ESCOLA UNIVERSITÁRIA VASCO DA GAMA

MESTRADO INTEGRADO EM MEDICINA VETERINÁRIA

DEOXYNIVALENOL AND ZEARALENONE NATURAL OCCURRENCE IN SWINE FEEDSTUFFS AND HISTOPATHOLOGICAL ANALYSIS

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List of Abbreviations and Acronyms

ADFI	Average daily feed intake
ADG	Average daily gain
BW	Body weight
DNA	Deoxyribonucleic acid
DON	Deoxynivalenol
ER	Endoplasmic reticulum
ER-α	Estradiol receptor-alfa
EC	European Commission
EU	European Union
H&E	Hematoxylin and eosin
INE	Statistics Portugal (from Portuguese *Instituto Nacional de Estatística*)
NaCl	Sodium chloride
PAS	Periodic acid Schiff
PB	Prussian blue
RNA	Ribonucleic acid
ZEA	Zearalenone

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ABSTRACT

Deoxynivalenol (DON) and Zearalenone (ZEA) are the most frequent and toxicologically important Fusarium mycotoxins detected in cereal grains and feedstuffs, causing toxic effects that impair the performance of farm animals, with pigs being the most susceptible species.

The objectives of this study were to evaluate the occurrence of DON and ZEA in feed cereal grains and compound feed destined to swine consumption, to perform a histopathological study using liver samples collected from gilts and sows fed with these compound feeds. Twelve ingredients (unprocessed maize, barley, wheat, bran, rapeseed, soybean) and twenty-six samples of swine compound feed were analyzed. The histopathological study was performed using samples from slaughtered pigs that had been fed the analyzed compound feed for at least two weeks.

Results showed that 71.1% of the samples were positive for ZEA and 78.9% for DON. Histologically, diffuse degenerative changes compatible with hepatosis were observed.

KEYWORDS
Deoxynivalenol; Feed; Histopathology; Mycotoxin; Swine; Zearalenone; Cereals.

Sumário

Desoxinivalenol (DON) e Zearalenona (ZEA) são as micotoxinas de Fusarium mais frequentes e toxicologicamente importantes detetadas em cereais e alimentos para animais, causando efeitos tóxicos que prejudicam o desempenho de animais de produção, sendo os suínos a espécie mais suscetível.

Este estudo teve como objetivo avaliar a ocorrência de DON e ZEA em grãos de cereais e alimentos compostos destinados ao consumo de suínos, bem como realizar um estudo histopatológico com a utilização de amostras de fígado recolhidas de marrãs e porcas alimentadas com estes alimentos compostos. Foram analisados doze ingredientes (milho não processado, cevada, trigo, farelo, colza, soja) e vinte e seis amostras de alimento composto para suínos. O estudo histopatológico foi realizado utilizando amostras de suínos abatidos que haviam sido alimentados com os alimentos compostos analisados durante pelo menos duas semanas.

Verificou-se que 71,1% das amostras foram positivas para a ZEA e 78,9% para a DON. Histologicamente, foram observadas alterações degenerativas difusas compatíveis com hepatose.

Palavras-Chave

Desoxinivalenol; Alimento destinado ao consumo animal; Histopatologia; Micotoxina; Suíno; Zearalenona; Cereais

1. INTRODUCTION

Mycotoxins are low molecular weight natural products, produced as secondary metabolites by filamentous fungi (Bracarense et al., 2020; Brown et al., 2017; Puntscher et al., 2018, cit. by de Santis et al., 2019; Andersen et al., 2012, cit. by Schulz et al., 2019). In spite of the hundreds of mycotoxins described in the literature, the number of known mycotoxins with significant economic, agronomic and health significance to farm animals is quite limited (Weaver et al., 2014, cit. by Kim et al., 2019; Logrieco et al., 2018; Aravind et al., 2003, cit. by Weaver et al., 2020).

Deoxynivalenol (DON) is the most frequently detected trichothecene (Type B trichothecenes) in animal feeds worldwide (Van Der Fels-Klerx et al., 2012, cit. by Liu et al., 2016; Sobrova et al., 2010, cit. by Sayyari et al., 2018; Rocha et al., 2005, cit. by Schwartz-Zimmermann et al., 2017). DON was first isolated from barley in Japan and named "Rd-toxin" (Rodrigues & Naehrer, 2012 cit. by Kang et al., 2019), and shortly thereafter, isolated from maize associated with emesis in pigs and thus coined as "vomitoxin" (Vesonder et al., 1973, cit. by Tang et al., 2019). Besides vomiting, ingestion of DON-contaminated feed is associated with feed refusal or decreased feed intake and digestive disorders that reduces body growth and promotes a general loss of condition (Kahlert et al., 2019; Serviento et al., 2018). DON impairs the immune function in various livestock (Iqbal et al., 2020), induces apoptosis in hemopoietic progenitor cells (Parent-Massin, 2004, cit by Sun et al., 2014) inhibits protein, DNA and RNA synthesis and operates as an immunomodulator (Kahlert et al., 2019; Schwartz-Zimmermann et al., 2017). Therefore, organs/tissues showing high rate of cell turnover are regarded as particularly susceptible to trichothecenes, such as the lymphoid and hematopoietic tissues, and the gastrointestinal tract (Hascheck et al., 2002; Rocha et al., 2005, cit. by Gerez et al., 2015). At certain doses, DON can trigger leukocytosis, hemorrhages, endotoxemia, circulatory shock, and even death (Iqbal et al., 2020; Shalapy et al., 2020). Wang et al. (2020), also mention brain damage, stating that the morphology of nerve cells in the piglet hippocampus is affected by low doses of DON, inhibiting their proliferation and leading to apoptosis.

Zearalenone (ZEA), a macrocyclic lactone previously known as F-2 toxin, was first reported to be associated with estrogenism in pigs in 1928 (McNutt et al., 1928, cit. by Morgavi & Riley, 2007). The basis for the estrogenic effect is now well established and explained to a close structural similarity between zearalenone (and many of its metabolites) and estradiol (Osweiler, 2000, cit. by Morgavi & Riley, 2007) that permits a binding affinity to estrogen receptors (17β-estradiol receptor), higher for ER-α (Pistol et al., 2015;Tiemann cit. by Catteuw et al., 2019). The most common toxic effects include decreased fertility, anoestrus, abortion, and increased embryonic and foetal death. Also, ZEA toxicity is associated with reduced litter size, changed weight of adrenal, thyroid, pituitary glands in offspring and change in serum levels of progesterone and oestradiol (Pistol et al., 2015).

DON and ZEA are amongst the most frequent and toxicologically important *Fusarium* toxins (produced mainly by *Fusarium graminearum*) detected in cereal grains, and thus co-occurrence is regularly observed worldwide (Alexandre et al., 2018; Sayyari et al., 2018; Serviento et al., 2018) in both feed ingredients/ raw materials and feed intended for different animal species. Nevertheless, swine are

more susceptible to both *Fusarium* mycotoxins than other species. For DON the susceptibility is partly explained by differences in the metabolism of DON due to their limited metabolic capacity to biotransform DON into less toxic metabolites and possibly also to their high exposure to cereal-rich diets (Sayyari et al., 2018; Pinton & Oswald, 2014, cit. by Serviento et al., 2018). There are derived forms of DON from fungal acetylation which are 3-acetyl-deoxynivalenol and 15-acetyl-deoxynivalenol, from plant production such as deoxynivalenol-3-β-D-glucopyranoside and from bacterial transformation, namely de-epoxy-deoxynivalenol, 3-epi-deoxynivalenol and 3-keto-deoxynivalenol, called masked mycotoxin (Bracarense et al., 2020). Regarding ZEA, the relative binding affinity for estrogen receptors was greater in pig than in other species, particularly female pigs, which may explain the interspecies differences in sensitivity to the estrogenic effects (Fitzpatrick et al., 1989, cit. by Morgavi & Riley, 2007). After oral exposure, ZEA is rapidly absorbed (85%), being metabolized at intestinal level and in hepatic tissue. The biotransformation of this toxin leads to generation of its metabolites, (α- and β-zearalenol, α- and β-zearalanol), all of them having biological activity (Pistol et al., 2015; Catteuw et al., 2019). As for DON, liver is the target organ in all animal species examined so far (Lim et al., 1996, cit. by Tiemann et al., 2006).

Pork is one of the main sources of meat in the human diet, and its consumption provides important nutrients such as vitamin B12, iron, amino acids, and others (Wolk, 2017; Yardımcı, 2020). Pork is the second most consumed meat by the Portuguese (41.4 kg/year per capita), after poultry and, according to INE, the value of pig production in 2020 represented 8% of all national agricultural production (INE, 2020).

The objectives of this study were firstly to evaluate the occurrence of DON and ZEA in feed cereal grains and compound feed destined to swine consumption. In addition, the study aimed to perform a histopathological study using liver samples collected from gilts and sows fed with these compound feeds.

2. EXPERIMENTAL

2.1. Sampling

The follow up of the feed production chain was carried out from the cereal grains up to the final compound feed in a feed factory supplying several swine farms (integrated production system) of the Portuguese region producing the highest number of swine. The twelve ingredients analyzed included unprocessed maize, barley, wheat, bran, rapeseed, and soybean. Incremental samples of these ingredients were collected before incorporation in the factory mixing machine. The compound feed was sampled at the feed facility exit (in trucks; n=6), in the farm silo (n=6) and at the feeder (n=14) of the pavilions of gilts and sows, in different production steps (breeding, gestation and lactation). In the period of sampling and throughout the study, no chelating/adsorbent agent was added to the feed. Sampling occurred during January and February 2013 and was performed in accordance with EU requirements (EC, 2006).

The histopathological study was performed in 90 slaughtered pigs (Landrace xs Largewhite; 36 gilts and 54 sows) reared with the analyzed compound feed for at least two weeks. All liver samples were randomly collected after regular post-mortem veterinary inspection of the carcasses and animal organs, in the regional slaughterhouse receiving animals from the entailed farms. None of the slaughtered animals showed signs of hyperestrogenism or uterotrophic changes, and none of the sampled livers or remaining organs featured any sign of macroscopic lesions. The study was authorized by the Scientific Council of Escola Universitária Vasco da Gama. None of the animals was slaughtered exclusively for the purpose of being part of the study.

2.2. Mycotoxin analysis

The incremental ingredient or feed samples were mixed carefully to form an aggregate sample, in order to make it homogenous, and then finely grinded. Fifty grams of ground sample was weight and 10 g of NaCl and 250 ml of 70% methanol (diluted in distilled water) were added. For wheat samples, no NaCl was added. The mixture was shaken thoroughly for three minutes in a magnetic stirrer, and the sample was filtered in Whatman filter paper, and the resulting filtrate collected.

2.2.1. ZEA

Determination of ZEA was carried out by means of a commercial competitive ELISA (CELER ZON V2, TECNA, Italy), according to the test kit instructions. Briefly, to each premixing well, 100 µL of enzyme conjugate were added. Fifty microliters of each standard/ filtered sample were added into the corresponding premixing wells. Using the micropipette, this mixture was pipetted up and down three times and 100 µL of the content from each premixing well was immediately transferred into the corresponding anti-zearalenone antibody coated microwell. The plate was incubated at room temperature for 10 minutes. At the end of incubation, the liquid was poured out from the wells, which were then filled completely with working washing buffer. The liquid was poured out of the wells. This washing sequence was repeated for a total of three times. The remaining droplets were removed by tapping the microplate upside down vigorously against absorbent paper. After that, 100 µL of development solution was added to each well and mixed thoroughly with rotatory motion for few seconds, before a 10-minute incubation period at room temperature. Fifty microliters of stop solution was added to each well and mixed thoroughly with rotatory motion for few seconds. Absorbance was measured at 450 nm against an air blank, within 60 minutes.

The absorbance value of each standard and sample was divided by the absorbance of the Standard 0 (B_0) and multiplied by 100; the Maximum Binding (B_0) was thus made equal to 100% and the absorbance values were quoted in percentage:

$$\frac{\text{Standard (or sample) absorbance}}{\text{Standard 0 } (B_0) \text{ absorbance}} \times 100 = \frac{B}{B_0}$$

The B/B0 values calculated for each standard (0, 10, 50, 200, 1000 µg/kg) were entered in a semi-logarithmic system of coordinates against the zearalenone standard concentration and the standard curve was drawn. The B/B0 value for each sample were interpolated to the corresponding concentration in the calibration curve. According to the manufacturer's description, the cut-off value was 10 µg/kg.

2.2.2. DON

Determination of DON was carried out by means of a commercial competitive ELISA (Celer DON v2, TECNA, Italy), according to the test kit instructions. Briefly, to each premixing well, 100 µL of enzyme conjugate were added. Fifty microliters of standard and samples were added into the corresponding premixing wells. Using a micropipette, the content of each premixing well was mixed, by pipette up and down three times) and 100 µL were immediately transferred into the corresponding anti-DON antibody coated microwell. Then, a 10-minute incubation period at room temperature followed. At the end of incubation, the liquid was poured out from the wells, which were then completely filled with washing buffer. The liquid was poured out from the wells. This washing sequence was carried out for a total of three times. All the remaining droplets were removed by tapping the microplate upside down vigorously against absorbent paper. Afterwards, 100 µL of development solution was added to each well and mixed thoroughly with rotatory motion for few seconds, before an incubation period of 10 minutes at room temperature, protected from light. Fifty microliters of stop solution was added to each well, and then mixed thoroughly with rotatory motion for few seconds. Within 60 minutes, absorbances were measured at 450 nm.

The absorbance value of each standard and sample was divided by the absorbance of the Standard 0 (B_0) and multiplied by 100; the Maximum Binding (B_0) was thus made equal to 100% and the absorbance values were quoted in percentage:

$$\frac{\text{Standard (or sample) absorbance}}{\text{Standard 0 } (B_0) \text{ absorbance}} \times 100 = \frac{B}{B_0}$$

The B/B_0 value provided for each standard (0; 40; 250; 1250; 5000 µg/kg) was entered in the kit lot conformity certificate in a semi-logarithmic system of coordinates against the DON standard concentration and the standard curve was drawn. The B/B_0 value for each sample was interpolated to the corresponding concentration in the calibration curve. According to the manufacturer's description, the detection limit for the analyzed samples was 40 µg/kg.

2.3. Histopathology

After collection, liver tissue samples (5-8 cm) were immediately fixed in 10% buffered formalin (for 24h), in a relation of about 1:20 (volume of tissue/ volume of fixative) to minimize post-mortem changes in cell structure. Samples were then dehydrated, cleared and paraffin embedded. Tissue samples were sectioned with 3 µm thickness and placed on glass slides. Mounted glass slides were

immersed in xylene (twice for 10 min) followed by rehydration with graded ethanol (five minutes in 100%, five minutes in 95%, five minutes in 70%) and transferred to deionized water. Each tissue sample was stained with routine haematoxylin and eosin (H&E) (10 minutes in haematoxylin, followed by a wash with distilled water and four minutes in eosin). Finally, they were washed and dehydrated with increasing alcohol (i. e. 70, 95, 100%). The mounting was performed with DPX. Basic structures like the nuclei were stained purple/violet, and acidic structures like the cytoplasm were stained pink. Routine H&E staining performed for examination by light microscopy was adapted from Siddiqui et al. (2016).

Prussian blue staining was performed for iron-loading as described by Han et al. (2017). Briefly, equal parts of 20% aqueous solution of Hydrochloric Acid and 10% aqueous solution of Potassium Ferrocyanide were mixed immediately before staining. Tissue slides were immersed in this solution for 20 minutes, after which a three-washing step was performed. Counterstain was carried out with nuclear fast red (safranin 0.05%) for five minutes, before dehydration, clearing and mounting procedures. The result was iron deposits stained blue, the cytoplasm pink, and the nuclei red (Han et al., 2017).

3. RESULTS & DISCUSSION

3.1. Mycotoxins

All the tested samples featured a widespread occurrence (71.1% for ZEA and 78.9% for DON), although more evident in compound feed, for both ZEA (84.6%) and DON (100%) as shown in Tables 1-3.

Nevertheless, the average contamination levels were higher in cereal grains than in compound feed for DON (644.9 vs. 286.4 µg/kg) (Table 1-3). It was noteworthy the high contamination of this toxin in the bran samples analyzed, up to 1433 µg/kg. Cereal bran is the grain constituent with the highest risk of presenting high levels of mycotoxin contamination, especially DON (Cheli et al., 2010, cit. by Lippolis et al., 2018) which was also evident in the results obtained in the present study (Table 1).

In the cereal samples analyzed, the bran and soybean samples had levels of DON and ZEA above the detection limit. Whereas bran featured the higher contamination of DON, soybean featured the highest ZEA contamination (Table 1 and 2). It should be noted that the bran samples were contaminated throughout the different sample collection moments in contrast to the maize samples contaminated by DON only in the first week (Table 2). The rapeseed samples also remained contaminated with ZEA at the different sample collection moments performed. Although the bran and rapeseed were contaminated by ZEA at different sample collection moments, the levels varied, which implies the influence of other factors (Table 2). Probably, the various factors present in the first week of harvest predisposed better conditions for mycotoxin production in the rapeseed sample, in contrast to those present in the second week, which increased the contamination levels of the bran.

Table 1. Contamination levels and occurrence of ZEA and DON in the cereal grain samples.

Sample	**ZEA**				**DON**			
Cereal grains (n)	**Incidence (%)**	**Range (µg/kg)**	**Mean ± SD (µg/kg)**	**EU ML (µk/kg)**	**Incidence (%)**	**Range (µg/kg)**	**Mean ± SD (µg/kg)**	**EU LM (µg/kg)**
Maize	0/3 (0%)	n.d.	n.d.	2000	1/3 (33.33%)	n.d.-67.83	67.83	8000
Wheat	0/2 (0%)	n.d.	n.d.		0/2 (0%)	n.d.	n.d.	
Rapeseed	2/2 (100%)	14.65-35.80	25.22±14.95		0/2 (0%)	n.d.	n.d.	
Soy	1/1 (100%)	54.56	54.56		1/1 (100%)	49.90	49.90	
Barley	0/2 (0%)	n.d.	n.d.		0/2 (0%)	n.d.	n.d.	
Bran	2/2 (100%)	10.21-25.80	18.01±11.03		2/2 (100%)	1028.10-1433.69	1230.89±286.79	
TOTAL	**5/12 (41.67%)**	**n.d.-54.56**	**28.20±17.79**		**4/12 (33.33%)**	**n.d.-1433.69**	**644.88±696.67**	

Table 2. Contamination levels and occurrence of ZEA and DON in the cereal grain samples, according to weeks of sample collection.

Mycotoxin		ZEA	DON
Samples (n)		Range (µg/kg)	Range (µg/kg)
Maize (1)	1st Week	n.d.	67.83
Maize (2)	2nd Week	n.d.	n.d.
Wheat (1)	1st Week	n.d.	n.d.
Wheat (1)	2nd Week	n.d.	n.d.
Rapeseed (1)	1st Week	35.80	n.d.
Rapeseed (1)	2nd Week	14.65	n.d.
Soy (1)	1st Week	54.56	49.90
Soy (0)	2nd Week	-	-
Barley (1)	1st Week	n.d.	n.d.
Barley (1)	2nd Week	n.d.	n.d.
Bran (1)	1st Week	10.21	1028.10
Bran (1)	2nd Week	25.80	1433.69

The compound feed analyzed was collected from six silo farms (Farm A to H). All the farms had their compound feed contaminated with DON and ZEA, except for some samples from farm G and F that were negative for ZEA (Table 3).

Maternity feed samples and those from pregnant sows were also found to be contaminated with ZEA and DON. The concentration of both remained almost the same, with ZEA having an average of contamination of 28.72 µg/kg in the maternity feed and 27.22 µg/kg in that of pregnant sows whereas DON featured 301.99 µg/kg and 280 µg/kg, respectively (Table 3). In turn, the silo samples showed slightly higher contamination values, meaning that neither site stands out for its degree of contamination, although in the silo samples the content is higher. Increasing levels were observed starting in the factory (14.1 µg/kg and 188 µg/kg for ZEA and DON, respectively) toward the farms' silos (31.9 µg/kg and 353 µg/kg, for ZEA and DON, respectively) suggesting that for these field mycotoxins the storage step could be a risk factor (Table 3). Mycotoxins can be produced in pre-harvest, harvest, handling, transport, and in storage under favorable conditions (Abd-Elsalam & Rai, 2020). The study conducted by Wu et al. (2017) showed that the environmental pH value plays an important role in the production of *Fusarium* spp. mycotoxins, with an alkaline pH promoting ZEA production while an acidic pH contributing to DON production. Temperature, relative humidity, and water activity also influence mycotoxin production (Lahouar, 2015, cit. by Peter Mshelia et al., 2020; Wu et al., 2017; Zhang et al., 2019). Previous studies reviewed by Zhang et al. (2019), reported that low temperatures inhibit the reproduction and development of most fungi, while high temperatures produce spores and mycotoxins. In addition, both low and high temperatures (15ºC and 17ºC, and 20-8ºC, respectively) can promote the synthesis of ZEA (Wu et al., 2017; Kokkonen et al., 2010, cit. by Zhang et al., 2019). In turn, DON production is enhanced by high temperatures between 23-25°C (Wu et al., 2017). Peter Mshelia et al. (2020), found an increase in ZEA and DON synthesis at 30°C with a water activity of 0.98, but suggests that temperature may have a greater effect compared to the influence of water activity.

Table 3. Contamination levels and occurrence of ZEA and DON in the compound feed contaminated with ZEA and DON.

Samples (n)		ZEA				DON			
		Incidence (%)	Range (µg/kg)	Mean ± SD (µg/kg)	EU ML (µk/kg)	Incidence (%)	Range (µg/kg)	Mean ± SD (µg/kg)	EU LM (µg/kg)
Compound feed (26)		22/26 (84.62%)	n.d.–58.91	28.21±14.00	100 (Gilts)	26/26 (100%)	110.44-669.98	286.37±165.25	900
Farms	A	5/5 (100%)	13.27-49.20	30.50±13.65	250 (Pigs)	5/5 (100%)	158.471-669.976	479.14±210.29	
	B	2/2 (100%)	16.38-23.57	23.48±5.08		2/2 (100%)	257.289-307.473	347.97±35.48	
	C	6/6 (100%)	11.85-58.91	34.57±17.13		6/6 (100%)	110.440-557.413	232.68±173.41	
	D	3/3 (100%)	19.73-27.04	26.36±3.67		3/3 (100%)	135.925-339.035	269.72±115.89	
	E	0/1 (0%)	n.d.	n.d.		1/1 (100%)	-	135.925	
	F	3/3 (100%)	13.78-50.00	26.97±20.02		3/3 (100%)	120.330-338.062	241.06±110.79	
	G	1/3 (33.33%)	n.d.-17.61	-		3/3 (100%)	119.248-385.844	237.93±135.68	
	H	2/3 (66.66%)	11.46-41.18	26.32±21.02		3/3 (100%)	124.754-292.812	235.67±96.07	
Location	Truck (Feed facility exit)	3/6 (50%)	n.d.–17.14	14.13± 2.86		6/6 (100%)	119.25-292.81	188.44±78.25	
	Feeder	13/14 (92.86%)	n.d.-50	29.77±14.24		14/14 (100%)	110.44-628.93	299.59±154.49	
	Farm silo	6/6 (100%)	21.91–58.91	31.88±13.84		6/6 (100%)	117.64-669.98	353.46±225.75	
Production stage	Silo	6/6 (100%)	21.91-58.91	31.88±13.84		6/6 (100%)	117.64-669.98	353.46±225.75	
	Gestation	6/6 (100%)	13.27-49.20	27.22±14.55		6/6 (100%)	149-556	280±158.97	
	Maternity	4/5 (80%)	n.d.-45.74	28.72±14.63		5/5 (100%)	110-629	300±207.48	
Weeks	1st	1/3 (33.33%)	n.d.-17.14	-		3/3 (100%)	n.d.-265	170±82.48	
	2nd	2/3 (66.67%)	n.d.-13.78	12.62±1,63		3/3 (100%)	n.d.-293	207±86.25	
	3rd	6/7 (85.71%)	n.d.-58.91	43.86±9.57		7/7 (100%)	136-670	430±225.33	
	4th	13/13 (100%)	11.85-50.00	20.63±10.68		13/13 (100%)	110-386	229±104.92	

Implementing preventive measures in the grain sector that minimize the risk of mycotoxin contamination in silo feed will be of great importance. Before performing good manufacturing practices, good agricultural practices must first be performed, considering the type of crop and the climatic conditions. Growing wheat after maize increases DON levels, since maize is a major DON producer, but if the soil is plowed, DON levels decrease significantly. Regarding storage conditions, cereals should be dried at a relative humidity that is not conducive to fungal growth and the temperature should be checked frequently. During transport, temperature fluctuations and condensation should be avoided (CE, 2006).

In the present study, animals were exposed to relatively low doses of two major Fusarium mycotoxins (ZEA and DON) at levels commonly found in feeds.

For several years, different studies performed in the Asian continent reported a very high frequency of ZEA contamination of swine compound feed. However, since 2017, the reported values seem to be decreasing; for example, in China, in 2016 Li et al. (2019) reported an incidence of 75%, but since 2017, the values decreased to 18.1% and to 22.1% in 2018 (Table 4).

The Iberian Peninsula showed much lower incidence of contamination, between 7.02% and 33.3% (Almeida et al., 2011; Arroyo-Manzanares et al., 2019; Table 4). In Portugal, the study by Almeida et al. (2011), reported a 26.5% frequency of ZEA contamination, while in the present study much higher levels (84.6%) were determined in samples collected during 2013 (Table 3 and 4). The maximum ZEA levels did not varied between both studies (73 µg/kg and 58 µg/kg; Table 3 and 4) and did not exceed the recommended limits. As in the previous studies conducted in Portugal, the frequency and concentration levels of ZEA remain similar (Almeida et al., 2011). In one of the most recent studies conducted in Spain (Muñoz-Solano & González-Peñas, 2020) there was an increase in the frequency of ZEA positive samples in compound feed (Table 4). Although between 2012 and 2014, Romera et al. (2018) obtained an incidence of 10% and in 2017, Arroyo-Manzanares et al. (2019) showed a rate of 7.02%, in 2019 the frequency close to 33.3% (Table 4). Many samples had ZEA levels higher than the recommended levels (Arroyo-Manzanares et al., 2019).

In Costa Rica, in the study conducted from 2012 to 2017 by Molina et al. (2019) the frequency was 11.2%, but the average ZEA contamination levels were well above the recommended limits, with a value of 518 µg/kg (Table 4).

As for DON, both Portugal and Spain showed a much lower frequency compared to ZEA contamination. Spanish studies reported an incidence of 5% between 2012 and 2014, and of 4.39% in 2017 (Almeida et al., 2011; Arroyo-Manzanares et al., 2019; Romera et al., 2018; Table 5). In turn, in Portugal, Almeida et al., (2011) reported a frequency of 16.9%. In comparison, in the present study, with less samples, the value of DON contamination was 100%.

It is important to note that the different values reported in the mentioned studies may be due to the difference in sample size analyzed but also to the analytical method used, among many other factors. For example, according to the literature, HPLC-UV is one of the most sensitive analytical method for the quantification of type B trichothecenes such as DON and ZEA (Kiseleva et al., 2020).

In the study by Wellington et al. (2020), a reduction in BW (body weight) was reported in pigs subjected to a DON-contaminated diet (>1000, 3000 and 5000 µg/kg) for 42 days, along with a decrease in ADG (average daily gain) and ADFI (average daily feed intake). Starting on day 28, ADG and ADFI are recovered, which may indicate an adaptation to the mycotoxin. BW remained reduced compared to pigs fed DON up to 1000 µg/kg.

Table 4. Reported occurrence of ZEA (µg/kg) in raw materials and feed destined to swine.

Matrix	Country (year)	Frequency of contamination (%)	Mean±¯SD	Range [min; max]	Analytical	LOD/LOQ	Reference
TOTAL:	Portugal (2013)	27/38 (71.05%)	28.21±14.38	n.d.-58.91	ELISA	10 (LOD)	Present study
Cereal grains:		5/12 (41.67%)	28.20±17.79	n.d.-54.56			
Maize		0/3 (0%)	n.d.	n.d.			
Wheat		0/2 (0%)	n.d.	n.d.			
Rapeseed		2/2 (100%)	25.22±14.95	14.65-35.80			
Soybean		1/1 (100%)	54.56	54.56			
Barley		0/2 (0%)	n.d.	n.d.			
Bran		2/2 (100%)	18.01±11.03	10.21-25.80			
Compound Feed		22/26 (84.62%)	28.21±14.00	n.d.–58.91			
- Silo		6/6 (100%)	31.88±13.84	21.91-58.91			
- Gestating		6/6 (100%)	27.22±14.55	13.27-49.20			
- Maternity		4/5 (80%)	28.72±14.63	n.d.-45.74			
TOTAL:	Spain (2017)	16/226 (7.02%)	741	101-7681	UHPLC-MS/MS and LC-MS/MS	30 (LOD) 99 (LOQ)	(Arroyo-Manzanares et al., 2019)
Compound feed (226):							
Presentation:							
Flour		n. a./183					
Pellet		n. a./43					
Type of animal:							
Fattening pigs		n. a./71					
Sows		n. a./42					
Piglets		n. a./111					
Gilts		n. a./2					
TOTAL:	China (2016)	54/133 (40,6%)	217.9	1.8-1100.0	BAS-MBI	0.98 (LOD)	(M. Li et al., 2019)
Corn		14/56 (25%)	144.1	1.8-950.4			
Corn products (Corn flour, corn bran, corn germ meal, corn starch)		18/48 (37.5%)	315.0	2.3-958.1			
Feed		22/29 (75.9%)	185.4	8.8-1100.0			
TOTAL:	China (2017)	35/143 (24.5%)	166.7	1.1.-722.6	BAS-MBI	0.98 (LOD)	(M. Li et al., 2019)
Corn		11/48 (22.9%)	87.2	1.1-468.5			
Corn products (Corn flour, corn bran, corn germ meal, corn starch)		11/23 (47.8%)	205.3	9.4-722.6			
Feed		13/72 (18.1%)	201.3	13.1-652.1			
TOTAL:	China (2018)	41/129 (31.8%)	157.0	1.3-947.8	BAS-MBI	0.98 (LOD)	(M. Li et al., 2019)
Corn		7/26 (26.9%)	24.1	1.3-76.8			
Corn products (Corn flour, corn bran, corn germ meal, corn starch)		19/35 (54.3%)	176.7	1.4-947.8			
Feed		15/68 (22.1%)	193.9	5.6-567.0			

Table 4. Reported occurrence of ZEA (µg/kg) in raw materials and feed destined to swine (continued).

Matrix	Country (year)	Frequency of contamination (%)	Mean±¨SD	Range [min; max]	Analytical	LOD/LOQ	Reference
TOTAL:	Taiwan (2015-2017)	n. a./820 (70.2%)	n. a.	n. a.-> 1000	ELISA	20 (LOD) 25-1000 (LOQ)	(Yang et al., 2019a)
	2015						
Feed and feed ingredients		n. a. (65.6%)	31.9	n. a.-362.0			
	2016		48.5 (+)				
		n. a. (85.2%)	51.9	n. a.-406.0			
	2017		61.0 (+)				
		n. a. (61.1%)	65.4	n. a.-> 1000			
	2015-2017		107.1 (+)				
	↓ (USA, Brazil)	n. a. (70.2%)	51.9	n. a.-> 1000			
Cornmeal	2015		73.8 (+)				
		n. a. (64.7%)	40.0	n. a.-116.0			
	2016		56.9 (+)				
		n. a. (72.9%)	48.9	n. a.-330.0			
	2017		67.0 (+)				
		n. a. (49.5%)	88.2	n. a.-> 1000			
	2015-2017		178.0 (+)				
		n. a. (56.1%)	68.2	n. a.-> 1000			
Nursery diet	2015		114.5 (+)				
		n. a. (69.9%)	30.0	n. a.-111.0			
	2016		43.0 (+)				
		n. a. (93.4%)	52.8	n. a.-326.0			
	2017		57.0 (+)				
		n. a. (68.1%)	49.6	n. a.-210.0			
	2015-2017		73.0 (+)				
		n. a. (75.0%)	43.5	n. a.-326.0			
Pregnancy diet	2015		57.1 (+)				
		n. a. (67.0%)	29.4	n. a.-147.0			
	2016		44.0 (+)				
		n. a. (86.1%)	54.2	n. a.-406.0			
	2017		63.0 (+)				
		n. a. (68.0%)	61.5	n. a.-742.0			
	2015-2017		90.0 (+)				
		n. a. (73.3%)	49.8	n. a.-742.0			
			67.6 (+)				
Feed	75 countries: Europe, America, Russia, Asia, Africa. Australia and others * (2014-2019)	836/1141 (73.3%)	126	n. a. – 9905	LC-MS/MS	n. a.	(Novak et al., 2019)

Table 4. Reported occurrence of ZEA (µg/kg) in raw materials and feed destined to swine (continued).

Matrix	Country (year)	Frequency of contamination (%)	Mean±¨SD	Range [min; max]	Analytical	LOD/LOQ	Reference
TOTAL:	China (2016-2017)	1155/1569 (73.6%)	2.3-729.2	1363.2	HPLC-FD	10 (LOD) 24 (LOQ)	(Ma et al., 2018)
Feed ingredients:		596/742 (80.3%)					
Corn	2016	183 (93.4%)	104.1	n. a.-624.3			
	2017	86 (90.7%)	55.0	n. a.-296.8			
Domestic DDGS#	2016	46 (100%)	299.4	n. a.-956.7			
	2017	1 (100%)	49.3	n. a.-49.3			
Imported DDGS#	2016	33 (100%)	274.8	n. a.-1169.2			
	2017	3 (100%)	204.0	n. a.-378.1			
Corn bran	2016	6 (100%)	432.1	n. a.-1268.6			
	2017	3 (100%)	231.5	n. a.-456.6			
Corn germ meal	2016	10 (100%)	316.5	n. a.-1363.2			
	2017	8 (100%)	129.6	n. a.-325.4			
Corn gluten meal	2016	5 (100%)	494.9	n. a.-1095.1			
	2017	2 (100%)	729.2	n. a.-1006.3			
Wheat	2016	14 (100%)	2.3	n. a. – 4.9			
Barley	2016	14 (100%)	154.4	n. a. – 393.8			
Wheat bran	2016	50 (88.0%)	94.0	n. a.-439.3			
	2017	20 (95%)	89.3	n. a.-304.7			
Wheat middlings	2016	32 (96.9%)	179.5	n. a.-1195.9			
	2017	5 (100%)	81.4	n. a.-180.8			
Wheat flour	2016	8 (90%)	111.7	n. a.-330.9			
	2017	2 (50%)	37.2	n. a.-79.2			
Broken rice	2016	13 (100%)	68.7	n. a.-257.3			
Rice bran	2016	13 (100%)	282.3	n. a.-879.8			
	2017	4 (100%)	169.1	n. a.-280.0			
Soybean meal	2016	27 (96.3%)	76.9	n. a.-202.4			
	2017	8 (100%)	38.8	n. a.-56.9			
Complete feed:		559/827 (67,6%)	31.7				
Pellet	2016	123 (100%)	210.7	n. a.-916.5			
	2017	9 (100%)	55.7	n. a.-138.5			
Powder	2016	187 (99.5%)	129.3	n. a.-1109.7			
	2017	240 (99.6%)	65.1	n. a.-597.8			

Table 4. Reported occurrence of ZEA (µg/kg) in raw materials and feed destined to swine (continued).

Matrix	Country (year)	Frequency of contamination (%)	Mean±¨SD	Range [min; max]	Analytical	LOD/LOQ	Reference
Feed (sows)	Belgium (2014-2015)	5/20 (25%)	137±73	54-214	LC-MS/MS	n. a.	(van Limbergen et al., 2017)
Soybean seeds	Poland (2015-2017)	24/32 (75%)	42.1	n. a. – 529	HPLC-MS/MS	0.07 (LOD) 0.2 (LOQ)	(Zaworska-Zakrzewska et al., 2020)
Feed	Spain (2019)	1/3 (33.3%)	n. a.	n.a.-178	LC-FLD	42 (LOD)	(Muñoz-Solano & González-Peñas, 2020)
TOTAL: **Compound feeds:** **Pigs:** **- Early growing** **- Late growing** **Sows:** **- Gestating** **- Lactating** **- Breeding gilt** **Piglets:** **- Sucking** **-Weanling**	South Korea (2009, 2010, 2012, 2014, 2016)	152/160 (95%) 30 18 32 25 1 54 8 46	31.70± 36.44% 25.9 31.2 48.5 32.5 25.7 10.9 27.2	n. a. 250 132	IAC, HPLC and LC-MS	0.1-3 (LOD) 0.3-8 (LOQ)	(Chang et al., 2017)
Complete feed, feed mixture (lactating sows) **- Barley** **- Corn** **- Soybean meal** **- Wheat byproducts**	Italy (n. a.)	2/19 (11%)	32	n. a.	ELISA and LC-MS	25 (LOQ)	(Trevisi et al., 2020)
Feed	Spain (2012-2014)	2/20 (10%)	n. a.	n. a.	UHPLC− MS/MS, UHPLC−QTOF−MS and UHPLC−ESI−MS/MS	25 (LOD) 50 (LOQ)	(Romera et al., 2018)
Compound feed (Lactating and gestating sows and pig grower)	Costa Rica (2012-2017)	2/18 (11.2%)	518±1327	n. a.	HPLC	0.072 (LOQ)	(Molina et al., 2019)
Finished Feed	Worldwide (2014-2018)	502/524 (96%)	n. a.	n. a.	LC-MS/MS and UHPLC	n. a.	(Khoshal et al., 2019)

Table 4. Reported occurrence of ZEA (µg/kg) in raw materials and feed destined to swine (continued).

Matrix	Country (year)	Frequency of contamination (%)	Mean±¨SD	Range [min; max]	Analytical	LOD/LOQ	Reference
Complete feed	China (2013-2015)				HPLC	1.5 (LOD) 4 (LOQ)	(Wu et al., 2016)
- Powder	2013	9/10 (90%)	214.7	n. a.-455.8			
	2014	2/2 (100%)	253.0	n. a.-287.1			
	2015	13/13 (100%)	348.6	n. a.-835.4			
- Pellet	2013	16/19 (84.2%)	232.5	n. a.-435.8			
	2014	22/33 (66.7%)	197.6	n. a.-862.4			
	2015	36/38 (94.7%)	375.0	n. a.-1296.5			
TOTAL:	Croatia (2014)	9/253 (3.6%)	325?		ELISA		(Pleadin et al., 2015)
Feed materials:							
- Maize		5/151 (3.3%)	411±860	2.21-5522		1.9 (LOD) 2.4 (LOQ)	
- Wheat		1/17 (5.9%)	275±832	4.72-3366			
- Barley		0/13 (0%)	1.78±2.46	2.30-8.07			
- Silage		0/14 (0%)	102±203	3.28-753			
Feedstuff piglets		0/20 (0%)	21.5±21.8	2.04-63.2		2.0 (LOD)	
Fattening pigs		3/38 (7.9%)	117±355	2.17-1949		2.8 (LOQ)	
TOTAL:	Hungary (n. a.)	n. a./45	39±48	18-192	ELISA and HPLC-UV	17 (LOD)	(Tima et al., 2016)
Feedstuff/grain (fine powder)							
	Manufacturer X	n. a./15					
- Sow		n. a.	26±7	18-35			
- Boars		n. a.	23±3	19-28			
- Piglet		n. a.	20±2	18-22			
	Manufacturer Y	n. a./15					
- Sow		n. a.	19±1	18-21			
- Boars		n. a.	172±18	153-192			
- Piglet		n. a.	21±4	18-26			
	Manufacturer Z	n. a./15					
- Sow		n. a.	20±1	19-22			
- Boars		n. a.	19±1	18-21			
- Piglet		n. a.	33±8	20-40			

Table 4. Reported occurrence of ZEA (µg/kg) in raw materials and feed destined to swine (continued).

Matrix	Country (year)	Frequency of contamination (%)	Mean±¨SD	Range [min; max]	Analytical	LOD/LOQ	Reference
TOTAL:	China (2011)	126/131 (96.2%)	n. a.	<LOD-2,958	HPLC-FD	1.5 (LOD)	(Li et al., 2014)
Feed ingredients		50/55 (91%)				4 (LOQ)	
Corn		14/14 (100%)	109.1	6.25-321			
Wheat bran		13/13 (100%)	14.9	<LOD-44.4			
Soybean meal		6/11 (54%)	9.2	<LOD 35.4			
DDGS#		17/17 (100%)	882.7	48.89-2,958			
Complete feeds		76/76 (100%)	58.9	<LOD-232			
Creep feeds		7/7 (100%)	39	4.7-107			
Starter feeds		14/14 (100%)	54	<LOD-115			
Grower		14/14 (100%)	67	7.59-132			
Grower-finisher		18/18 (100%)	59	7.1-150			
Gestating sows		10/10 (100%)	63	9.2-149			
Lactating sows		13/13 (100%)	76	7.4-231			
Feed	Austria (n. a.)	n. a./27	40±39	n. a.-126	HPLC	n. a.	(Weissenbacher-Lang et al., 2012)
TOTAL:	Portugal (n. a.)	107/404 (26.5%)	n. a.	5-73	HPLC-FD	5 (LOD)	(Almeida et al., 2011)
Feed:							
- Fattening pigs (maize, wheat, barley, soy husks, wheat flour, colza husks, toasted soy seeds)		69/277 (24.9%)	18.9±17.9	5-73			
- Sows (barley, maize, colza husks, wheat, wheat husks, soy husks, citrine pulp, soy oils)		38/127 (29.9%)	19.4±14.5	5-57.7			
Feed (fine powders)	China (2013 and 2014)	28/30 (93.3%) (swine and poultry feed)	n. a.	16-57	LC-MS/MS and HPLC-TSQ	3 (LOD)	(Zhao et al., 2015)
						10 (LOQ)	
		n. a./17 (swine feed)					
			n. a.	n. a.		5 (LOD)	
						10 (LOQ)	
Feedstuff	China (2010)	358/420 (85.2%) (swine and poultry feedstuff)	410±319	35-1478	RP-HPLC/FD	5.7 (LOD)	(Wang et al., 2013)
- Compound						19 (LOQ)	
- Concentrated							
- Premixing		201/244 (82.4%) (swine feedstuff)	n. a.	n. a.		n. a.	
Feed mixtures:	Croatia (2011)	28/30 (93.3%)	184±214	8.93-866	ELISA	1.8 (LOD)	(Pleadin et al., 2012)
- Fattening pigs							
Feedstuff	Croatia (2010)	44/64 (68.7%) (commodities, pig and cattle feed)	n. a.	n. a.-577	ELISA	n. a.	(Vulic et al., 2012)
		90.5% (pig feed)					

Table 4. Reported occurrence of ZEA (µg/kg) in raw materials and feed destined to swine (continued).

Matrix	Country (year)	Frequency of contamination (%)	Mean±¨SD	Range [min; max]	Analytical	LOD/LOQ	Reference
Feed	Thailand (n. a.)	91/100 (91%)	17.4	0.53-169.2	LC-MS/MS, UHPLC-MS/MS and LC-ESI-MS/MS	0.25-40.0 (LOD) 0.5-100.0 (LOQ)	(Nualkaw et al., 2020)
Hay pellet:	Germany (n. a.)	n. a.	n. a.	479 - ZEA 1040 - ZEA+its metabolites	ELISA and LC-MS/MS	n. a.	(Hennig-Pauka et al., 2018)
<u>Sows</u>:				72.6			
- Gestating				51.9			
- Lactating							
Feed	Korea (2018)	4/17 (23.5%)	106.44±0.55	124.78	IAC, HPLC and LC-MS/MS	1.1 (LOD) 3.1 (LOQ)	(Lee & Kim, 2018)
Fattening pig feed:	Argentina (2008)	50/90 (55.6%)	n. a.	n. a.	TLC	50 (LOD)	(Pereyra et al., 2011)
Raw materials:		10/40 (25%)					
- Milled maize		n. a./10	ND				
- Soybean		n. a./10	ND				
- Wheat bran		10/10 (100%)	153±26.2				
- Soybean pellets		n. a./10	ND				
Finished feed:		40/50 (80%)					
- Suckling pig		10/10 (100%)	306±95.3				
- Piglet		10/10 (100%)	153±66.1				
- Weaner		n. a./10	ND				
- Growing		10/10 (100%)	153±70.2				
- Boar		10/10 (100%)	178.5±89.6				

n. a. – Not available. SD – Standard deviation. LOD – Limit of detection. LOQ – Limit of quantitation.
BAS-MBI – Magnetic bead immunoassay-coupled biotin-streptavidin system; ELISA – Enzyme-linked immunosorbent assay; HPLC – High performance liquid chromatography; HPLC-MS/MS – High performance liquid chromatography with tandem mass spectrometry; HPLC-TSQ – High performance liquid chromatography with triple stage quadrupole; HPLC-UV – High performance liquid chromatography with ultraviolet; IAC – Immunoaffinity column; LC-ESI-MS/MS – Liquid chromatography electrospray ionization with tandem mass spectrometry; LC-FLD – Liquid chromatography with fluorescence detection; LC-MS – Liquid chromatography-mass spectrometry; LC-MS/MS – Liquid chromatography with tandem mass spectrometry; RP-HPLC/FD – Reverse phase high performance liquid chromatography with fluorescence detection; TLC – Thin-layer chromatography; UHPLC – Ultra high performance liquid chromatography; UHPLC-MS/MS – Ultra high performance liquid chromatography with tandem mass spectrometry; UHPLC-QTOF-MS – Ultra high performance liquid chromatography quadrupole time-of-flight mass spectrometry.
*** Europe – 77.7% (Germany – 22.4%; Denmark – 15.3%; Austria – 14.1%); Central and South America – 9%; Russia and Asia – 5.6%; North America – 3.5%; Africa – 2.4%; Australia – 0.6%; Others (13 samples) – 1.1%.**
 # Dried distillers grains with solubles.

Table 5. Reported occurrence of DON (µg/kg) in raw materials and feed destined to swine.

Matrix	Country (year)	Frequency of contamination (%)	Mean±¨SD	Range [min; max]	Analytical	LOD/LOQ	Reference
TOTAL:	Portugal (2013)	30/38 (78.95%)	334.17±298.52	n.d.-1433.69	ELISA	40 (LOD)	Present study
Cereal grains:		4/12 (33.33%)	644.88±696.67	n.d.1433.69			
Maize		1/3 (33.33%)	67.83	n.d.-67.83			
Wheat		0/2 (0%)	n.d.	n.d.			
Rapeseed		0/2 (0%)	n.d.	n.d.			
Soybean		1/1 (100%)	49.90	49.90			
Barley		0/2 (0%)	n.d.	n.d.			
Bran		2/2 (100%)	1230.89±286.79	1028.10-1433.69			
Compound Feed		26/26 (100%)	286.37±165.25	110.44-669.98			
- Silo		6/6 (100%)	353.46±225.75	117.64-669.98			
- Gestating		6/6 (100%)	280±158.97	149-556			
- Maternity		5/5 (100%)	300±207.48	110-629			
Complete feed, feed mixture (lactating sows) **- Barley** **- Corn** **- Soybean meal** **- Wheat byproducts**	Italy (n. a.)	17/19 (89.5%)	0.375	n. a.	ELISA, LC-MS and UHPLC	125 (LOQ)	(Trevisi et al., 2020)
Feed	Thailand (n. a.)	43/100 (43%)	215	20.1-631.9	LC-MS/MS, UHPLC-MS/MS and LC-ESI-MS/MS	0.25-40.0 (LOD) 0.5-100.0 (LOQ)	(Nualkaw et al., 2020)
Soybean seeds	Poland (2015-2017)	22/32 (68.75%)	23.6	n. a.-244 (2016, Aligator)	HPLC with MS/MS	1.0 (LOD) 3.0 (LOQ)	(Zaworska-Zakrzewska et al., 2020)
Feed	n. a. (2017, 2018)	99 (poultry and swine) (85%)	511±94.0	10.1-1709	IAC, LC-MS/MS, HILIC and UHPLC-MS/MS	10.1 (LOD) 33.3 (LOQ)	(Panasiuk et al., 2020)
Compound feed (Lactating and gestating sows and pig grower)	Costa Rica (2013-2017)	6/17 (35.3%)	6302±14,932	n. a.	HPLC	10.00 (LOQ)	(Molina et al., 2019)
Finished Feed	Worldwide (2014-2018)	463/524 (88%)	367	n. a.	LC-MS/MS and UHPLC	n. a.	(Khoshal et al., 2019)
Feed	75 countries: Europe, America, Russia, Asia, Africa. Australia and others * (2014-2019)	879/1141 (77%)	634	n. a.-34.862	LC-MS/MS	n. a.	(Novak et al., 2019)
TOTAL:	Spain (2017)	10/226 (4.39%)	237	153-555	UHPLC-MS/MS and LC-MS/MS	26 (LOD) 86 (LOQ)	(Arroyo-Manzanares et al., 2019)
Compound feed (226):							
Presentation:							
Flour (183)		n. a./183					
Pellet (43)		n. a./43					
Type of animal:							
Fattening pigs (71)		n. a./71					
Sows (42)		n. a./42					
Piglets (111)		n. a./111					
Gilts (2)		n. a./2					

Table 5. Reported occurrence of DON (µg/kg) in raw materials and feed destined to swine (continued).

Matrix	Country (year)	Frequency of contamination (%)	Mean±¨SD	Range [min; max]	Analytical	LOD/LOQ	Reference
TOTAL:	Taiwan (2015-2017)	n. a./820 (91.4%)	n. a.	n. a.	ELISA	200 (LOD) 250-5000 (LOQ)	(Yang et al., 2019b)
Feed and feed ingredients	2015	n. a. (85.7%)	534.2 623.0 (+)	n. a.-4208.0			
	2016	n. a. (92.6%)	879.9 950.0 (+)	n. a.-> 5000			
	2017	n. a. (94.2%)	872.3 923.0 (+)	n. a.-> 5000			
	2015-2017	n. a. (91.4%)	782.6 855.5 (+)	n. a.-> 5000			
Cornmeal	2015	n. a. (79.4%)	625.6 788.0 (+)	n. a.-2421.0			
	2016	n. a. (87.9%)	785.4 893.0 (+)	n. a.-2690.0			
	2017	n. a. (92.4%)	967.9 1038.0 (+)	n. a.-> 5000			
	2015-2017	n. a. (88.8%)	854.6 928.7 (+)	n. a.-> 5000			
Nursery diet	2015	n. a. (86.0%)	455.0 529.0 (+)	n. a.-1370.0			
	2016	n. a. (92.1%)	634.0 688.0 (+)	n. a.-2652.0			
	2017	n. a. (94.5%)	774.9 820.0 (+)	n. a.-2595.0			
	2015-2017	n. a. (91.1%)	619.3 682.3 (+)	n. a.-2652.0			
Pregnancy diet	2015	n. a. (87.2%)	500.2 573.0 (+)	n. a.-1349.0			
	2016	n. a. (93.5%)	726.4 777.0 (+)	n. a.-2956.0			
	2017	n. a. (95.2%)	842.5 885.0 (+)	n. a.-3591.0			
	2015-2017	n. a. (90.5%)	705.8 764.2 (+)	n. a.-3591.0			
Feed	Spain (2012-2014)	1/20 (5%)	n. a.	n. a.	UPLC− MS/MS, UPLC−QTOF−MS and UPLC−ESI−MS/MS	125 (LOD) 250 (LOQ)	(Romera et al., 2018)

Table 5. Reported occurrence of DON (µg/kg) in raw materials and feed destined to swine (continued).

Matrix	Country (year)	Frequency of contamination (%)	Mean±¨SD	Range [min; max]	Analytical	LOD/LOQ	Reference
TOTAL:	China (2016-2017)	1271/1569 (74.5%)	450.0-4381.5		HPLC-UV	100 (LOD)	(Ma et al., 2018)
Feed ingredients:		687/742 (92.6%)				260 (LOQ)	
Corn	2016	187 (98.4%)	857.4	n. a.-4590.8			
	2017	97 (97.9%)	750.3	n. a.-2250.9			
Domestic DDGS#	2016	48 (100%)	2599.7	n. a.-6044.7			
	2017	2 (100%)	3547.2	n. a.-5406.8			
Imported DDGS#	2016	34 (100%)	1855.4	n. a.-4044.7			
	2017	55 (74.5%)	872.8	n. a.-7297.8			
Corn bran	2016	6 (100%)	2943.2	n. a.-4710.7			
	2017	3 (100%)	1295.3	n. a.-1916.7			
Corn germ meal	2016	10 (100%)	1426.5	n. a.-2900.6			
	2017	7 (100%)	1206.9	n. a.-2374.6			
Corn gluten meal	2016	4 (100%)	1688.1	n. a.-2229.1			
	2017	2 (100%)	559.9	n. a.-620.3			
Wheat	2016	14 (100%)	3613.8	n. a.-6595.6			
Barley	2016	15 (100%)	2635.8	n. a.-11,028.9			
Wheat bran	2016	53 (100%)	2304.2	n. a.-6054.4			
	2017	23 (100%)	1394.6	n. a.-5642.1			
Wheat middlings	2016	34 (100%)	2961.2	n. a.-12,633.3			
	2017	7 (100%)	1543.0	n. a.-3363.0			
Wheat flour	2016	9 (100%)	4381.5	n. a.-9556.8			
	2017	3 (100%)	450.0	n. a.-736.9			
Broken rice	2016	13 (100%)	1607.3	n. a.-4075.4			
Rice bran	2016	16 (100%)	1532.7	n. a.-3148.5			
	2017	4 (100%)	1271.6	n. a.-1900.0			
Soybean meal	2016	29 (96.6%)	451.6	n. a.-1171.4			
	2017	12 (100%)	610.7	n. a.-1478.5			
Complete feed:		584/827 (70.6%)					
Pellet	2016	128 (99.2%)	1194.0	n. a.-4279.3			
	2017	9 (100%)	753.1	n. a.-1690.9			
Powder	2016	195 (100%)	1018.1	n. a.-3400.9			
	2017	252 (98%)	876.3	n. a.-10,437.6			

Table 5. Reported occurrence of DON (µg/kg) in raw materials and feed destined to swine (continued).

Matrix	Country (year)	Frequency of contamination (%)	Mean±¨SD	Range [min; max]	Analytical	LOD/LOQ	Reference
Feed (Sows)	Belgium (2014-2015)	20/20 (100%)	371±196	104-884	LC-MS/MS	n. a.	(van Limbergen et al., 2017)
TOTAL: **2 compound feeds**	South Korea (2009, 2010, 2012, 2014 and 2016)	160/n. a. (93.1%)	231.47±235.30	n. a.-900	IAC, XIC, HPLC and LC-MS/MS	1-10 (LOD) 3-35 (LOQ)	(Park et al., 2018)
<u>Pigs</u>:							
- Early grower		30	201.8±174.3				
- Later grower		18	217.6±167.8				
<u>Sows</u>:							
- Gestating sows		32	392.1±338.8				
- Lactating sows		25	225.7±221.0				
- Breeding gilts		1	162.8±0.0				
<u>Piglets</u>:							
- Sucking piglets		8	55.1±38.8				
- Weanling piglets		46	179.7±164.5				
Complete feed	China (2013-2015)	97/115 (84.4%)	n. a.	n. a.	HPLC	0.02 (LOD) 0.06 (LOQ)	(Wu et al., 2016)
- Powder	2013	9/10 (90%)	791.3	n. a.-1602.6			
	2014	2/2 (100%)	523.4	n. a.-623.6			
	2015	13/13 (100%)	1216.3	n. a.-2767.6			
- Pellet	2013	19/19 (100%)	660.3	n. a.-946.6			
	2014	22/33 (66.7%)	537.7	n. a.-2478.3			
	2015	32/38 (84.2%)	704.0	n. a.-3346.0			
TOTAL: **Feedstuff/grain (fine powder)**	Hungary (n. a.)	n. a./45	261±224	137-997	ELISA and HPLC-UV	13 (LOD)	(Tima et al., 2016)
	Manufacturer X	n. a./15					
- Sow		n. a.	196±25	169-223			
- Boars		n. a.	203±74	162-335			
- Piglet		n. a.	186±9	170-192			
	Manufacturer Y	n. a./15					
- Sow		n. a.	191±28	160-224			
- Boars		n. a.	872±139	681-997			
- Piglet		n. a.	159±24	137-190			
	Manufacturer Z	n. a./15					
- Sow		n. a.	191±27	167-225			
- Boars		n. a.	173±27	156-220			
- Piglet		n. a.	180±8	170-190			

Table 5. Reported occurrence of DON (µg/kg) in raw materials and feed destined to swine (continued).

Matrix	Country (year)	Frequency of contamination (%)	Mean±¨SD	Range [min; max]	Analytical	LOD/LOQ	Reference
TOTAL:	China (2011)	124/131 (95%)	n. a.	<LOD-2.31	HPLC-UV	0.02 (LOD)	(Li et al., 2014)
Feed ingredients		48/55 (87%)	(ppm)	(ppm)		0.06 (LOQ)	
Corn		13/14 (93%)	1.01	<LOD-2.13			
Wheat bran		12/13 (92%)	0.44	<LOD-0.89			
Soybean meal		6/11 (54%)	0.05	<LOD-0.21			
DDGS#		17/17 (100%)	1.36	0.85-1.72			
Complete feeds		74/76 (97%)	0.65	<LOD-2.31			
Creep feeds		6/7 (86%)	0.28	<LOD-0.53			
Starter feeds		14/14 (100%)	0.85	0.16-2.31			
Grower		14/14 (100%)	0.62	0.12-1.14			
Grower-finisher		17/18 (94%)	0.58	<LOD-1.44			
Gestating sows		10/10 (100%)	0.82	0.13-1.45			
Lactating sows		13/13 (100%)	0.62	0.07-1.52			
Feed	Austria (n. a.)	n. a./27	251±254	n. a.-1243	HPLC	n. a.	(Weissenbacher-Lang et al., 2012)
Feed: **- Fattening pigs (maize, wheat, barley, soy husks, wheat flour, colza husks, toasted soy seeds)**	Portugal (n. a.)	47/277 (16.9%)	223.2±181.1	100-864	HPLC-UV	100 (LOD)	(Almeida et al., 2011)
Feed (fine powders)	China (2013 and 2014)	27/30 (90%) (swine and poultry feed)	n. a.	38-928	LC-MS/MS and HPLC-TSQ	3 (LOD) 10 (LOQ)	(Zhao et al., 2015)
		n. a./17 (swine feed)	n. a.	n. a.		10 (LOD) 25 (LOQ)	
Feed mixtures: **- Fattening pigs**	Croatia (2011)	29/30 (96.7%)	817±447	156-1,864	ELISA	150 (LOD)	(Pleadin et al., 2012)

n. a. – Not available. SD – Standard deviation. LOD – Limit of detection. LOQ – Limit of quantitation.
ELISA – Enzyme-linked immunosorbent assay; HILIC – Hydrophilic interaction liquid chromatography; HPLC – High performance liquid chromatography; HPLC-TSQ – High performance liquid chromatography with triple stage quadrupole; HPLC-UV – High performance liquid chromatography with ultraviolet; IAC – Immunoaffinity column; LC-ESI-MS/MS – Liquid chromatography electrospray ionization with tandem mass spectrometry; LC-MS – Liquid chromatography-mass spectrometry; LC-MS/MS – Liquid chromatography with tandem mass spectrometry; UHPLC – Ultra high performance liquid chromatography; UHPLC-MS/MS – Ultra high performance liquid chromatography with tandem mass spectrometry; UHPLC-QTOF-MS – Ultra high performance liquid chromatography quadrupole time-of-flight mass spectrometry; XIC – Extracted ion chromatogram.
*** Europe – 77.7% (Germany – 22.4%; Denmark – 15.3%; Austria – 14.1%); Central and South America – 9%; Russia and Asia – 5.6%; North America – 3.5%; Africa – 2.4%; Australia – 0.6%; Others (13 samples) – 1.1%.**
 # Dried distillers grains with solubles.

In the study by Skiepko et al. (2020), microscopic changes in liver architecture were observed in a single administration of DON and another combined with ZEA at a concentration of 12 µg/kg BW and 40 µg/kg BW, respectively, for six weeks. The changes were visible from the very first week (Skiepko et al., 2020).

Reddy et al. (2018) identified several altered gene expressions in liver samples from pigs subjected to ZEA- and DON-contaminated feed (800 µg/kg, 8000 µg/kg, for four weeks). One of the identified genes, GAS1, is implicated in growth suppression, inhibition of the cell cycle, and apoptosis. IGF1 coding gene has also been shown to be dysregulated. IGF1 is a protein produced in the liver that functions as an endocrine hormone and is related to growth retardation. However, the most downregulated gene was GALP, which implicated in appetite regulation and associated with some inflammatory processes, stress situations, and sexual behavior in animals. Reddy et al. (2018) state that GALP downregulation might be caused by high concentrations of *Fusarium* mycotoxins.

Previous experimental studies have shown that exposure to mycotoxins can cause reproductive toxicity, and Gerez et al. (2017) reported that exposure to DON in pigs can also have this toxic effect.

3.2. Histopathology

Histological analysis is a method for examining the physiological structure of organ tissues and the state of their cells, enabling, in the event of pathological changes, a pathological anatomical diagnosis (Feldman & Wolfe, 2014; Ozawa & Sakaue, 2020). In the histopathological study of the liver sections, the H&E staining was performed to highlight the morphology of tissues and their cells (Ozawa & Sakaue, 2020).

H&E-stained sections showed swollen hepatocytes, masking the sinusoids architecture. This swelling corresponds to cellular edema due to acute cellular injury by toxins, bacterial or viral infections, among others. Most of the nuclei were normal, some were pyknotic and others were in karyolysis, which evidences a hydropic degeneration where there is a complete breakdown of the hydro electrolytic mechanism. In addition to hydropic degeneration, some cells also showed microvacuolar degeneration. Hydropic and microvacuolar degeneration are the step following cell swelling and occur mainly in ER-rich cells, such as hepatocytes (Pires et al., 2004; Skiepko et al., 2020). Perhaps due to DON and ZEA intoxication, the hepatocytes have exhausted their available ATP resources, so the water and ion pump is blocked, leaving an accumulation of free water in the cytoplasm instead of being routed to the ER and mitochondria. In this way, the hepatocytes lose their functional capacity and end up in apoptosis. Numerous binucleated hepatocytes were observed, however, several authors mention that it is a common finding (de Santis Puzzonia et al., 2016; Mescher, 2018; Pires et al., 2004; Young et al., 2007). Regarding the cytoplasm, some hepatocytes with cloudy swelling images, others more acidophilus cytoplasm. Generally, hepatocytes have eosinophilic cytoplasm due to the high presence of mitochondria and have a slight basophilic granulation that corresponds to the many free ribosomes and the ER. The appearance of the cytoplasm will depend on the nutritional status of the animal. Well-

nourished animals will show higher amounts of glycogen compared to malnourished ones, but in the H&E preparation, the glycogen is removed, forming discolored areas (Young et al., 2007). However, Skiepko et al. (2020) reported finding changes in the first week with respect to glycogen with PAS staining. Subsequently, it will be important to perform a histopathological analysis with this staining. Regarding the stroma, the capsule, the interlobular septa and the portal triads did not show any change, maintaining their normal architecture. Skiepko et al. (2020) reported an increase in septal thickness in a gilt treated with DON for six weeks with onset of changes in the first week (Skiepko et al., 2020). During our experiment, the tissue samples showed no changes at the septal level, which may be due to the stipulated dose of DON, the combination of DON and ZEA, the age at which the animals were treated, the breed used, the body weight, the sex, the degree of stress, the endocrine function, the genetic and the diurnal factors (Brown et al., 2017; Sayyari et al., 2018) . Whenever liver damage occurs, stellate cells produce ECMP in association with vitamin A loss. However, when there is chronic injury, they constantly form collagen fibers and other ECMP compounds that develop fibrosis, which explains the thickness of the interlobular septa described by Skiepko et al. (Brown et al., 2017; Skiepko et al., 2020).

In sections of the sow's liver, a discrete granular brownish pigment was noticeable, evidencing the presence of hemosiderin. Hemosiderin is a granular physiological iron-containing pigment that results from the degradation of ferritin, but when it accumulates excessively, it becomes a pathological pigment (Brown et al., 2017; Grotto, 2008; Pires et al., 2004). In the liver, hemosiderin is usually present in Kupffer cells and hepatocytes in cases of hemolysis (Peleteiro & Carvalho, 2011).

Histologically, Prussian Blue (PB) staining is used to demonstrate the presence of iron deposits (ferric iron pigment) in tissues and is valuable in liver tissues for cases of diagnosing hemochromatosis or hemosiderosis, where iron tends to be located near periportal hepatocytes and/or in the lining of sinusoids (Gurina & Simms, 2020; Orah et al., 2016). Hepatic iron overload can also be found in Kupffer cells and in the cells of endothelia (Han et al., 2017). The stored ferric iron pigment is bound to hemosiderin, myoglobin, and hemoglobin, but when its accumulation is excessive, it ends up being stored only in its ferric state, as hemosiderin. Under these circumstances, the PB reaction becomes useful in identifying hemosiderin, staining it with a brilliant blue precipitate (Rowatt et al., 2018). On the PB preparations there were deposition of hemosiderin within the hepatocytes and at Kupffer cells, predominantly at the perilobular area, particularly in sows, which can be explained by the concept of the hepatic acinus. This current concept shows that the blood flow from the portal vein and the hepatic artery flows into the sinusoids, beginning at the periphery of each lobule (Young et al., 2007). In gilts, eight slides (out of 36) did not present any hemosiderin deposition, while in the remaining the pigment was only slightly deposited in the hepatocytes and kupffer cells. At average, deposition of hemosiderin was observed in two cells per optic field, at 40x magnification. In summary, predominantly diffuse degenerative changes observed were compatible with hepatosis.

4. CONCLUSIONS

The raw materials used for swine feed feature a widespread ZEA and DON contamination. Bran may have a higher predisposition to DON contamination while soybeans may be more predisposed to ZEA contamination. Good agricultural practices remain of major importance in reducing mycotoxin contamination of raw materials, however, after collection, the storage step becomes the biggest challenge in their prevention, and more efficient measures need to be established. DON and ZEA at low doses promote degenerative changes in the liver of sows and gilts in the short term, that required further studies.

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