slovak origin were analysed for fumonisin B_1 and B_2 (FB_1, FB_2) and moniliformin (MON) using SAX-clean up procedure being detected by high pressure liquid chromatography with mass spectrometry (HPLC-MS) and diode array detection (HPLC-DAD), respectively. The samples were also simultaneously investigated for *Fusarium* species occurrence, and for the capability of *Fusarium* isolates recovered to produce FB_1 and MON *in vitro*. FB_1 was detected in 49 samples (98%) in concentrations ranging from 43 to 798 $\mu g.kg^{-1}$, and FB_2 in 42 samples (84%) in concentrations ranging from 26 to 362 $\mu g.kg^{-1}$. MON was detected in 26 samples (52%) in concentrations that ranged from 42 to 1,214 $\mu g.kg^{-1}$. Only two *Fusarium* populations were encountered, namely *F. proliferatum* and *F. subglutinans*, of which the former was the most dominant and frequent. All 86 *F. proliferatum* isolates tested for FB_1-production ability proved to be producers of the toxin although none of them produced MON. On the contrary, MON production was observed in a half out of 16 *F. subglutinans* isolates tested, yet no FB_1 production was detected in this case. Despite the limited number of samples investigated during this study, it is obvious that poultry feed mixtures may represent a risk from a toxicological point of view and should be regarded as a potential source of the *Fusarium* mycotoxins in central Europe. This is the first reported study dealing with fumonisin and moniliformin contamination of poultry feeds from Slovakia.

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INTRODUCTION

The International Agency for Research on Cancer has classified the fumonisins as carcinogenic to animals and possibly carcinogenic to humans, being placed in group 2 B of potential carcinogens [4, 6]. Since the first report on fumonisins by Gelderblom and co-workers in 1988, a lot of attention has been given to these highly carcinogenic compounds [16]. Despite the established carcinogenicity of the fumonisins, statutory regulations do not exist for these *Fusarium* mycotoxins. Limits have been set in some countries to protect susceptible animals such as horses (5 $mg.kg^{-1}$) and pigs (50 $mg.kg^{-1}$), and temporary guidelines have been introduced in a few countries to limit the

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exposure for humans [14]. For example, Switzerland has set a limit for the amount of FB_1 and FB_2 at 1,000 $\mu g.kg^{-1}$ in maize intended for human consumption [11, 14]. Feeds contaminated with FB_1 are known to cause leukoencephalomalacia in horses and rabbits, pulmonary edema, hydrothorax and hepatic syndrome in swine, hepatotoxic and carcinogenic effects and apoptosis in the liver of rats, poor performance in poultry, and alteration in hepatic and immune function in cattle [4, 25]. In poultry, the fumonisins have been implicated in unusual disease outbreaks characterized by black, sticky diarrhoea, severe reduction in food intake, egg production and body weight, followed by lameness and death [33]. Furthermore, FB_1 in chick feed diet evoked thymic cortical atrophy, multifocal hepatic necrosis, biliary hyperplasia and rickets [23]. Significant decreases in red blood cell counts, hemoglobin, packed cell volume and white blood cell count have been noted [19], and also abnormalities in shapes of red blood cells and reduced lymphocyte viability were reported [13]. Embriopathic toxicity of the fumonisin was extensively studied by Javed *et al.* [20]. A FB_1 exposure may result in increased susceptibility of chickens to bacterial infections [34]. In addition to inducing mortality and to its putative interaction with fumonisins, MON is endowed with cardio-toxic effects in a wide range of laboratory and domesticated animals, including rats, chickens, turkeys and ducks [12]. Chronic exposure of poultry to MON in diets may evoke cardiac injury with subsequent alterations in cardiac electric conductance, and cause the sudden death of the animals [36]. Acute cardiotoxicity of MON in broiler chicken was described by Marasas [26]. The toxin was suspected of causing keshan disease, a myocardic human impairment occurring in rural areas of China and South-Africa (Transkei), regions with a high maize consumption [6]. MON and FB_1 were found to occur naturally at biologically significant levels in maize and in a variety of maize-based human foods and animal feeds worldwide [6, 16]. As for fumonisins occurrence, data reported by Rumbeiha et Oehme [37] from the USA and Patel *et al.* [31] from the UK demonstrated that certain corn products contain relatively high levels of fumonisins: corn meal (up to 349 $ng.g^{-1}$), polenta (up to 2,124 $ng.g^{-1}$), and corn flour (up to 167.7 $ng.g^{-1}$). Levels of 29–63 $mg.g^{-1}$ FB_1 were detected in corn-based feeds associated with animal disease outbreaks and averages of 1–3 $mg.g^{-1}$ in apparently healthy corn kernels [28]. Despite the fact that corn and other small-kernel cereals have been suspected of fumonisin and/or moniliformin contamination in a relatively extensive range in Europe [6, 25], there exist no available reports dealing with the mycotoxins in poultry feed mixtures (corn-based commodities) from this area at all. Moreover, Labuda *et al.* [22] recently reported a high frequency and number of *F. proliferatum* isolates in poultry feed mixtures that originated from Slovakia. Thus, a main goal of this study was focused on the investigation of poultry feed mixtures from Slovakia in terms of occurrence of FB_1 and FB_2, moniliformin, as well as *Fusarium* species incidence in the samples tested and their ability to produce the mycotoxins *in vitro*.

MATERIAL AND METHODS

Samples. A total of 50 randomly collected samples of feed mixtures designed for poultry feeding were obtained from the State Veterinary Institution in Nitra (Slovak Republic) during September 2003–April 2004. The samples represented the following three poultry categories: HYD 01 (chicken broilers - starting, 37 samples), HYD 02 (chicken broilers - growing, 7 samples), HYD 03 (chicken broilers - finishing, 6 samples).

Isolation of *Fusarium* species. Standard dilute plate method was used for isolation of fusaria. A part of poultry feed sample weighing 20 g was mixed with 180 ml of saline solution on a horizontal shaker for ca 30 minutes. One ml of the diluents (made up to 10^{-5}) was then inoculated on Dichloran Chloramphenicol Peptone agar (DCPA, [3]) in triplicate. After 5–7 days of incubation at 25ºC in the dark, resulting colonies were counted and transferred onto identification media.

Identification of *Fusarium* species. Identification of fusaria encountered was carried out according to [9, 29, 38] using potato dextrose agar (PDA) for observation of colony characteristics. Synthetic nutrient agar (SNA) and carnation leaf agar (CLA) was used for observation of micro-morphological features. Plates were incubated at 25ºC during 7 days in the dark (PDA) and under UV-light (CLA, SNA). All formulae are those given in [38].

FB_1 and MON screening *in vitro*. The method was carried out according to Mubatanhema *et al.* [27], consisting of cutting a small agar plug from the growing *Fusarium* colony on yeast extract agar (YES), prepared according to [38], after 7-, 14- and 30-day-incubation at 25ºC in the dark, using a steel-tube (i.d. 9 mm). The plug was then placed into an 8 ml screw vial. One ml of extraction solvent (acetonitril/water, 50:50, v/v) was added to the bottle containing the agar block. The content was shaken for a few minutes (2–3). Five microlitres of aliquot was then applied onto the thin layer chromatography plate (TLC; Kieselgel 60, Merck, Darmstadt, Germany). Butan-1-ol/acetic acid/water (BAW; 20:10:7) as a solvent system gave average R_f values of 0.64 for FB_1 and 0.68 for MON. FB_1 was visualized by spraying with 0.5% p-anisaldehyde in methanol/acetic acid/oleum (17:2:1; v/v/v) and heating at 120ºC for 2–3 min. Consequently, it appeared as a reddish-purple spot under daylight. MON was viewed by spraying with 0.5% methylbenzothiazolone-hydrochloride (MBTH; Merck, Darmstadt, Germany) followed by heating at 120ºC for 2–3 min. It appeared as very faint yellowish-orange spots under daylight, becoming bluish after next 15º - 20 min. FB_1 and MON standards were purchased from Sigma (St. Louis, USA).

Determination of MON in the samples. The method was carried out according to Parich *et al.* [30]. Altogether, 10.0 g of a sample were extracted with 100 ml of acetonitril/water (84:16) on a horizontal shaker at 170 rpm for 30 min. The resulting extract was filtered over 15 cm folded paper filter (Schleicher & Schüll, BioScience GmbH, Dassel, Germany) into a 100 ml Erlenmeyer-flask. Twenty ml of the filtrate was then evaporated on a rotary evaporator (Heidolph VV2011, Mueller-Scherr, Linz, Austria) at 50ºC (300–400 hPa). The evaporated extract was dissolved with twice 2 ml methanol (HPLC grade, Merck), shaken for 60 secs. on a vortex mixer (type VF2, IKA, Hamburg, Germany) and transferred onto a SAX-SPE column (500 mg, 3 ml; Cat. No. 500-0050-B; IST International, Foster City, USA;) conditioned with 2 ml methanol, 2 ml water and 2 ml 0.1 M phosphoric acid. The column was washed by 2 ml 0.1 M phosphoric acid and 2 ml water immediately after the extract passed through the column and dried under vacuum. Subsequently an ion pair solution buffer was prepared by mixing 50 ml of 20% (w/w) tetrabutylammonium hydroxide and 100 ml of 1.1 M potassium dihydrogen orthophosphate. The toxin was eluted with 2 ml elution solution prepared by diluting the ion pair solution buffer with water (1:1). Finally, the eluted moniliformin was retained in a 4 ml vial. Prior to HPLC analyzing, the eluate was shaken and filtered through a 0.22 µm filter (Millex GV_{13}, Milipore, Bedford, USA). A high-pressure liquid chromatograph HPLC (type 1090, Hewlett Packard, Toronto, Canada) equipped with diode array detector at 228 nm and 260 nm (HPLC-DAD; Hewlett Packard) was used for the detection. The separation was performed with a Hypersil ODS C18 column (150 × 2.1 mm, 5 µm; Hewlett Packard) and guard column ODS-Hypersil (20 × 2.1 mm, 5 µm; Hewlett Packard). The mobile phase was an ion pair buffer (10 ml of ion pair solution and 50 ml acetonitril were diluted with water to 1,000 ml). A flow of 0.4 ml per min, a column temperature of 22ºC, an injection volume of 20 µl and isocratic conditions were used. The quantitative evaluation was performed by signal high at 228 nm (Reference-Wavelength 450 nm, calibration curve: 0.25–10.5 $\mu g/ml^{-1}$). The limit of detection for MON was 39 $\mu g.kg^{-1}$. MON standard was purchased from Sigma (St. Louis, USA).

Determination of FB_1 and FB_2 in the samples. The extraction and clean-up was a modification of methods given by Sydenham *et al.* [40] and Thiel *et al.* [42]. Altogether, 25.0 g of a sample were extracted with 100 ml of methanol/water (3:1) by blending on an Ultra Turrax (Type T25; Jepson Bolton Co. Ltd., Watford, UK) for 5 min at 13.500 rpm. The sample was then centrifuged at 4,500 rpm for 10 min at room temperature and filtered over 15 cm folded paper filter (Schleicher & Schüll, BioScience GmbH). The extract was adjusted to a pH of 6-8 using 1M NaOH and pH-stripes (Macherey-Nagel, Düren, Germany) and 5 ml of adjusted extract (2 × 2.5 ml) was loaded onto a previously conditioned SAX-SPE column with 5 ml MeOH and 5 ml MeOH/water (3:1). Then the samples were washed twice with 6 ml MeOH/water (3:1), 3 ml MeOH followed by elution with 10 ml glacial acetic acid/MeOH (1:99) into 10 ml eprouvettes. The eluate was dried under nitrogen at 50ºC. Re-dissolution of sample was carried out in 500 µl buffer/acetonitril (70:30) and then vortexed for 1 min, centrifuged in Eppendorf plastic vessels for 10 min at 12,500 rpm, filtrated trough Millex GV_{13} (0.22 µm, Milipore) and transferred to HPLC-vials. A high-pressure liquid chromatograph HPLC Type HP 1100 equipped with mass spectrometer Type 1100 Series LC/MSD (Hewlett Packard) was used for the detetection. The separation was performed with an Agilent eclipse XDB-C8 (150 × 4.6 mm, 5 µm; Hewlett Packard). A buffer consisting of 385 mg of ammonium formate in 1 L with 385 µl formic acid in 1 L of water in Chanal A and acetonitril in Chanal B were used. A flow of 0.6 $ml.min^{-1}$, a column temperature of 30ºC, an injection volume of 50 µl and a gradient of the solvents were used (0-9 min: 35% B, 9-11 min: 35 to 45% B, 15-17 min: 45 to 35% B, 17-30 min: 35% B). For the MS detection, API-ESI positive mode was used (Nebulizer Pressure: 35 psig, Drying Gas flow: 9.0 $L.min^{-1}$, Drying Gas temperature: 350ºC, Quadrupole temperature: 100ºC, Capillary Voltage: 3,000 V, Fragmentor Voltage: 160 and 240 V for FB_1, 200 and 280 V for FB_2). The calibration ranged from 25-1,000 $ng.ml^{-1}$ toxin; the limit of detection for FB_1 was 29 $\mu g.kg^{-1}$ of sample and for FB_2 24 $\mu g.kg^{-1}$ of sample. The peak areas of the protonated ions $[M+H]^+$ were used to quantify the toxins using the single ion mode (FB_1: *m/z* 722.3; RT=3.9 min; FB_2: *m/z* 706.3; RT=10.0 min). FB_1 and FB_2 standards were purchased from Sigma (St. Louis, USA).

RESULTS

FB_1 was detected in 49 samples (98%) in concentrations ranging from 43–798 $\mu g.kg^{-1}$ (average 235 $\mu g.kg^{-1}$) and FB_2 in 42 samples (84%) in concentrations ranging from 26–362 $ug.kg^{-1}$ (average 87 $\mu g.kg^{-1}$). MON was detected in 26 samples (52 %) in concentrations from 42–1,214 $ug.kg^{-1}$ (average 217) $\mu g.kg^{-1}$. Concerning *Fusarium* species, only two populations were recovered from the samples studied, namely *Fusarium proliferatum* (Matsushima) Nirenberg and *Fusarium subglutinans* (Wollenw. & Reinking) Nelsom, Toussoun & Marasas. The *F. proliferatum* isolates were encountered in 19 samples (38%). The isolates belonging to *F. proliferatum* taxon were characteristic by the formation of clavate microconidia with a flattened base being produced in various long chains and in false heads from both monophialides and polyphialides. The *F. subglutinans* isolates were found in 7 samples (14%). They were characteristic by the formation of oval to ellipsoidal micro-conidia produced only in false heads (not in chains) from rich-branched polyphialides. Total counts of *F. proliferatum* isolates

Table 1. Occurrence of FB_1, FB_2, and MON (µg.kg^{-1}), *Fusarium* species and their counts (cfu × 10^4.g^{-1} of sample) in poultry feed mixture samples from Slovakia investigated during September 2003–April 2004.

No.	HYD	FB_1	FB_2	MON	fusaria	cfu
1	1	179	46	nd	–	–
2	1	338	64	71	–	–
3	1	202	68	203	–	–
4	1	96	nd	nd	–	–
5	1	51	nd	nd	–	–
6	1	145	nd	nd	F. prol	10
7	1	43	nd	nd	–	–
8	1	73	nd	nd	F. prol + F. sub	6/2
9	1	387	94	190	–	–
10	1	626	179	nd	–	–
11	1	523	95	59	–	–
12	1	nd	nd	nd	–	–
13	1	82	32	nd	F. prol + F. sub	7/1
14	1	304	140	182	–	–
15	1	213	70	nd	F. prol	0.5
16	1	196	54	nd	–	–
17	1	292	110	nd	–	–
18	1	207	66	280	–	–
19	1	244	74	216	–	–
20	1	127	49	nd	F. prol	0.5
21	1	107	39	nd	–	–
22	1	273	94	305	F. prol + F. sub*	0.9/0.2
23	1	543	167	682	–	–
24	1	88	nd	73	F. prol + F. sub	1/1
25	1	244	85	297	F. prol	8
26	1	74	34	nd	F. prol	1
27	1	101	45	nd	–	–
28	1	164	63	nd	F. prol	0.9
29	1	392	147	nd	–	–
30	1	388	147	73	F. prol + F. sub*	1/0.03
31	1	401	167	126	F. prol + F. sub	9/1
32	1	147	57	80	F. sub	6
33	1	106	45	77	–	–
34	1	85	27	nd	–	–
35	1	307	119	492	–	–
36	1	158	56	174	F. prol	0.08
37	1	143	55	63	–	–
38	2	385	137	nd	–	–
39	2	798	362	nd	F. prol	0.5
40	2	460	131	185	F. prol	0.5
41	2	649	204	42	F. prol	0.4
42	2	36	nd	nd	F. prol	0.01
43	2	78	30	146	–	–
44	2	109	34	1214	–	–
45	3	239	66	nd	–	–
46	3	297	84	nd	F. prol	0.1
47	3	119	41	46	–	–
48	3	132	48	87	–	–
49	3	74	26	170	F. prol	0.5
50	3	92	29	110	–	–

nd - mycotoxin under detection limit (FB_1=29 µg.kg^{-1}, FB_2=24 µg.kg^{-1}, MON=39 µ.kg^{-1}); – - the fungi not found in the all dilutions used; HYD type of the feed mixture: 1 - chicken broilers - starting, 2 - chicken broilers - growing, 3 - chicken broilers-finishing; F. prol - *Fusarium proliferatum*, F. sub - *Fusarium subglutinans*; F. sub* - proved moniliformin producers; cfu - colonies forming units of the fusaria.

Table 2. Capability of fusaria recovered from samples of Slovak poultry feed mixtures investigated during September 2003–April 2004 to produce FB_1 and MON after 7-, 14- and 30-day-incubation on YES at 25ºC in the dark.

Species	No. of isolates	FB_1			MON		
		7d	14d	30d	7d	14d	30d
F. proliferatum	86	86/86	86/86	86/86	0/86	0/86	0/86
F. subglutinans	16	0/16	0/16	0/16	8/16	8/16	8/16

ranged from 0.08–10 × 10^4 colonies forming units per g of the sample (cfu. g^{-1}), while those for *F. subglutinans* were found to range from 0.03–4 × 10^4 cfu.g^{-1} of sample. The outcomes from the mycotoxin detection along with appropriate *Fusarium* species findings are listed in Table 1. FB_1-production was observed in all out of 86 *F. proliferatum* isolates after 7-day-incubation on YES. At least 4 isolates of the species representing each *F. proliferatum* positive sample was tested for the fumonisin potency. No MON was detected in any out of *F. proliferatum* isolates tested during this study, even after 30-day-incubation. No FB_1 was observed to be produced in any out of 16 *F. subglutinans* tested, whereas MON was detected in 8 *F. subglutinans* isolates after 7-day-incubation on YES (Tab. 2).

DISCUSSION

The American Association of Veterinary Laboratory Diagnosticians has recommended a maximum level for FB_1 of 50 mg.g^{-1} in feed for poultry [11]. As for MON, its quantities in diet of broiler chickens should remain below 50 mg.g^{-1} [24]. The outcomes from the present study have clearly shown comparatively lower levels of the fumonisins (maximum level of 798 µg.kg^{-1}) as well as MON (maximum level of 1.214 µg.kg^{-1}) in the investigated samples. However, a high incidence of the mycotoxins in the samples (98%, 84% and 52% for FB_1, FB_2 and MON incidence, respectively) should not be neglected in any case. It is also notable that all of the *F. proliferatum* tested (86 isolates) have been proved to produce FB_1 after 7 days cultivation on YES at 25ºC. This finding is in concordance with [e.g. 1, 8, 10, 39, 44] who recovered *F. proliferatum* isolates from corn-based feeds and corn, or both, and all isolates tested appeared to be FB_1-producers *in vitro*. In Slovakia, there is also known a report dealing with FB_1-production observed in all *F. moniliforme* isolates tested (obsolete name for *F. verticillioides*, a species ecologically and morphologically closely related to *F. proliferatum*, readily distinguishable by absence of polyphialides [9, 29, 38]), which were recovered from stored corn [32]. Accordingly, it is highly recommended to consider all isolates of *F. proliferatum* and/or *F. verticillioides* species recovered from cereal related habitats, including feeds and foods, as relevant producers of carcinogenic fumonisins. This is supported by the fact that in all samples investigated during this study, the FB_1 was detected also in those contaminated with *F. proliferatum* simultaneously. Concerning ecological con-

ditions versus the *F. proliferatum* occurrence, unusually drier and warmer summers, like those prevailing in the 1990s, led to an increase of *F. proliferatum* in central Europe [25]. According to Šrobárová *et al.* [41] in Slovakia, and Adler et al. [2] in Austria, the overall number of *F. proliferatum* infections rose from less than 1% in the 1980s to 2–11% towards the end of the 1990s, leading to an expected increase of fumonisins in contaminated maize samples. Thus, findings of *F. proliferatum* isolates in substrates such as feed mixtures may indicate a heightened suspicion of the presence of these mycotoxins directly in the feeds. In addition to *F. proliferatum*, a total of 16 *F. subglutinans* isolates originated from the samples investigated in this study were tested for their ability to produce MON. Of these, 8 isolates (50%) were revealed as being producers of the toxin. Likewise, in the studies [14, 18, 27, 35, 45] the production of MON by *F. subglutinans* isolates encountered in cereals and/or cereals-based products has been reported, although in comparatively higher percentage of 70–100%. Interestingly, as can be seen from Table 1, in five samples there is no connection between the most contaminated samples, i.e. 35, 23 and 44 with levels of MON reaching 492, 682 and 1,214 $\mu g.kg^{-1}$, respectively, and the presence of potentially MON producing species, even in the sample showing the highest MON contamination, i.e. in sample No. 44. Other *Fusarium* species being renowned as significant contaminants of cereals including corn in the conditions of the central Europe [7, 25] and proven MON producers are mainly *F. avenaceum* and *F. acuminatum* [15, 38, 43]. Especially *F. avenaceum* is responsible for so-called red rot in corn ears [25] as well as head blight in wheat [7]. Moreover, this species has shown to be one of the most frequently encountered species on asymptomatic grains of wheat collected in Slovakia [17]. Severe infections of small-grain cereals such as barley and wheat caused by *F. avenaceum* in central Europe (Austria) and northeastern Europe (Poland) have been usually responsible for the high content of MON in infected grains [6]. Hence, MON or any other mycotoxin, may subsequently outlast in food or feed even though its producers are no longer viable, due to either storage conditions, during processing of a commodity, or due to effect of chemical additives such as propionic acid [21, Šrobárová, personal communication].

CONCLUSION

The present study revealed that 50 randomly collected samples of poultry feed mixtures from Slovakia were contaminated with significant *Fusarium* mycotoxins, i.e. FB_1, FB_2, and MON with 98, 84 and 52% incidence, respectively, albeit in relatively low levels. Concerning the incidence of *Fusarium* species, *F. proliferatum* has shown to be an important fungus, being encountered at a comparatively high frequency and account. Moreover, all of the *F. proliferatum* isolates tested have been proved to be toxinogenic for FB_1, while none of them produced MON. On the other hand, the later mycotoxin has been detected only in a half of the *F. subglutinans* isolates tested *in vitro*. Despite the limited number of samples studied, it might be concluded that poultry feed mixtures represent a risk from the toxicological point of view as a potential source of the fumonisins and moniliformin in central Europe.

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