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Original article

Cecal enzyme activity in gilts following experimentally induced *Fusarium* mycotoxicosis

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Abstract

The objective of the presented study was to examine the influence of *Fusarium* mycotoxins (zearalenone – ZEN and deoxynivalenol – DON), administered separately and in combination, on the activity of cecal enzymes (β-glucosidase and β-glucuronidase) in gilts which were fed fodder contaminated with these mycotoxins. The activity of β-glucosidase and β-glucuronidase varied in the range of 0.170-1.236 μmol · h^{-1} · mg^{-1} and 8.701-96.704 μmol · h^{-1} · mg^{-1}, respectively. In the first two weeks, the toxins had no significant effect on the activity of β-glucosidase and β-glucuronidase in the ascending and descending colon. After week 3 and later on, ZEN and DON administered as a mixture led to the highest increase in the activity of both enzymes. Administered separately, DON affected the activity of enzymes more than ZEN. From the third week of the experiment, an increase in the activity of CW β-glucosidase and β-glucuronidase was observed.

Key words: zearalenone, deoxynivalenol, gilts, β-glucosidase, β-glucuronidase

Introduction

Mycotoxins are toxic products generated by naturally occurring metabolic processes in fungi, which fungi can contaminate various feed components, such as maize, wheat, barley, millet, peanuts, peas, and oily feedstuffs (Antonissen et al. 2014). No geographic region is free from the occurrence of mycotoxins. According to Lawlor and Lynch (2005), 25% of global crops are contaminated with mycotoxins. Out of the 7049 maize, soybean meal, wheat, and finished feed samples analyzed in the years 2009-2011 for the presence of aflatoxin, zearalenone, deoxynivalenol, fumonisins, and ochratoxins in the Americas, Europe, and Asia, 81% were positive for at least one mycotoxin; aflatoxins were present in 33%, deoxynivalenol in 59%, and fumonisins in 64% (Rodrigues and Naehrer 2012). A report by the Food and Agriculture Organization of the United Nations (FAO 2004) on mycotoxin regulations around the world revealed that at least 77 countries have now specific rules concerning acceptable concentrations of mycotoxins in foods and feeds. Regardless of the geographical area (temperate, subtropical, or tropical zone), a filamentous fungus infection of grain is likely if humidity is high at the time of harvest.

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Although the number of isolated and chemically characterized mycotoxins reaches 300, research usually focuses on those most affecting animal production, such as deoxynivalenol (DON), zearalenone (ZEN), trichothecenes, ochratoxins, aflatoxins, and fumonisins (Rohweder 2013).

DON and ZEN are among the most frequently detected toxins in Europe. They are produced by toxicogenic fungi of the genus *Fusarium*, attacking cereals, grains, and corn cobs (Park et al. 1996). They are characterized by high toxicity, directly related to the presence of a highly reactive epoxide ring in their structure (D'Mello et al. 1993, Grenier and Applegate 2013). At the cellular level, trichothecenes are responsible for the inhibition of protein biosynthesis, reduction of enzymatic activity, abnormal cytoplasmic membrane permeability, abnormal cell division, and cell cycle progression (Hughes and Rowland 2000). In infected wheat and triticale grains, DON content reaches on average 30 mg/kg (Bottalico and Perrone 2002), while the average ZEN content is in the range of 1000-2000 µg/kg of grain (Swamy et al. 2003). The Joint FAO/WHO Expert Committee on Food Additives (JECFA) has set the maximum tolerable daily intake (PMTDI) of ZEN at 0.5 µg/kg BW, considering its estrogenic activity and metabolism. Chronic and acute exposure of animals by DON were estimated to be between 3.9 and 43.3 µg/kg BW per day and between 11.6 and 137.9 µg/kg BW (FAO/WHO 2000).

The consumption of DON-contaminated feed affects the GI tract in pigs, causing epithelial injuries of the stomach and the intestine, leading to intestinal inflammatory response. *In vitro* and *in vivo* studies have also demonstrated that DON inhibits intestinal nutrient absorption, alters intestinal cell function, and compromises the intestinal barrier function (Wache et al. 2009). ZEN is quickly absorbed from the GI tract. ZEN is metabolized in hepatocytes, peripheral blood erythrocytes, and epithelial cells of the GI tract (Malekinejad et al. 2006). It is toxic, especially for gilts, when administrated *per os*. According to Obremski et al. (2008), ZEN administered at a dose of 400 µg/kg BW contributes to aberrant crypt foci within the intestinal mucosa.

Feeding animals with mycotoxin-contaminated fodder may contribute to the accumulation of carcinogens in animal tissues, and may also have a harmful effect on human health after consumption of contaminated meat. ZEN has been shown to cause a variety of toxic effects in both experimental and farm animals, and is also suspected to cause toxicity in humans (Gajęcki et al. 2010).

Due to the procarcinogenic and proinflammatory properties of both examined toxins, attempts were made to evaluate their effects in the feces of gilts by determining the activity of two enzymes, β-glucuronidase and β-glucosidase, which are factors initiating the creation of unfavorable changes in the gut. It has been repeatedly shown that increased concentrations of these enzymes are directly correlated with increased production of harmful metabolites and more severe inflammations (Hughes and Rowland 2000, Beaud et al. 2005, De Moreno and Perdigón 2005). Therefore the objective of the present study was to determine the activity of β-glucuronidase and β-glucosidase in the cecal feces of gilts after exposing the animals to zearalenone (ZEN) and deoxynivalenol (DON) separately and as a mixture (ZEN+DON). The presented study is fully innovative, because analysis of the activity of β-glucosidase and β-glucuronidase in the course of exposure to *Fusarium* mycotoxins has not been the subject of any investigation to date.

Materials and Methods

Animal treatment

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 ± 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, α-ZEL and DON. Mycotoxin levels in the diets were estimated by common separation techniques with the use of immunoaffinity columns (Zearala-Test™ Zearalenone Testing System, G1012, VICAM, Watertown, USA and DON-Test™ 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.

All the experimental procedures involving animals were carried out in compliance with 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 16 December 2009).

Study design

The animals were divided into three experimental groups (ZEN, DON, and ZEN+DON; n = 18 in each group) and one control group (C; n = 21). Animals in the ZEN group were orally administered 40 µg ZEN/kg BW, animals in the DON group were orally

administered 12 DON μg/kg BW, and animals in the ZEN+DON group were orally administered a mixture of ZEN and DON (40 μg ZEN/kg BW + 12 μg DON/kg BW). The control animals were fed a placebo. In all experimental groups, the mycotoxins were administered at doses below the NOAEL (Boermans and Leung 2007). Both mycotoxins were synthesized and standardized by the Department of Chemistry of the Poznan University of Life Sciences under the supervision of Professor Piotr Goliński. The experiment covered a period of 42 days. Three animals from each group were sacrificed on days 1, 7, 14, 21, 28, 35, and 42 (a total of 12 gilts on each day), excluding day 1, when only three control animals were scarified.

Cecal water preparation (CW)

Cecal samples were collected in plastic containers. For testing cecal enzyme activity, 0.7 g aliquots of each cecal sample was transferred into a tube containing 7 mL of 0.2 mol/L phosphate buffer (pH 7.0). The samples were homogenized, disintegrated by ultrasound for 2 minutes (impulse length 6 s, amplitude 60) at 0C (ice bath). The cell debris were separated by centrifugation (10.700 × *g*, 20 min, 4°C). The supernatant fractions were filtered (0.20 μm, *Millipore*) and used for analysis.

Determination of β-glucuronidase and β-glucosidase activity

Enzymatic activity was measured by the spectrophotometric method. The activity of β-glucuronidase (phenolphthalein mono-β--D-glucuronic acid substrate, Sigma) and βglucosidase (p-nitrophenyl-β-D-glucopyranoside substrate, Sigma), was determined at 37°C as described by Freeman (1986) with some modifications (Ling et al. 1994). Activity units were calculated as the amount (μmol/L) of phenolphthalein (for β-glucuronidase) and nitrophenyl (for β-glucosidase) released per 1 h per 1 mL (U) per 1 mg of protein (U/mg). Protein content was determined using Lowry's method.

Statistical analysis

Statistical analysis was performed using two-way analysis of variance (ANOVA, $P<0.05$). Results are presented as means of three replications.

Results

Activity of β-glucosidase

The activity of β-glucosidase, throughout the study period, varied in the range of 0.170-1.236 $\mu mol \cdot h^{-1} \cdot mg^{-1}$. In the first two weeks of the experiment, the tested toxins had no effect on the activity of the enzymes (activity of β-glucosidase was 0.170-0.421 $\mu mol \cdot h^{-1} \cdot mg^{-1}$). Indeed, there were no statistically significant differences between the three study groups (ZEN, DON, and ZEN+DON) and the control group either in the proximal or distal colon (Fig. 1A). After the third week of the experiment, the activity of β-glucosidase increased threefold as compared to the week before and differed between all the experimental groups. The highest activity of this enzyme was found in the proximal colon contents of gilts from the ZEN+DON group (0.866 $\mu mol \cdot h^{-1} \cdot mg^{-1}$). A lower activity was found in the DON (0.694 $\mu mol \cdot h^{-1} \cdot mg^{-1}$) and ZEN (0.512 $\mu mol \cdot h^{-1} \cdot mg^{-1}$) groups, and the lowest in the control group (0.306 $\mu mol \cdot h^{-1} \cdot mg^{-1}$). This trend continued in the subsequent weeks of the experiment (Fig. 1A).

A similar effect was observed for the activity of β-glucosidase in the distal colon, with the difference being that after week three the highest enzymatic activity was found in the DON group, while in the subsequent weeks it was found in the mixed group (ZEN+DON). As in the proximal colon, the lowest activity of β-glucosidase was observed in the control group (Fig. 1B).

After week three (in the proximal colon) and week four (in the distal colon), and then in the subsequent weeks of the experiment, ZEN and DON administered as a mixture (ZEN+DON) caused the highest increase in the activity of β-glucosidase. In the case of these toxins administered separately, DON was observed to activate β-glucosidase more than ZEN, with the exception of week five, when the trend was reversed in the distal colon.

In the proximal colon, the activity of β-glucosidase increased every week in the ZEN and ZEN+DON groups, reaching the highest activity after week six. In case of DON, the highest activity of the enzyme was found after week five, and then decreased slightly in the subsequent week. In the distal colon, the highest activity of β-glucosidase was found in the DON and ZEN+DON groups after week three and in the ZEN group after week four. In the subsequent weeks, the activity of this enzyme in the above-mentioned groups slightly declined.

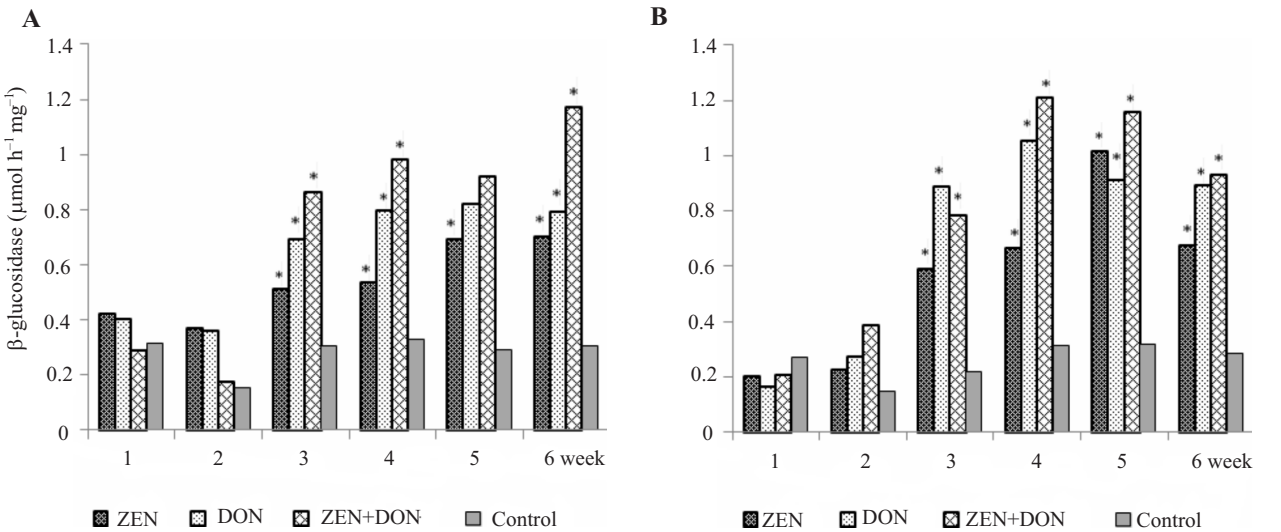

Fig. 1. Activity of β-glucosidase in the proximal (A) and distal colon (B) of gilts after experimentally induced *Fusarium* mycotoxicosis. Results significantly different (*) from the control in a given week of experiment, ANOVA (P<0.05).

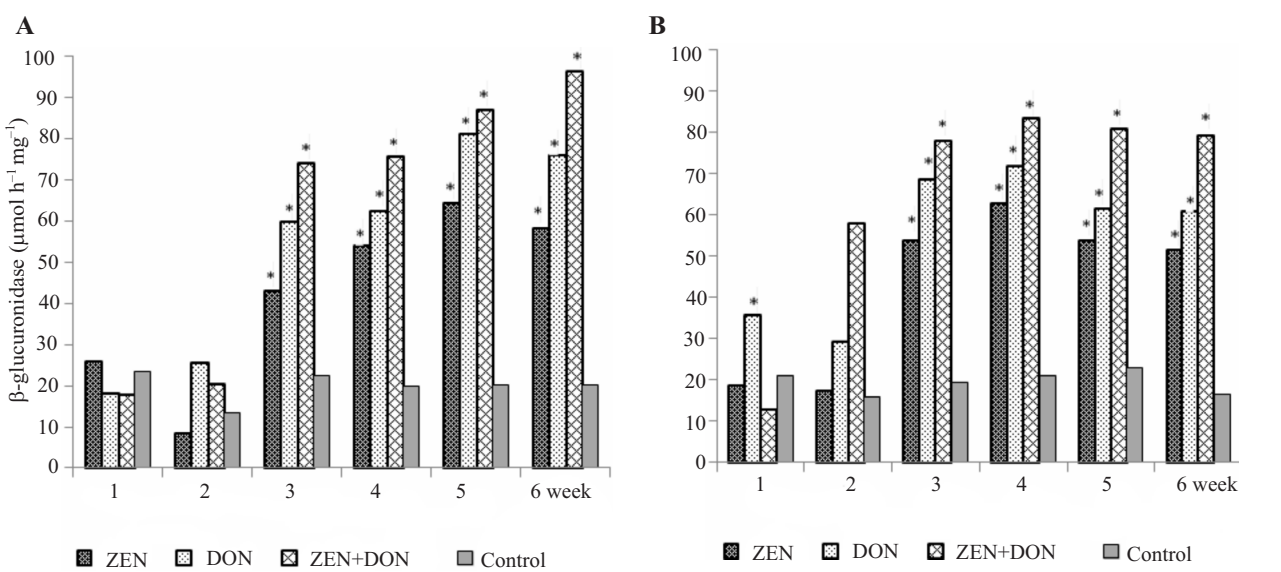

Fig. 2. Activity of β-glucuronidase in the proximal (A) and distal colon (B) of gilts after experimentally induced *Fusarium* mycotoxicosis. Results significantly different (*) from the control in a given week of experiment, ANOVA (P<0.05).

It was also found that after week three and in the subsequent weeks of the experiment, the type of the toxin used had a statistically significant effect (ANOVA, P<0.05) on the activity of β-glucosidase (both in the proximal and distal colon) in comparison with the control. The results were statistically significant for all the experimental groups (ZEN, DON, ZEN+DON).

β-glucuronidase activity

The activity of β-glucuronidase, was significantly lower than β-glucosidase and throughout the period study was in the range 8.701-96.704 μmol · h^{-1} · mg^{-1}. In the case of β-glucuronidase, the change in its enzymatic activity occurred after the first week of the experiment, especially in the distal colon. The highest activity was observed in the DON group (35.89 μmol · h^{-1} · mg^{-1}).

In the ascending colon, the activity of this enzyme increased after week three and remained at a similar level over the next few weeks of the experiment (Fig. 2A).

After week two (in the distal colon) and three (in the proximal colon), the highest activity of β-glucuronidase was observed in the ZEN+DON group, and this trend continued until the end of the

experiment. In the groups in which the toxins were administered separately, it could be seen that DON activated β-glucuronidase more than ZEN.

In the proximal colon, the activity of β-glucuronidase increased every week in the mixed group (ZEN+DON), reaching the highest activity after week six. For the DON and ZEN groups, the highest activity of this enzyme was observed after the fifth week of the experiment, and then decreased slightly over subsequent weeks. In the distal colon, the highest activity of β-glucuronidase in the ZEN, DON, and ZEN+DON groups was observed after week three. In subsequent weeks, there was a slight decrease in the activity of this enzyme in all the groups (Figs. 2A and B).

It was also found that after week three and in the subsequent weeks the type of toxin used had a statistically significant effect on the activity of β-glucuronidase (in the proximal and distal colon) in comparison with the control group. Statistically significant results were also found between the ZEN, DON, ZEN+DON groups and the control group.

Discussion

The enzymes selected for this study are known to be potential mediators of colon carcinogenesis. β-glucuronidase is involved in the hydrolysis of glucuronides in the gut and releasing free compounds which can cause CRC (De Moreno de LeBlanc and Perdigón 2005). β-glucosidase hydrolyses glycosides to sugar groups (glycones) and non-sugar groups (aglycones). Glycosides that are undigested in the upper parts of the gastrointestinal tract are hydrolyzed by enzymes in the colon, forming toxic and carcinogenic aglycones (Nakamura et al. 2002). The highest amounts of fecal enzymes are produced by anaerobic colon bacteria such as: *Bacteroides* (*B. vulgatus*, *B. uniforme*, *B. fragilis*), *Clostridium* (*Cl. paraputrificum*, *Cl. clostridioforme*, *Cl. perfringens*), *Enterococcus*, *Peptostreptococcus*, *Staphylococcus*, *Ruminococcus* (*R. gnavus*), *Eubacterium*, and *Escherichia coli* (Nakamura et al. 2002, De Moreno de LeBlanc and Perdigón 2005).

The distal colon holds the most complex microbial ecosystem with an equally complex metabolic activity. In that part of the colon, proteolytic processes dominate over fermentation, which may lead to the generation of harmful substances, such as cresols, indoles, skatoles, ammonium, biogenic amines, and others. The concentration of proteolytic products in the distal colon can be even 100 times higher than that in the proximal colon. Mycotoxins can increase the number of mesophiles, especially *Escherichia coli*, which produce high activity fecal enzymes, transforming procarcinogens into carcinogens and thus increasing the genotoxicity of cecal or fecal water (Waché et al. 2009). Additionally, the colon microbiota can activate and transform potentially harmful substances into their equally or more toxic derivatives.

Feed contaminated with ZEN can be the cause of still birth, neonatal mortality, splay legged postpartum piglets, and repetition of estrus. This could be caused by the direct similarity of the chemical structure of ZEN to that of sex hormones, interfering with estrogens in females (De Moreno de LeBlanc and Perdigón 2005). ZEN in an amount of 1-5 mg/kg BW can cause swelling and redness of the reproductive organs in pigs, while 100 mg/kg BW dosage in feed can cause complete infertility. It has been found that at concentrations of 50-100 μg/kg in pig diet, ZEN affects oocyte implantation, ovulation, and fertilization. Similar changes were observed by Andretta et al. (2010) and Teixeira et al. (2011), who administered ZEN in an amount of 2 mg/kg BW and 0.75 mg/kg BW, respectively. Hyperemia and swelling of the vulva were observed in the first and third weeks of the experiment, respectively. Similar results were obtained by Aucock et al. (1980), Kordic et al. (1992), and Biehl et al. (1993). They observed changes in the reproductive organs, uterine metaplasia, and hyperplasia of the vulva. They found that piglets may be exposed to ZEN in their embryonic life and directly by contact with the mother's milk. ZEN was found to be toxic and carcinogenic. In gilts, experimental administration of ZEN or contaminated fodder at relatively low doses (from 1.5 to 2 ppm) leads to swelling and thickening of the vaginal and vulvar wall, increased uterus mass, and atrophic ovaries, but without the standing reflex (Andretta et al. 2010).

Rotter et al. (1996) demonstrated that the animals most sensitive to DON are pigs. DON content in the range of 1-2 mg/kg of fodder resulted in decreased appetite, while a dose of 3 mg/kg of fodder was the direct cause of reduced body temperature and changes in the gastric wall in piglets. Symptoms of total refusal of feed intake occurred beyond 12 mg DON/kg of fodder, and vomiting was observed at a concentration of 20 mg DON/kg of feed (Rotter et al. 1996). Smith et al. (1997) and Danicke et al. (2004) demonstrated that with increasing DON concentrations in fodder, its consumption by pigs decreases. This effect is usually observed in the presence of DON in feed at concentrations from 1000 to 2000 mg/kg. Feeding pigs with DON-contaminated fodder can compromise their immune system (cellular and humoral), leads to metabolic disturbances in the liver and spleen, mainly due to inhibition of RNA, DNA, and protein synthesis (Smith and Az-Llano 2009), and

causes reproductive alterations, resulting in decreased oocyte and embryo development (Tiemann and Danicke 2007, Ranzenigo et al. 2008, Kanora and Maes 2009).

In our study, ZEN and DON administered to gilts at doses of 40 µg/kg BW and 12 µg/kg BW, respectively, stimulated the activity of fecal enzymes, which increased gradually, especially from the third week of administration. Therefore, the acceptable levels of mycotoxin intake with feed should be verified.

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