

## The genotoxicity of caecal water from gilts following experimentally induced *Fusarium* mycotoxicosis

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**ABSTRACT:** The examination of caecal contents allows one to investigate the exposure of colon mucosa to dietary toxins and the chemical contaminants present in animal feed. The objective of the present study was to examine the influence of *Fusarium* mycotoxins (zearalenone, ZEN, and deoxynivalenol, DON), administered separately and as a mixture, on the genotoxicity of caecal water (CW) from gilts fed these mycotoxins. CW genotoxicity was evaluated with the comet assay using the LLC-PK1 porcine epithelial kidney cell line. It was shown that after the first week of the experiment, the presence of DON in animal feed led to increased CW genotoxicity in the proximal colon, while the presence of DON and ZEN + DON had a similar effect in the distal colon (ANOVA,  $P < 0.05$ ). After the third week of experiment, elevated genotoxicity in the distal colon was observed for all experimental groups of gilts, and it was 62%, 52.4%, and 52.8% higher for ZEN-, DON-, and ZEN + DON-fed animals, respectively, than for control group animals. However, no effect on CW genotoxicity in the proximal colon was seen. After six weeks, the presence of ZEN in the proximal colon increased CW genotoxicity by 103% in comparison with the control group. In the distal colon, after the sixth week, CW genotoxicity in all groups of animals fed with mycotoxin was significantly (by 80% to 116%) higher than in the control group. ZEN and DON administered as a mixture did not lead to an increased genotoxicity compared to either agent administered separately. Generally, the mycotoxins clearly started to increase the genotoxicity of CW from the third week of administration.

**Keywords:** zearalenone; deoxynivalenol; pigs; genotoxicity; caecal water

### List of abbreviations

CW = caecal water; DON = deoxynivalenol; FBS = foetal bovine serum; GI = gastrointestinal; HPLC = high performance liquid chromatography; ZEN = zearalenone; ZOL = zearalenol

Fungi can contaminate various feed components, such as maize, wheat, barley, millet, peanuts, peas, and oily feedstuffs (Rohweder et al. 2013). The most important mycotoxin-producing fungi belong to the genera *Aspergillus*, *Penicillium*, *Fusarium*, *Alternaria*, and *Claviceps* (Doll and Danicke 2011). *Fusarium* mycotoxins are secondary metabolites produced by several species of *Fusarium* molds. They exhibit both acute and chronic aspects of toxicity and have been shown to have a broad variety

of toxic effects in animals (Cortinovis et al. 2013). Deoxynivalenol (DON) and zearalenone (ZEN) are two major mycotoxins produced by *Fusarium* fungi. Because of the natural co-occurrence of these mycotoxins in food and the globalisation of food markets, there is an increasing concern about health hazards linked to human and animal exposure to mycotoxin mixtures (Ma and Guo 2008; Wan et al. 2013). DON and ZEN are among the most frequently detected toxins in Europe. The results of a global

survey show that the *Fusarium* mycotoxins DON, fumonisins, and ZEN contaminated 55%, 54%, and 36% of feed and feed ingredients, respectively, in the years 2004–2011. Nevertheless, the majority of samples were found to comply with even the most stringent European Union regulations or recommendations on maximal tolerable concentrations (Antonissen et al. 2014). Exposure to these mycotoxins has been linked to reproductive disorders in animals. In farm animals, ZEN interferes with reproduction, and pigs have been found to be particularly susceptible to ZEN as typical signs of hyperoestrogenism have been described in them following the ingestion of ZEN-contaminated feed (Schoevers et al. 2012). *In vivo*, 22 mg ZEN/kg bw in the diet causes alterations in the reproductive tract of pigs (including the uterus), and affects follicular and embryo development (Tiemann and Danicke 2007). ZEN and its metabolites have been shown to bind competitively to oestrogen receptors in an *in vitro* system. Feeding a diet contaminated with 9 mg DON/kg bw to pigs can disturb protein synthesis as well as humoral and cellular immune response, depending on the dose, exposure, and timing of the functional immune assay, and can affect liver and spleen cell structures (Tiemann and Danicke 2007). ZEN has been shown to be hepatotoxic, haematotoxic, immunotoxic, and genotoxic (Wan et al. 2013).

DON usually enters the body via the oral route and subsequently encounters intestinal epithelial cells, which represent the primary target for alimentary intoxication (Savard et al. 2014). One effect of this mycotoxin on the intestine is a reduction in the expression of proteins at cell junctions (claudin-4, E-cadherin, occludin), resulting in changes in paracellular and transcellular permeability, favouring the penetration of pathogens (Basso et al. 2013). Exposure to 10 µM of DON induces a significant decrease in normal intestinal morphology and in the number of goblet cells (Basso et al. 2013).

Carcinogens and toxins may enter the animal colon directly with the ingested feed. Faeces are a complex mixture that reflects the diet consumed, so examination of caecal or faecal contents is a non-invasive way to investigate the exposure of the colon mucosa to putative risk factors in dietary intervention studies (Klinder et al. 2007). This biomarker approach is mainly used to detect DNA damage and the mutagenic effects of faecal/caecal samples (Pearson et al. 2009). Faecal or caecal wa-

ter genotoxicity is also a biomarker of the harmful action of the colon microbiota. The aim of the present study was to determine the genotoxicity of caecal water in caecal contents from gilts after exposure of the animals to zearalenone (ZEN) and deoxynivalenol (DON) separately and as a mixture (ZEN + DON).

## MATERIAL AND METHODS

**Animal treatment.** All experimental procedures involving animals were carried out in compliance with the Polish legal regulations determining the terms and methods of performing experiments on animals (opinion of the Local Ethics Committee for Animal Experimentation No. 88/N of December 16, 2009).

The experiment was conducted at the Department of Veterinary Prevention and Feed Hygiene, Faculty of Veterinary Medicine, University of Warmia and Mazury in Olsztyn, Poland, on 75 clinically healthy gilts with an initial body weight of  $25 \pm 2$  kg. The gilts were penned in groups with *ad libitum* access to water. The administered feed was tested for the presence of mycotoxins: ZEN,  $\alpha$ -ZEL, and DON. Mycotoxin levels in the diets were estimated by common separation techniques with the use of immunoaffinity columns (Zearala-Test<sup>TM</sup> Zearalenone Testing System, G1012, VICAM, Watertown, USA and DON-Test<sup>TM</sup> DON Testing System, VICAM, Watertown, USA) and high performance liquid chromatography (HPLC) (Hewlett Packard, type 1050 and 1100) (Obremski et al. 2003) with fluorescent and/or UV detection techniques.

**Study design.** A total of 39 animals were divided into two groups: control ( $n = 21$ ) and experimental ( $n = 18$ ). The animals from the experimental group were administered DON daily at a dose of 12 µg/kg body weight *per os* for 42 days. Analytical samples of the mycotoxin were applied in gelatin capsules before morning feeding. Mycotoxin samples were diluted in 300 µl 96% ethyl alcohol (96% ethyl alcohol, SWW 2442-90, Polskie Odczynniki Chemiczne SA, Poland) to obtain the required doses (subject to body weight). The resulting solutions were stored at room temperature for 12 h to evaporate the solvent. The animals from the control group were orally administered placebo (gelatin capsules without mycotoxin) for the same period of time as the experimental group. Amounts of toxin application were dependent on the body weight and

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updated weekly. The animals were weighed every seven days to update mycotoxin doses for each gilt. Both mycotoxins were synthesised and standardised by the Department of Chemistry of the Poznan University of Life Sciences under the supervision of Professor Piotr Golinski. Three animals from both groups (experimental and control) were sacrificed on Days 1, 7, 14, 21, 28, 35 and 42 (a total of six gilts on each day), excluding Day 1 when only three control group animals were sacrificed. Each time, the contents of the ascending colon were collected from three gilts from each experimental group. In all experimental groups, the mycotoxins were administered at NOAEL (no observable adverse effect level) doses (Schlatter 2004; Boermans and Leung 2007; Laufersweiler et al. 2012). The experiment was conducted over 42 days.

**Preparation and administration of diets.** The animals were kept in cages with *ad libitum* access to water and were fed standard diets tested for the presence of the following mycotoxins: aflatoxin, ochratoxin, ZEN,  $\alpha$ -zearalenol ( $\alpha$ -ZEL),  $\beta$ -zearalenol ( $\beta$ -ZEL) and deoxynivalenol. Mycotoxin concentrations in the diet were evaluated with the use of common separation techniques involving immunological affinity columns and high-performance liquid chromatography (HPLC) (Hewlett Packard, type 1050 and 1100) (Zwierzchowski et al. 2004) with fluorescent and/or UV detection techniques.

**Caecal water preparation.** Tissue samples were taken after resection of two 10 cm-long pieces of the colon. To preserve the tissues undestroyed, all pieces, before resection, were ligatured at both ends. First moiety – the proximal colon – was achieved by ligation of the first piece, five centimetres from the caecal junction; the second was done ten centimetres further. In the case of the distal colon, the first ligation was performed ten centimetres towards the caecum and rectum; the second was ten centimetres further, towards the head. Then, the tissues were collected in plastic containers, stored at  $-20^{\circ}\text{C}$  and immediately transported to the laboratory. For genotoxicity testing, freshly obtained caecal contents (20%) were mixed with sterile phosphate-buffered saline (pH 7.0) (80%), homogenised for 2 min, and centrifuged ( $10,700 \times g$ , 40 min,  $4^{\circ}\text{C}$ ). The supernatant fractions were filtered ( $0.45 \mu\text{m}$  pore size, Millipore) and stored at  $-20^{\circ}\text{C}$  until analysis.

**Cell culture and treatment.** The porcine kidney cell line LLC-PK1 (ATCC CL-101) was used in this

work. The cell line is often used as a model for mycotoxin eno- and cytotoxicity testing (Gutleb et al. 2002). The cells were cultured in Roux flasks as a monolayer in 199 Medium (Sigma) with the addition of 10% FBS (Invitrogen), 200mM L-glutamine (Sigma), and 25mM HEPES (Sigma). The cells were incubated in a  $\text{CO}_2$  incubator at  $37^{\circ}\text{C}$  under 5%  $\text{CO}_2$  for seven to 10 days. After reaching confluence, the cells were sub-cultivated every week. The medium was changed every three to four days.

LLC-PK1 cells were detached with TrypLE Express (Invitrogen) for 15–20 min and gently shaken off the plastic flask. As this reagent is of plant origin, neutralisation with FBS was not necessary. After detaching, the cell suspension in PBS was transferred into a 15 ml Falcon tube, centrifuged ( $182 \times g$ , 5 min), decanted, and resuspended in 199 Medium. After determining the cell number and viability by trypan blue exclusion (min. 85%), the cells were ready to use.

**Genotoxicity testing (single cell gel-electrophoresis assay).** The final concentration of LLC-PK1 cells in each sample was adjusted to  $10^5$  cells/ml. The cells ( $800 \mu\text{l}$ ) were incubated with CW ( $200 \mu\text{l}$ ) at  $37^{\circ}\text{C}$  for 1 h. Each experiment included a negative control (LLC-PK1 cells in Medium 199) and positive control, which was hydrogen peroxide at  $40 \mu\text{M}$  for 5 min on ice.

The comet assay was performed under alkaline conditions ( $\text{pH} > 13$ ) (Singh et al. 1988) with some modifications (Klaude et al. 1996; Blasiak and Kowalik 2000). After incubation, the cells were centrifuged ( $182 \times g$ , 15 min,  $4^{\circ}\text{C}$ ), resuspended in 0.75% LMP agarose (Sigma), layered onto slides precoated with 0.5% agarose, and lysed at  $4^{\circ}\text{C}$  for 1 h in a buffer consisting of 2.5M NaCl, 1% Triton X-100, 100mM EDTA, and 10mM Tris, pH 10. After the lysis, the slides were placed in an electrophoresis unit and DNA was allowed to unwind for 20 min in an electrophoretic solution containing 300mM NaOH and 1mM EDTA. Electrophoresis was conducted at  $4^{\circ}\text{C}$  for 20 min under an electric field strength of 0.73 V/cm (300 mA). Then, the slides were neutralised with 0.4 mol/l Tris, stained with  $2.5 \mu\text{g/ml}$  propidium iodide, and covered with cover slips. The slides were examined at  $\times 200$  magnification under a fluorescence microscope (Nikon, Japan) connected to a video camera and a personal computer-based image analysis system – Lucia-Comet v. 7.0 (Laboratory Imaging, Prague, Czech Republic). Fifty images were randomly selected

from each sample and the percentage of DNA in the comet tail was measured. Two parallel tests with aliquots of the same sample were performed for a total of 100 cells and the mean percentage of DNA in the tail was calculated and taken as a measure of DNA damage. Comet data were analysed using two-way analysis of variance (ANOVA), while a particular mode of interaction time was used to compare the effects induced by chemicals at this mode of interaction. Differences between means were compared using Scheffe's multiple comparison test. The results are presented as mean  $\pm$  SEM.

## RESULTS

### Analysis of mycotoxin levels in feed

The values reported in the analysis of mycotoxins in levels in feed were below the sensitivity of the method.

### Genotoxicity of caecal water in the proximal and distal colon

The non-exposed sample (LLC-PK1 cells in Medium 199, negative control) exhibited DNA damage of  $4.15\% \pm 0.56$  (as expressed by DNA percentage in the comet tail). The treatment of the cells with  $10\mu\text{M}$  hydrogen peroxide resulted in  $42.96\% \pm 3.11$  damage.

On the first day of the experiment (before the application of mycotoxins), the genotoxicity of CW

was  $15.26\% \pm 2.90$  and  $8.05\% \pm 1.67$  for the proximal and distal colon of gilts, respectively.

After the first week of the exposure of gilts to mycotoxins, the genotoxicity of proximal colon CW ranged from  $5.40\% \pm 0.79$  to  $39.43\% \pm 4.63$  and that of distal colon CW ranged from  $12.07\% \pm 2.10$  to  $50.39\% \pm 4.59$  (Table 1). After the second week, genotoxicity ranged from  $12.57\% \pm 2.02$  to  $64.53\% \pm 4.14$  in the proximal colon and from  $8.71\% \pm 1.76$  to  $46.04\% \pm 4.3$  in the distal colon (Table 1). Generally, in the first two weeks, the genotoxicity of CW from both parts of the colon was comparable, and the administration of mycotoxins did not influence the genotoxicity of CW, with the exception of DON (after the first week) and ZEN + DON (after the first and second weeks), which significantly increased genotoxicity (ANOVA,  $P < 0.05$ ) (Table 1).

From the third to sixth weeks of mycotoxin administration, the average genotoxicity of proximal colon CW did not change (Figure 1). The dispersion of genotoxicity values among individuals in all groups of gilts was high. In the proximal colon, the average increase was significant only for the ZEN group ( $39.56\% \pm 4.65$ ) after the sixth week, when in the control group the corresponding value amounted to  $19.48\% \pm 2.48$ . In the distal colon, a visible gradual increase (relative to the control group) in average CW genotoxicity following the administration of ZEN, DON, and ZEN + DON, was observed from the third week. In the sixth week, CW genotoxicity levels were significantly elevated (ANOVA,  $P < 0.05$ ), by about 80% to 116% for all the groups administered mycotoxins (Figure 1). In the control group, the average CW genotoxicity of the proximal

Table 1. Genotoxicity of caecal water from both the proximal and distal colon of gilts in the first and second week of the experiment, expressed as percentage of DNA in the tail of comets in the LLC-PK1 cell line, in an alkaline comet assay. The number of cells analysed in each treatment was 50. Data are mean values  $\pm$  SEM for three gilts

Week			DNA (%) in comet tail ( $\pm$ SEM)			
			control	ZEN	DON	ZEN + DON
1	Proximal colon	range	8.15–31.64	15.49–36.49	5.40–39.67	9.29–29.83
		mean	$21.21 \pm 2.76$	$26.54 \pm 3.62$	$28.17 \pm 3.31^*$	$19.56 \pm 3.21$
		range	17.60–37.59	13.44–64.53	12.57–22.21	25.41–28.00
		mean	$19.98 \pm 2.78$	$32.14 \pm 3.26$	$18.72 \pm 2.68$	$33.02 \pm 3.82^*$
2	Distal colon	range	15.37–26.67	12.07–13.58	18.16–50.39	20.90–31.58
		mean	$19.98 \pm 2.78$	$13.07 \pm 2.19$	$33.38 \pm 3.83^*$	$27.81 \pm 3.70^*$
		range	18.90–45.87	8.71–44.28	14.26–21.72	33.49–46.04
		mean	$36.40 \pm 4.27$	$25.10 \pm 2.99$	$17.25 \pm 3.01$	$39.75 \pm 4.17^*$

\*significantly different from the control in a given week, ANOVA ( $P < 0.05$ )



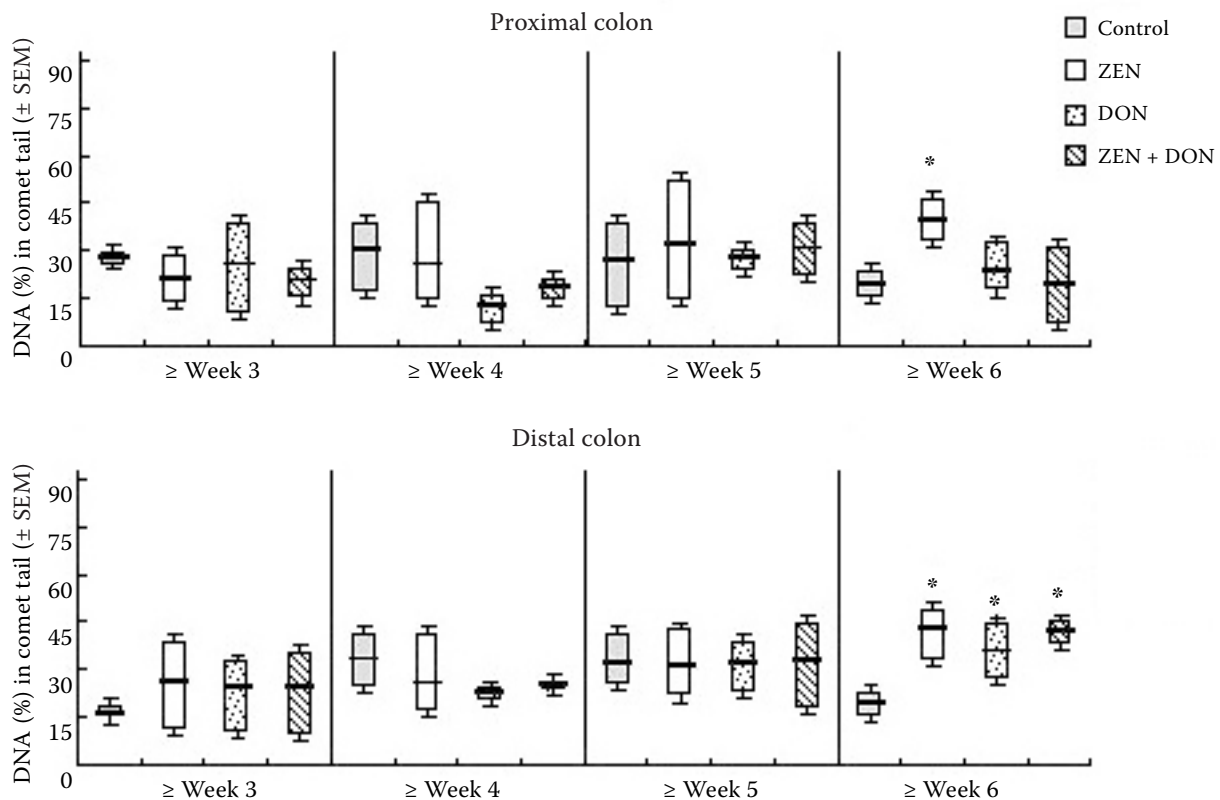


Figure 1. Genotoxicity of caecal water from the proximal and distal colon of pigs. The mean values for three gilts in each group  $\pm$  SEM are given, expressed as percentage of DNA in the tail of comets in an alkaline comet assay. The number of cells analysed in each treatment was 50

\*results significantly different from the control in a given week, ANOVA ( $P < 0.05$ )

colon was similar to that of the distal colon after the sixth week ( $19.48\% \pm 2.48$  and  $19.70\% \pm 2.92$ , respectively).

## DISCUSSION

Feeding animals fodder contaminated with mycotoxins can contribute to the accumulation of carcinogens in animal tissues, which might also have a harmful effect on human health after the consumption of contaminated meat. ZEN was shown to cause a variety of toxic effects in both experimental and farm animals, and is also suspected of being toxic to humans (Gajecki et al. 2010). Following oral intake of low to moderate amounts of the studied mycotoxins, the gastro-intestinal epithelial cell layer will be affected first. The intestinal mucosa acts as a barrier, preventing the entry of foreign antigens including food proteins, xenobiotics (such as drugs and toxins), commensal microbiota, and pathogens into the underlying tissues (Antonissen

et al. 2014). According to the literature, ZEN is quickly absorbed from the GI tract. ZEN metabolism in monogastric animals takes place in the liver and intestinal mucosa, where it is hydroxylated to  $\alpha$ - and  $\beta$ -zearalenol (ZOL) (Tiemann and Danicke 2007). ZEN,  $\alpha$ -ZOL, and  $\beta$ -ZOL are quickly absorbed (in less than 30 min) from the GI tract into the blood. The metabolism of ZEN occurs also in peripheral blood erythrocytes and epithelial cells of the GI tract (Malekinejad et al. 2006). It is toxic when administered *per os*, especially to gilts. It was reported (Obremski et al. 2005) that ZEN administered at a dose of  $400 \mu\text{g/kg bw}$  contributed to aberrant crypt foci within the intestinal mucosa.  $\alpha$ -ZOL binds more effectively to receptors than the parent toxin and might therefore explain the sensitivity of pigs to this mycotoxin. An accumulation of  $\alpha$ -ZOL might inhibit cell proliferation in the system, which might be mediated by apoptotic mechanisms of cell death (Luongo et al. 2006). The other major biotransformation pathway for ZEN is conjugation with glucuronic acid. These hydroxylation reactions

compete in the intestines and liver with conjugation reactions, particularly following oral ingestion of the toxin with feeds (Malekinejad et al. 2006).

The intestines are the main site of DON absorption. In pigs, DON is rapidly and efficiently absorbed, most probably in the upper part of the small intestine, and is mainly excreted in the urine. The consumption of DON-contaminated feed in pigs impacts the GI tract, causing epithelial damage in the stomach and intestines and leading to intestinal inflammatory response. *In vitro* and *in vivo* studies have also demonstrated that DON inhibits intestinal nutrient absorption, alters intestinal cell functions, and compromises the intestinal barrier (Wache et al. 2009). DON is also implicated in reduced reproductive performance in pigs, due to its ability to impair oocyte maturation and embryo development (Cortinovis et al. 2013). DON can inhibit translation and induce chromosome aberrations – mostly chromatid breaks. Results from a comet assay showed that DON increased the mean tail moment in human Caco-2 cells in a dose-dependent manner at concentrations of 0.01–0.05  $\mu$ M (3–15 ng/ml), which did not induce apoptosis (Sobrova et al. 2010).

In our research, high week-to-week variations in genotoxicity, even in the control groups were observed. The reason could be that immature gilts, which do not possess stable intestinal microbiota, were used in the experiment. The genotoxicity of CW varies among individuals and many factors can influence it. These factors could include stress, internal metabolism of the dietetic compounds, quality and quantity of gastrointestinal microbiota and their metabolites. Both mycotoxins, ZEN and DON, increased the genotoxicity of CW. The most significant increase in genotoxicity was observed after the sixth week of the experiment in the distal colon, which was probably caused by the accumulation of ZEN, DON, and ZEN + DON in porcine tissues. In our previous study (Waskiewicz et al. 2014) it was shown that DON, even when applied for a short period, resulted in its presence in gastrointestinal tissues. The concentrations of DON reported in the large intestine samples increased gradually from the second (in the proximal part) and from the fourth (in the distal part) week of experiment, and ranged from 2.5 to 20.52 ng/g (in ascending colon) and from 6.75 to 20.0 ng/g (in descending colon).

In the proximal colon, the transit time of intestinal contents is faster than in the distal colon, and

that is why a gradual increase in genotoxicity was not observed in the former (except for the ZEN group). A fast transit time shortens the contact of carcinogens with the colon epithelium. While in the proximal colon genotoxicity remained at a similar level in the control group throughout the experiment, in the distal colon it increased. Thus, harmful metabolites accumulate even as a result of the consumption of food without any toxins. The longer the transit time was, the longer the exposure of cells to genotoxins and their harmful interaction with the colon. The intestinal transit time in the distal colon is the longest out of all parts of the GI tract. Slow transit of intestinal contents, especially following the consumption of feed contaminated with carcinogens, e.g., mycotoxins, increases the risk of colon diseases, including tumours.

Furthermore, the distal part of the colon is the region with the most complex microbial ecosystem and metabolic activity. In the distal colon, proteolytic processes dominate over fermentation. As the result of proteolysis, harmful substances can be produced: cresols, indoles, skatoles, ammonium, biogenic amines, and others. The concentration of proteolytic products in the distal colon can be even 100 times higher than in its proximal part. Mycotoxins can increase the number of mesophiles, especially *Escherichia coli*, with very active faecal enzymes, which transform pro-carcinogens into carcinogens, increasing the genotoxicity of caecal and faecal water (Wache et al. 2009). Additionally, the colon microbiota can activate and transform potentially harmful substances to their equally or more toxic derivatives.

In previous tests, our analysis revealed the predominance of lactic acid bacteria in all groups of pigs. ZEN administered separately and together with DON, was found to have an adverse effect on mesophilic aerobic bacteria, but only after long exposure to this mycotoxin. During the six weeks of the experiment, the amount of *Clostridium perfringens* and *Escherichia coli* was most considerably reduced in the experimental groups exposed to zearalenone, both separately and together with DON (Piotrowska et al. 2014).

ZEN and DON administered to gilts (40  $\mu$ g/kg bw and 12  $\mu$ g/kg bw, respectively) stimulated the genotoxicity of CW, which increased gradually, especially beginning in the third week of administration. ZEN and DON administered as a mixture did not lead to increased genotoxicity compared to either agent administered separately. The toxicity

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of DON is partially explained by the ability of the compound to bind to eukaryotic ribosomes and to induce rapid activation of the mitogen-activated protein kinases via a process termed the “ribotoxic stress response” (Pinton and Oswald 2014). Our unpublished histopathologic data of the jejunum demonstrate that the antiproliferative effects in relation to goblet cells, T- and B-lymphocytes and plasma cells is much stronger after administration of a single type of mycotoxin than after administration of their mixture. The latter observation suggests that the effects of mycotoxins alone and in combination need to be better understood to assess health risks. Although the applied doses were small and acceptable in feeds, the studied *Fusarium* mycotoxins could probably cause pathological changes in porcine bodies. Consequently, the acceptable mycotoxin intake levels with feeds should be verified.

In our research, the feed intake may have had an impact on the results. In deoxynivalenol mycotoxicosis a reduction in feed intake is proportional to the dosage. In the case of low doses of ZEN we observed an increase in feed intake only at the beginning of administration of mycotoxin. In mixed mycotoxicosis DON is dominant so a decrease in feed intake was observed. It seems possible that differences in the feed form could modulate the bioavailability of DON, as it could affect the liberation of the toxin from the matrix and thereby influence residue concentration in the animal tissue. In this study, the daily DON intake during the whole period of the experiment reflects slight differences between animals because of DON doses converted into body weight and precisely administered by a small portion of the feed matrix (Waskiewicz et al. 2014).

The present study on the genotoxicity of CW from the colon contents of gilts fed with *Fusarium* mycotoxins (ZEN, DON, and ZEN + DON) utilises an innovative approach and reports novel findings. The increasing genotoxic tendency of CW after the administration of mycotoxins to gilts, which was observed mostly from the third week in the distal colon, would have probably been more pronounced if the experiment had been prolonged, e.g., to 12 weeks. This remains a subject for further research.

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