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Investigations of time-dependent effects of dietary deoxynivalenol and zearalenone exposure on female piglets and *in vivo* evaluation of a feed decontamination procedure

Dissertation

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ABBREVIATIONS

15-ADON	15-acetyl-deoxynivalenol
Alb	Albumin
ANOVA	Analysis of Variance
AST	Aspartate amino-transferase
Bili	Bilirubin
BW	Body weight
BWG	Body weight gain
$Ca(OH)_2$	Calciumhydroxide
Chol	Cholesterol
CON	Control maize
ConA	ConcanavalinA
de-DON	De-epoxy-deoxynivalenol
DM	Dry matter
DON	Deoxynivalenol
DON-3G	Deoxynivalenol-3-glucosid
DONS	DON-sulfonate
EC	The Commission of the European Communities
EFSA	European Food Safety Authority
FUS	*Fusarium* toxin-contaminated maize
GfE	Gesellschaft für Ernährungsphysiologie
GLDH	Glutamate dehydrogenase
Gluc	Glucose
γ-GT	γ-Glutamyltransferase
HPLC	High-performance liquid chromatography
IAC	Immuno-affinity column
LC-MS/MS	Liquid chromatography-mass spectrometry
LSmeans	Least Square means
MMA	Methylamine
OD	Optical density
PBMC	Peripheral blood mononuclear cells
Prot	Total protein
PSEM	Pooled standard error of means

RNA	Ribonucleic acid
RSD	Residual standard deviation
SBS	Sodium metabisulfite
SCOOP	Scientific cooperation on questions relating to food
SI	Stimulation index
Tgl	Triglycerides
VDLUFA	Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten
ZEA	Zearalenone
α-ZEL	Alpha-zearalenol
β-ZEL	Beta-zearalenol
ZEN	Zearalenone

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INTRODUCTION

> *"The worldwide contamination of foods and feeds with mycotoxins is a significant problem. […] The economic impact of mycotoxins include loss of human and animal life, increased health care and veterinary care costs, reduced livestock production, disposal of contaminated foods and feeds, and investment in research and applications to reduce severity of the mycotoxin problem. Although efforts have continued internationally to set guidelines to control mycotoxins, practical measures have not been adequately implemented."* Hussein and Brasel (2001)

Mycotoxins are toxic secondary metabolites of diverse molds that can be found on various agricultural plants and in derived feed and food products. Such contamination can induce diverse negative implications that include considerable limitations of the quality of obtained products for both human and animal nutrition purposes and a restricted efficiency of feed resources and livestock systems. Zearalenone (ZEN) and trichothecenes such as deoxynivalenol (DON) can be detected worldwide and are among the most important Fusarium mycotoxins. Even good management strategies cannot adequately prevent their occurrence because their formation is highly related to weather conditions during plant growth (Oldenburg et al., 2007). Though temporary outbreaks of *Fusarium* toxicosis have been reported in Europe, Asia, New Zealand and South America, chronic exposure may be more important as it was reported to occur on a regular basis (D'Mello et al., 1999). In contrast to ruminants and poultry, swine are very sensitive to intoxications with ZEN and DON, which are primarily synthesized by *Fusarium graminearum* and *F. culmorum* (Oldenburg et al., 2007). Both toxins were described to compromise animal performance either via feed intake depressions (DON) or reduced fertility (ZEN) (Döll and Dänicke, 2011). However, the carry-over of these substances in food products is usually considered negligible (Dänicke et al., 2008b). Therefore and in contrast to other mycotoxins suchs as aflatoxin B1, the concentration of both mycotoxins is not regulated by upper limits in animal feed but guidance values were recommended within the European Union (The Commission of the European Communities, 2006a). However, adverse effects on health and performance of pigs might be induced even by dietary concentrations below the given guidance values and the total time of exposure could influence the severity of a potential mycotoxicosis.

The widespread presence of ZEN and DON and the known adverse implications in swine production substantiate the ongoing need for efficient decontamination procedures in order to ensure both good animal health and expedient production systems.

BACKGROUND

1 Mycotoxins: occurrence, importance and regulation

The enormous global relevance of mycotoxins is substantiated by the fact that, according to the Food and Agriculture Organization of the United Nations approximately 25 % of the world's yearly harvested crops are contaminated with mycotoxins. Over 400 of these substances and about 350 mycotoxin-producing fungal species are known (Weidenbörner, 2001), of which the most important belong to the genera *Aspergillus, Penicilium, Fusarium, Alternaria* and *Claviceps* (Steyn, 1998). In the nutritional sector, the most important mycotoxins with regard to both human and animal health include aflatoxins, ochratoxin A, fumonisins, trichothecenes and ZEN. However, of special relevance are DON, a subtype of the trichothecenes, and ZEN, which are potently formed by *F.graminearum* and *F.culmorum*, the most frequently occurring fungi among the toxin-producing *Fusarium* species in central Europe (Chelkowski, 1998). In the case of these toxins, the fungal infection of the plant (mostly maize, wheat and triticale) and production of toxins has to be defined as a pre-harvest contamination as it occurs already in the field. These processes are hardly avoidable even at the best agricultural management because weather conditions during plant growth have a high impact on the risk of mycotoxin formation (Oldenburg et al., 2000). A recent screening on the occurrence of mycotoxins in European cereals and feed ingredients revealed that about 67 and 50 % of all samples were contaminated with DON and ZEN, respectively (Biomin, 2012). Maximum detected levels were 21.54 mg DON and 0.86 mg ZEN per kg.

Although the acute toxicity of DON and ZEN is considered relatively low in comparison with toxins such as aflatoxin, their frequent occurrence in high concentrations makes them toxicologically relevant for both human and animal nutrition purposes. While the concentrations of these toxins in food products are strictly limited by European regulations (The Commission of the European Communities, 2006b), for animal feed only guidance values for critical dietary concentrations were published (The Commission of the European Communities, 2006a) as shown in Table 1.

As reflected by the low guidance values, especially pigs are regarded very sensitive to these toxins in comparison with other livestock species such as ruminants or poultry. The sensitivity is considered to generally decrease in the order pigs >> poultry ~ ruminants (Rotter et al., 1996). Differences in the susceptibility between species may thereby depend on differences in the metabolism of the toxins. In the forestomach of ruminants, DON is almost completely converted into the less toxic metabolite de-epoxy-DON (de-DON) while ZEN is mainly

converted into the metabolites α- and β-zearalenol which are either less absorbable or less potent than the parent compound (Kiessling et al., 1984; Kuipergoodman et al., 1987; Binder et al., 1998).The rumen thus enables the detoxification of the toxins before they reach the following segments of the gastrointestinal tract where they can be absorbed. The susceptibility of poultry is also known to be relatively low which seems to be associated with a highly efficient renal first-pass elimination of the toxins that hinders the intestinally absorbed compounds from reaching the systemic circulation (Rotter et al., 1996).

Table 1. Guidance values for deoxynivalenol and zearalenone in products intended for animal feed (relative to feeding stuff with a moisture content of 12%) (The Commission of the European Communities, 2006a)

Mycotoxin	Products intended for animal feed	Guidance value (mg/kg)
Deoxynivalenol	Complementary and complete feeding stuffs with exception of:	5
	• complementary and complete feeding stuffs for pigs	0.9
	• complementary and complete feeding stuffs for calves (< 4 months), lambs and kids	2
Zearalenone	Complementary and complete feeding stuffs	
	• complementary and complete feeding stuffs for piglets and gilts (young sows)	0.1
	• complementary and complete feeding stuffs for sows and fattening pigs	0.25
	• complementary and complete feeding stuffs for calves, dairy cattle, sheep (including lamb) and goats (including kids)	0.5

2 Zearalenone

2.1 Chemical and physical characteristics

ZEN belongs to the group of β-resorcylates and is chemically described as 3,4,5,6,9,10-hexahydro-14,16-dihydroxy-3-methyl-1*H*-2-benzoxacyclotetradecin-1,7(8*H*)-dione ($C_{18}H_{22}O_{5;}$ molecular weight: 318.36; EFSA, 2011; Figure 1). It is white, crystalline in structure and has a melting point of 164 – 165 °C. It is insoluble in water, but soluble in aqueous alkali and organic solvents. The toxin is stable during storage, milling, processing and cooking and at high temperatures (EFSA, 2004b).

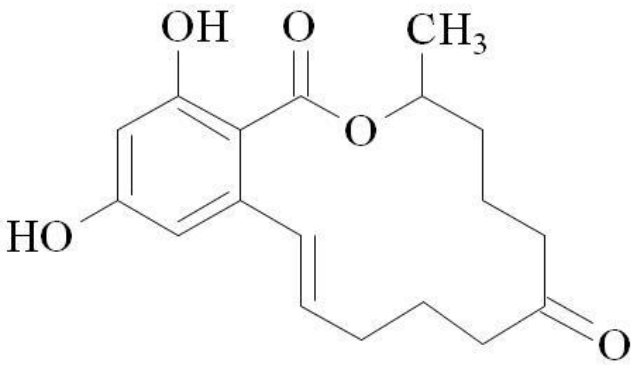

Figure 1. Chemical structure of zearalenone (according to Urry et al., 1966)

2.2 Mode of action

The chemical structure of ZEN and its metabolites mimics to that of natural oestrogens such as 17-beta-oestradiol (Figure 2). In target tissues that contain oestrogen receptors, ZEN has been shown to competitively bind to these receptors, justifying its assignment to the group of endocrine disruptors. While the binding affinity of ZEN itself to these receptors is low in comparison to that of 17-β-oestradiol, metabolization to α-zearalenol (α-ZEL) increases its oestrogenic potency by factor 92 in proliferation assays (Shier et al., 2001). Upon binding, the ZEN-receptor complex is translocated into the nucleus, where RNA and protein synthesis and subsequent cell proliferation is enhanced (Kuipergoodman et al., 1987). The presence of ZEN in the organism thus increases the level of oestrogenic compounds and may lead to both intensified reactions of oestrogen-sensitive tissues and disturbance of the sensitive hormone balance. This mainly results in reproductive disorders and hyperoestrogenism (Bauer et al., 1987b; EFSA, 2004b). Since its first isolation from molded corn in 1962, the presence of ZEN in feeding stuffs was related to vulvar hypertrophy and uterotrophic effects (Stob et al., 1962).

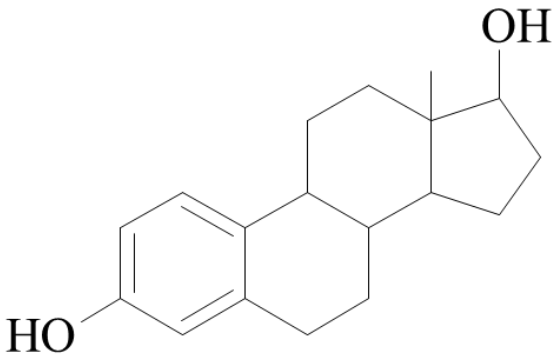

Figure 2. Chemical structure of 17-β-oestradiol

2.3 Toxicokinetics and metabolism

Ingested ZEN is quickly absorbed in the intestine by passive diffusion (Ramos et al., 1996) and detectable serum levels can be found within 30 min after feed intake. During the absorption process ZEN may partly be reduced to the stereoisomeric derivates α- and β-ZEL by the reduction of the keto-group at C-7 (Figure 3) in the intestinal mucosa with α-ZEL being the major metabolite (Olsen et al., 1985; Biehl et al., 1993). Further reduction leads to α- and β-zearalanol, which lack the 11,12-double bond. However, the latter two metabolites are of minor importance in pigs, as their presence in physiological samples is negligible.

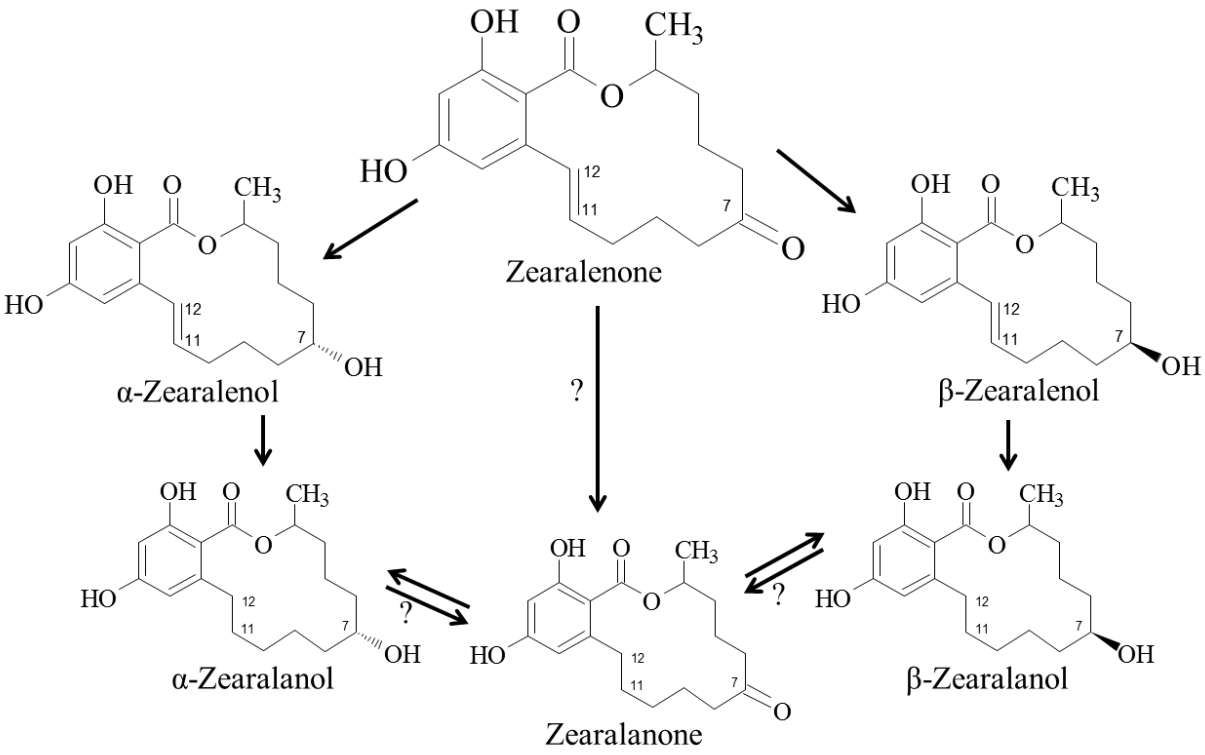

Figure 3. Zearalenone and its metabolites (according to Zöllner et al., 2002)

During the passage through the mucosa and the reduction of ZEN glucuronic acids may also be conjugated to the molecules (Biehl et al., 1993) as illustrated in Figure 4. Via the portal vein, ZEN and metabolites enter the liver, where ZEN may as well be reduced to α- and β-ZEL by 3α-hydroxysteroid dehydrogenase in hepatocytes (Hussein and Brasel, 2001). Phase I metabolization (oxidation, reduction and hydroxylation reactions) as well as phase II metabolization (conjugation reaction) thereby result in an increased polarity and solubility of the molecules to facilitate their excretion (Galtier, 1999). Following the passage through the liver ZEN and metabolites either enter the systemic circulation to be distributed in the

organism and reach target tissues or are excreted via bile in the first pass. Circulating ZEN and metabolites are primarily excreted via urine in their glucuronidated form (Olsen et al., 1985) but may also be extracted from the circulating blood by the liver to a high percentage to be excreted in bile fluid (Biehl et al., 1993). Almost 85% of ZEN and metabolites reaching the intestine via bile may be reabsorbed again, resulting in an entero hepatic cycling that extends the elimination of the toxin from the organism and may lead to a serum elimination half-life of up to 86.6 h (Biehl et al., 1993). Accordingly, Olsen et al. (1985) were able to detect the toxin in porcine plasma 5 days after oral ZEN-exposure. In contrast, the interruption of the entero hepatic cycling by the removal of the bile reduced the half-life to 3.3 h in a study by Biehl et al. (1993). This cycling is of special importance in terms of toxicology, as a continuous intake of the toxin may lead to an accumulation in bile fluid and might increase the oestrogenic activity in the organism. In general, only very few of ingested ZEN is recovered in faeces (Biehl et al., 1993).

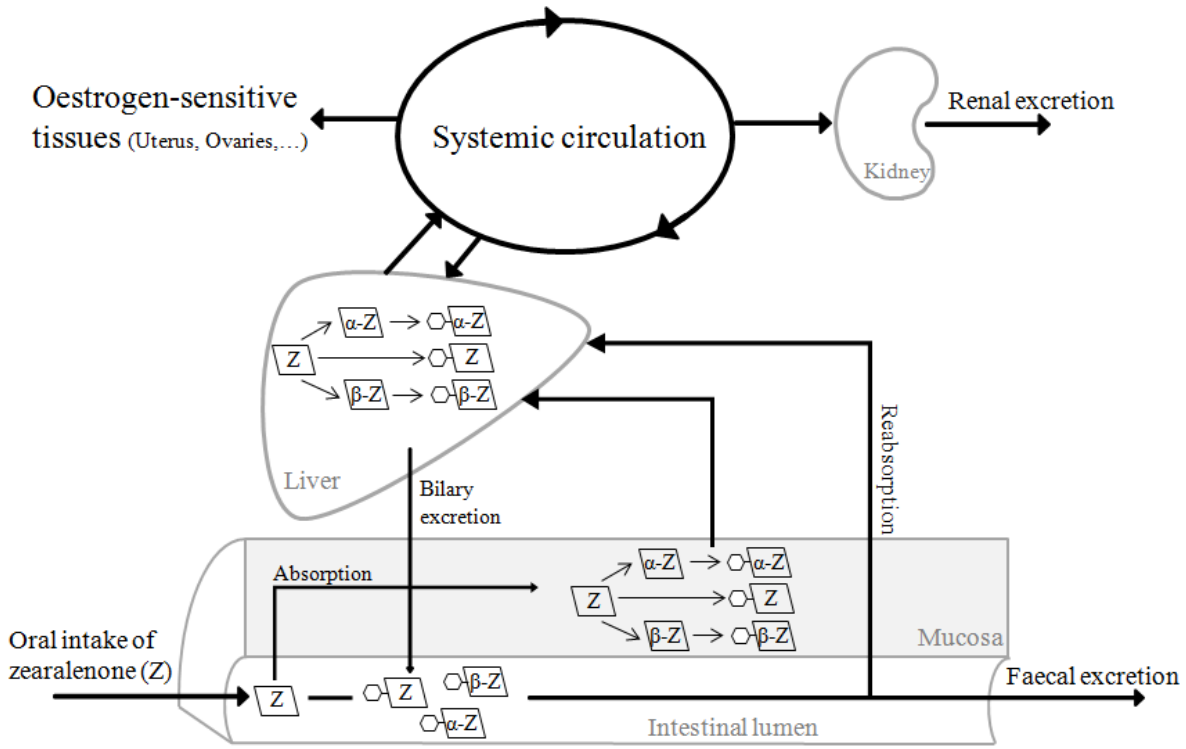

Figure 4. Metabolism and entero hepatic cycling of zearalenone (α-Z, α-zearalenol; β-Z, β-zearalenol; ⎔ ,glucoronide; adapted from Boyens, 2001)

3 Deoxynivalenol

3.1 Chemical and physical characteristics

DON belongs to the trichothecenes, a heterogeneous group of tetracyclic sesquiterpenes, which have a 12-13-epoxy moiety in common (EFSA, 2004a). The group is further divided into 4 subgroups (A, B, C and D) according to their fungal producer or chemical structure (Ueno et al., 1973; Ueno, 1984). DON is assigned to the B-type trichothecenes, characterized by a carbonyl function at the C8 position and is chemically described as 12,13-epoxy-3α,7α,15-trihydroxytrichothec-9-en-8-one ($C_{15}H_{20}O_6$, MW: 296.32, CAS 51481-10-8; EFSA, 2004a; Figure 5). It crystallizes as colorless needles and is tolerant to high temperatures (stable at 120 °C, moderately stable at 180 °C). This mycotoxin is soluble in water and in some polar solvents such as aqueous methanol, acetonitrile and ethyl acetate (EFSA, 2004a).

3.2 Mode of action

The toxicity of DON is closely linked to its 9,10-double bond and the 12,13-epoxide ring (Ehrlich and Daigle, 1987). It enables the binding to the 60S subunit of eukaryotic ribosomes and thereby interferes with the peptidyl transferase function (Rotter et al., 1996), which in turn results in the inhibition of protein synthesis. Binding of the ribosome has also been shown to trigger a ribotoxic stress response in mice, which leads via the activation of mitogen-activated protein kinases und subsequent signaling cascades to an upregulation of proinflammatory genes or apoptosis (Pestka et al., 2004). Especially leukocytes, representing the functional repertoire of the immune system, are considered as main target of DON, which can exert inhibitory or stimulatory effects, depending on dose and time of exposure (Pestka et al., 2004). However, the most apparent effect in different studies investigating DON exposure in pigs is a reduction in feed intake and subsequent decreases in weight gain and loss of performance. During numerous experiments in the last decades, wide concentration-ranges of DON, different toxin sources and several other experimental factors were investigated, partially resulting in high variations in this effect between these studies. Dänicke et al. (2008b) quantified the relation between the DON-concentration in feed and the extent of feed intake depression irrespective of experimental factors and found that voluntary feed intake decreased linearly with increasing DON-intake by approximately 5% per mg DON/kg feed. The nature of the anorectic properties of DON is still not completely elucidated. Some studies

imply that this effect may be induced on the level of the central nervous system, mediated through DON-induced alterations in the serotoninergic activity (Rotter et al., 1996) because serotonin is involved in neural processes that regulate feeding behavior (Silverstone and Goodall, 1986).

3.3 Toxicokinetics and metabolism

DON is nearly completely absorbed in the proximal small intestine and can be detected in serum approximately 15 min after oral exposure with a maximum level being reached between 0.8 and 4.1 h after the ingestion of the toxin (Dänicke et al., 2004a; Goyarts and Dänicke, 2006). During passage through the intestinal barrier DON may be conjugated to glucuronic acids and other compounds by phase II enzymes, which enhance its water solubility and facilitate the excretion (Starke et al., 2009). Metabolization of DON to de-DON seems to occur primarily in the intestine involving only the low amount of unabsorbed DON. As illustrated in Figure 5 it results in a cleavage of the C-12,13-epoxide ring (King et al., 1984) which implies a decrease in toxicity.

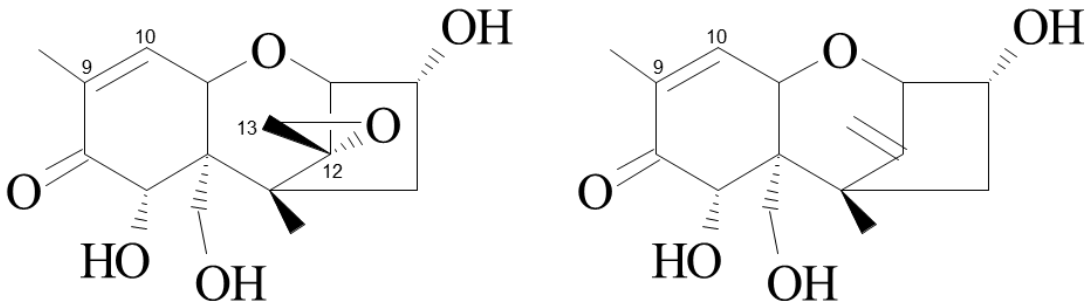

Figure 5. Chemical structure of deoxynivalenol and its metabolite de-epoxy-DON

The de-epoxydation rate thereby increases from stomach to large intestine, resulting in approximately 80% de-DON from total DON in faeces (Dänicke et al., 2004a).The ability to form de-DON, however, seems to depend on the presence of special DON-metabolizing microorganisms, which develop or increase their numbers at exposure to the toxin (Hedman and Pettersson, 1997). Since de-epoxidation in the liver seems to play a minor role (Côté et al., 1987), the low de-DON-concentrations that are usually detectable in serum and urine (about 5% of total DON; Razzazi et al., 2002) presumably originate from the digestive tract

(Dänicke et al., 2008b). Phase I metabolization thus seems not to relevantly contribute to a detoxification in pigs (Dänicke et al., 2004a). The excretion of DON via urine has been confirmed to be the main route of elimination from the organism with a high percentage of DON being present in its glucuronidated form (Dänicke et al., 2005a; Goyarts and Dänicke, 2006). Thereby the elimination half-life from the serum after oral exposure to the toxin was found to range between 2.8 and 9.95 h as reviewed by Dänicke and Brezina (2013).

4 Co-occurrence of different mycotoxins in feed

Under practical farm conditions, the co-occurrence of several toxic compounds on the same plant can be the natural result of the fact that many fungal species are able to produce several of the known 400 different mycotoxins (Oldenburg 2000). Therefore ZEN and DON are often simultaneously detected in contaminated plants used for feed production purposes. Although their mode of action is different, this co-occurrence has to be considered for the evaluation of toxic effects. The concentrations of the toxins but as well their ratio may influence the intensity of observable effects (Figure 6). Döll and Dänicke (2011) suggested combination experiments with pure toxins and thus defined contamination of feed as potential approaches to investigate the interaction of ZEN and DON. However, the same authors stated that both *Fusarium* toxins may impact the same target cells and tissues and therefore a precise assignment of the observable effects to one of the toxins is generally complicated. Moreover, unidentified other mycotoxins may be present in naturally contaminated feed and could also contribute to effects on the parameters illustrated in Figure 6.

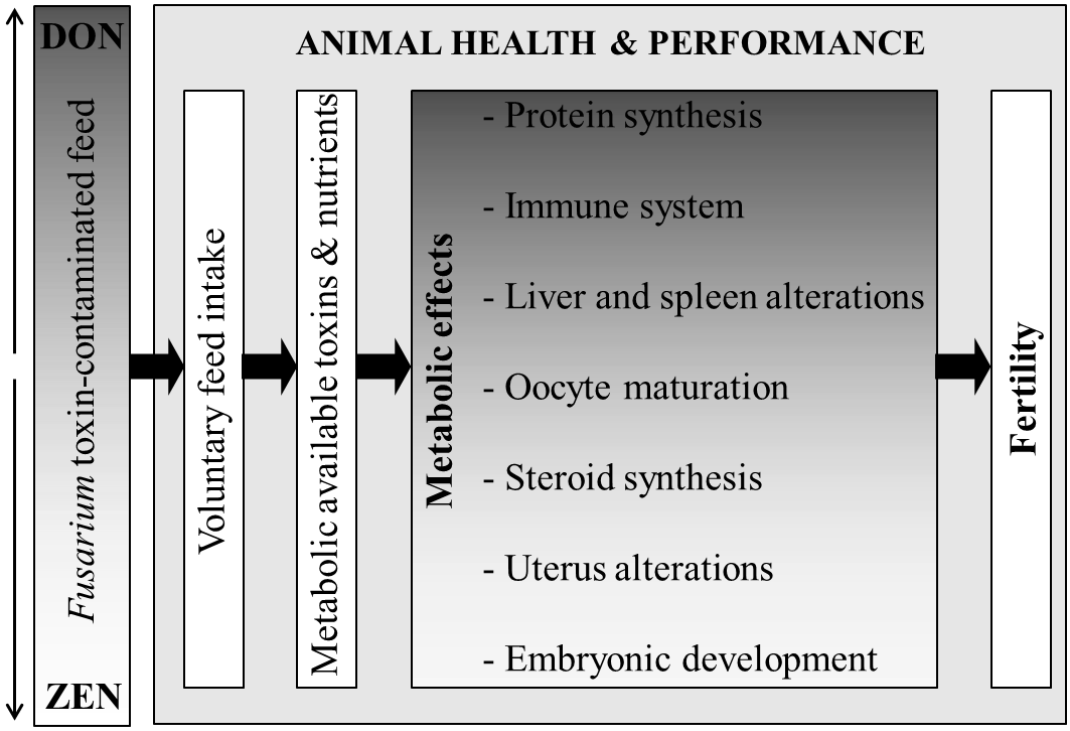

Figure 6. Synopsis of possible interactive effects of DON and ZEN: the effects of a diet contaminated with *Fusarium* toxins and containing predominantly DON and ZEN on the fertility of female pigs need to be viewed in the general context of the toxin effects on animal health and performance. Before the toxins are able to modify metabolic processes, they need to be consumed voluntarily by the pig. The effects on feed intake, which are mediated by DON, not only determine the amount of toxins entering the organism but also the amounts of metabolically available nutrients which might also markedly modulate processes involved in fertility. Although the primary molecular targets of DON (inhibition of protein synthesis) and ZEN (interference with oestrogen receptor) are different, they obviously act in a concerted manner with regard to health and fertility (according to Döll and Dänicke, 2011)

5 Relevance of exposure times for the development of effects

Beside the toxin concentrations, the time of exposure to ZEN and DON is of great importance for the occurrence and intensity of effects. While impacts on feed intake can promptly follow the initial consumption of contaminated diets, effects involving clinical signs such as alterations in organs or tissues are usually only detectable after longer exposure (Table 2). For example, liver weights of DON and ZEN exposed pigs were increased after 95 days of exposure (Bergsjø et al., 1993). In contrast, a feeding period of 37 days with comparable dietary toxin concentrations did not affect this parameter (Döll et al., 2003). Similarly, uterus weights were increased at day 21 of an exposure to 2 mg ZEN/kg (Wang et al., 2012), while a

diet containing only about one-tenth of this concentration led to increased uteri after a longer period of 35 days (Gutzwiller et al., 2007).

Table 2. Effects of deoxynivalenol (DON) and zearalenone (ZEN) on selected reproductive and non-reproductive parameters in pigs in dependence on dose and time of exposure (adapted from Tiemann and Dänicke, 2007)

Parameter	Exposure mg/kg diet; (µg/kg BW)		Duration	Effect	Reference
	DON	ZEN	[days]		
Protein synthesis	(77)		~35	+	(Dänicke et al., 2006)
	5.7		28-42	+	(Goyarts et al., 2006b)
Immune response	5.7		28	-	(Goyarts et al., 2006a)
	(53)		Acute oral	+	(Goyarts et al., 2006a)
	9.57	0.36	35	+	(Tiemann et al., 2006)
	3.9	0.42	34-37	+	(Döll et al., 2003)
	2-2.2	0.2-0.3	35	-	(Gutzwiller et al., 2007)
	1	0.25	42	+	(Cheng et al., 2006)
	2.8		35	+	(Bracarense et al., 2012)
	2-3		70	-	(Dänicke et al., 2012a)
	2.8		28	+	(Grenier et al., 2011)
	1.03	0.25	28	+	(Chen et al., 2008)
Serum biochemistry		3.2	18	+	(Jiang et al., 2011)
		0.75	21	-	(Teixeira et al., 2011)
		1.05	22	+	(Jiang et al., 2012)
	1	0.25	42	+	(Cheng et al., 2006)
	2-3		70	-	(Dänicke et al., 2012a)
	1.03	0.25	42	+	(Chen et al., 2008)
	3.9	0.42	34-37	+	(Döll et al., 2003)
	3.5	0.21	42	+	(Bergsjø et al., 1993)
	5.7		28-42	-	(Goyarts et al., 2006a)
Morphology/ histopathology of:					
• liver and spleen	9.57	0.36	35	+	(Tiemann et al., 2006)
	2.8		35	+	(Grenier et al., 2011)
	3.9	0.42	34-37	-	(Döll et al., 2003)
		2	28	-	(Andretta et al., 2010)
		3	18	+	(Jiang et al., 2011)
		1.05	22	+	(Jiang et al., 2012)
		2	21	-	(Wang et al., 2012)
	2-3		70	-	(Dänicke et al., 2012a)
	3.5	0.21	95	+	(Bergsjø et al., 1993)
• intestine	2.8		35	+	(Bracarense et al., 2012)
	2-3		70	+	(Dänicke et al., 2012a)
	2.29		28	+	(Pinton et al., 2012)
• uterus	3.9	0.42	34-37	+	(Döll et al., 2003)
		2	28	+	(Andretta et al., 2008)
		2	28	+	(Andretta et al., 2010)
	2-2.2	0.2-0.3	35	+	(Gutzwiller et al., 2007)
		(20)	48	+	(Gajecka et al., 2011)

Table 2 - Continued

		2	21	+	(Wang et al., 2012)
		0.5	21	-	(Wang et al., 2012)
		1.5	28	+	(Oliver et al., 2012)
Vulva morphology		0.25	5-7	+	Bauer et al., 1987)
		2	7	+	(Andretta et al., 2008)
		2	6	+	(Andretta et al., 2010)
	2-2.2	0.2-0.3	35	-	(Gutzwiller et al., 2007)
		(200)	4	+	(Zwierzchowski et al., 2005)
		1.1	18	+	(Jiang et al., 2011)
		0.75	21	+	(Teixeira et al., 2011)
	1	0.25	7	+	(Cheng et al., 2006)
		1.5	7	+	(Oliver et al., 2012)
		(195)	2	+	(Olsen et al., 1985)
Follicular development		0.25	11 days	+	(Bauer et al., 1987b)
		(200)	8	+	(Zwierzchowski et al., 2005)
	9.57	0.36	35	-	(Alm et al., 2006)

BW, body weight

6 Options for the reduction of DON and ZEN in feed

The frequent occurrence of mycotoxin-concentrations in cereals above given guidance values has promoted the development of strategies to reduce the toxin load of grain used as animal feed. According to Döll and Dänicke (2011) current possibilities include:

- blending of contaminated with uncontaminated feed stuff to dilute the concentration of toxins
- distribution according to species sensitivity (e.g. cattle and poultry is less sensitive than swine)
- decontamination procedures.

However, in dependence on the focus of production of a practical farm or the availability of uncontaminated feed, the first two options might not always be possible.

Decontamination procedures are unaffected by these factors. Within decontamination procedures pre-feeding and *in vivo* strategies can be differentiated. *In vivo* approaches mostly consist of feed additives that aim to degrade or transform the toxin into a less toxic metabolite or to bind it during the passage through the digestive tract and thereby hinder its absorption and related adverse effects. Most investigated additives include mycotoxin-degrading enzymes or microbes providing such enzymes and adsorbents including inorganic (eg. clay, betonite, zeolite) and organic (yeast or bacterial cell wall components) substances as reviewed

lately (Conte and Baricco, 2010; Kolosova and Stroka, 2011). However, the efficacy of such substances in diverse studies was shown to be questionable (e.g. Dänicke et al. (2004b, 2012a), Döll et al. (2005), Dänicke and Döll (2010)).

Diverse pre-feeding strategies were discussed and evaluated previously as summarized in Table 3. Abrasive methods, which aim to peel the outer layers of cereal grains, seem to be promising procedures as most of the toxins are located in these layers (Miller et al., 1985; Schaafsma et al., 2004). For example, dehulling reduced the concentrations of ZEN and DON in barley by 85 and 88%, respectively (Trenholm et al., 1991). Similarly, DON-concentrations in corn were reduced by 79% during density segregation in water and sucrose solution (Huff and Hagler, 1985). However, these methods always imply the removal of cereal fractions, which sometimes exceed even 50% of the cereal weight itself, making it extremely uneconomic and drastically impair the efficacy of the respective livestock systems. Cleaning-techniques with water seem to be generally ineffective. Although soaking in chemical solution can provide some good results (Rotter et al., 1995; Ragab et al., 2007), the efforts to dry cereals afterwards reduce its applicability when they cannot be processed wet and include high energy costs. The use of chemicals generally provides the most promising toxin-reductions. Most studies conducted in order to investigate the detoxification potential of different chemicals focused on the reduction of individual toxins, which often only solves a part of the problem, considering the co-occurrence of several toxins in naturally contaminated feed. Sodium metabisulfite (SBS), for example, is highly effective in converting DON to the non-toxic derivate DON-sulfonate (DONS), while ZEN remains totally uninfluenced by this chemical (Dänicke et al., 2008a).

Table 3. Pre-feeding procedures to reduce the toxin load of cereals

Procedure	Toxin	Highest achieved reductions	Cereal (kernels)	Reference
Dehulling with pearling machines				
	DON	66%	Barley	(House et al., 2003)
	DON	45%	Durum wheat	(Rios et al., 2009)
	DON, ZEN	84, 88%	Barley	(Trenholm et al., 1991)
	DON	52%	Wheat	(Trenholm et al., 1991)
Sorting				
Sieving	DON	6.2%	Barley	(Pei et al., 2005)
Sieving	DON, ZEN	67, 73%	Ground barley	(Trenholm et al., 1991)
Sieving	DON	83%	Ground wheat	(Trenholm et al., 1991)
Sieving	DON, ZEN	73, 79%	Ground corn	(Trenholm et al., 1991)
Density segregation	DON	52%	Corn	(Rotter et al., 1995)
Density segregation	ZEN	>99%	Sorghum	(Babadoost et al., 1983)
Density segregation	DON	79%	Corn	(Huff and Hagler, 1985)
Density segregation	DON	96%	Wheat	(Huff and Hagler, 1985)
Cleaning techniques with water				
Rinsing	DON, ZEN	44, 50%	Corn	(Rotter et al., 1995)
Washing	DON	6%	Barley	(Pei et al., 2005)
Soaking in chemical solution				
Sodium carbonate	DON, ZEN	79%	Corn	(Rotter et al., 1995)
Sodium carbonate	DON	93%	Wheat	(Ragab et al., 2007)
Sodium carbonate	DON	37%	Barley	(Pei et al., 2005)
Hydrothermal treatment with chemicals				
Methylamine, $Ca(OH)_2$	ZEN	85%	Ground barley	(Bauer et al., 1987a)
Sodium metabisulfite	DON	>99%	Ground wheat	(Dänicke et al., 2005b)
Sodium carbonate	ZEN	74%	Ground wheat	(Polak et al., 2009)
Ammonium carbonate	DON	92%	Ground Corn	(Young, 1986b)
Sodium carbonate	DON	99%	Barley	(Abramson et al., 2005)
Wet preservation				
Sodium metabisulfite	DON	99%	Wheat	(Dänicke et al., 2010b)
Sodium metabisulfite	DON	96-97	Triticale	(Dänicke et al., 2009)
Sodium metabisulfite	DON	>98%	Wheat	(Young et al., 1986)

SCOPE OF THE THESIS

Based on the current literature it can be deduced that the development and intensity of adverse effects of a consumption of *Fusarium* toxin-contaminated diets in swine may likely be influenced by the dosage and time of exposure. As a result, for the evaluation of decontamination procedures long-term effects such as the enlargement of the uterus are frequently used as parameters. Determining the onset of measurable effects of the most important *Fusarium* toxins ZEN and DON in long-term experiments is therefore crucial for both the understanding of the mode of action of these toxins and the evaluation of the efficiency of detoxification procedures. Moreover, there is an ongoing need for decontamination techniques that involve both ZEN and DON as they are often detected in toxicologically relevant concentrations in the same samples.

Therefore, the first aim of this thesis was to elucidate the potential progression of effects of continuous consumption of diets containing graded levels of *Fusarium* toxin-contaminated maize on health and performance of female weaned piglets in time (**Paper I**).

The efficiency of a hydrothermal treatment in the presence of different combinations and dosages of sodium metabisulfite, methylamine and calcium hydroxide was to be evaluated with regard to a simultaneous reduction of ZEN and DON (**Paper II**).

A variant with considerable reduction of both ZEN and DON from the hydrothermal treatment test (**Paper II**) was evaluated *in vivo* for its efficacy to alleviate the effects of the inclusion of *Fusarium* toxin-contaminated maize in diets for female piglets and for mycotoxin unspecific effects of the treatment itself (**Paper III**).

Paper I

Time-dependent effects of graded levels of *Fusarium* toxin-contaminated maize in diets for female piglets

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Abstract

The study was conducted to evaluate the effect of graded levels of the *Fusarium* toxins zearalenone (ZEA) and deoxynivalenol (DON) in diets for female piglets during 29 treatment days on the development of performance, organ weights, clinical serum characteristics, differential blood counts and vulva morphology. For this purpose, 120 female weaned piglets with an initial mean bodyweight of 8.9 ± 1.6 kg, were assigned to 5 feeding groups (n=24). The piglets of groups 1 to 5 received diets containing 0, 1.25, 2.5, 5 and 10 % naturally-contaminated maize instead of control maize, resulting in 0.01, 0.05, 0.08, 0.17, 0.29 mg ZEA/kg and 0.03, 0.59, 1.27, 2.01, 4.52 mg DON/kg feed, respectively. After periods of 1, 3, 8, 15, 22 and 29 days, 20 piglets (four piglets from each group) were slaughtered. Animals of group 5 consumed at least 20% less feed than animals of the other feeding groups. Body weight gain was significantly reduced in group 5 in the last week in comparison to groups 2 and 3. Relative visceral organ weights were not affected by treatment but mostly showed minimal time-dependent alterations. Uterus weights tended to be decreased in group 5 on day 15 of the experiment. Though not significant, signs of hyperoestrogenism, such as swelling of the vulva, became more pronounced with increasing time of exposure. Histopathological examination of organ specimens did not reveal any toxin-related lesions. Total leukocytes, differential blood count and stimulation index of peripheral blood mononuclear cells were not affected by diet, however, the proportion of lymphocytes increased while neutrophils decreased during the experiment independent of treatment. Clinical-chemical serum characteristics were not affected by treatments, however, they were somewhat affected by time. In conclusion, the dose-related effects of ZEA and DON on performance and signs of hyperoestrogenism seemed to be dependent on exposure time.

Keywords: mycotoxins, deoxynivalenol, zearalenone

1 Introduction

The secondary metabolites of several *Fusarium* species, deoxynivalenol (DON) and zearalenone (ZEA), are prevalent contaminations of maize (SCOOP, 2003). Their occurrence in toxicologically relevant concentrations raises a particular issue in animal feeding, where maize is a common component of diets for different species. Although adverse effects of both ZEA and DON have been observed in various livestock species (D'Mello et al., 1999), pigs are regarded as the most susceptible animals as they may show considerable responses to relatively low dosages of *Fusarium* toxins. DON is known to depress voluntary feed intake

and to inhibit protein synthesis at the cellular level by binding to the 60S subunit of eukaryotic ribosomes (Dänicke et al., 2006; EFSA, 2004a; Rotter et al., 1996). ZEA and its derivates, which are structurally related to natural oestrogens, were reported to bind to oestrogen receptors (Powelljones et al., 1981) and may hence be assigned to the group of endocrine disruptors. Their occurrence in feed stuff is related to hyperoestrogenism and reproductive disorders (Döll and Dänicke, 2011; Döll et al., 2003; EFSA, 2004b).

To avoid these adverse effects, guidance values for critical feed concentrations were introduced by the European Union in 2006, recommending values not exceeding 0.9 mg/kg for DON and 0.1 mg/kg for ZEA in piglet diets (EC, 2006). However, negative effects at dietary concentrations below the mentioned guidance values might occur due to high concentrations of other undetected fungal metabolites in contaminated feedstuff, the overall health status of the animals or the time of exposure to the toxins.

Effects on feed intake, mainly attributed to DON, seem to be quite constant during the course of experiments, when comparing exposed groups to control groups (Döll et al., 2003; Goyarts et al., 2005). Dietary ZEA was reported to be subjected to enterohepatic cycling and accumulation in bile fluid (Biehl et al., 1993). Hence, even low dietary concentrations may result in consecutive accumulation during the course of exposure. Whether a time-dependent accumulation of ZEA and its metabolites in bile is also associated with an increased systemic absorption and oestrogenic activity is not known so far (Dänicke et al., 2008b). By evaluating external symptoms of hyperoestrogenism such as swelling of the vulva, Andretta et al. (2010) detected increasing differences in vulva volumes of piglets fed diets containing 2 mg/kg ZEA compared to the control group during a 4-week experiment.

Very few studies focused on in practice relevant concentrations in diets for female piglets and most investigations were conducted exclusively at the end of the entire experimental period (Döll et al., 2003).

The present study aimed to elucidate the potential progression of effects of continuous consumption of diets containing graded levels of *Fusarium* toxin-contaminated maize on health and performance of female weaned piglets in time.

2. Materials and methods

2.1 Experimental diets

Naturally-contaminated maize used in the feeding trial was generated via inoculation. Briefly, maize (*Zea mays L.*, “Magister”) was cultivated on an experimental field site of the Friedrich-

Loeffler-Institute in Braunschweig, Germany in 2010 according to local farm practices. In lactic ripeness stage, maize was infected with *Fusarium graminearum* by applying a spore solution directly into the cob through the opening of the husk. The mycotoxin-contaminated maize was harvested two months later.

Table 1. Composition of the experimental diets, metabolisable energy and analyses of selected ingredients

	Group 1	Group 2	Group 3	Group 4	Group 5
Components (g/kg)					
Barley	350.0	350.0	350.0	350.0	350.0
Wheat	340.0	340.0	340.0	340.0	340.0
Contaminated maize	0.0	12.5	25.0	50.0	100.0
Control maize	100.0	87.5	75.0	50.0	0.0
Soybean extraction meal	150.0	150.0	150.0	150.0	150.0
Soya bean oil	10.0	10.0	10.0	10.0	10.0
Marker	10.0	10.0	10.0	10.0	10.0
Premix[1]	10.0	10.0	10.0	10.0	10.0
Calciumcarbonate	10.0	10.0	10.0	10.0	10.0
Sodium chloride	0.5	0.5	0.5	0.5	0.5
L-lysine-HCl	7.0	7.0	7.0	7.0	7.0
L-tryptophane	1.0	1.0	1.0	1.0	1.0
DL-methionine	2.9	2.9	2.9	2.9	2.9
L-threonine	2.3	2.3	2.3	2.3	2.3
Formic acid	6.0	6.0	6.0	6.0	6.0
Phytase	0.3	0.3	0.3	0.3	0.3
Analysed composition					
Zearalenone (mg/kg)	0.005	0.05	0.08	0.17	0.29
Deoxynivalenol (mg/kg)	0.03	0.59	1.27	2.01	4.52
Dry matter (g/kg)	888.9	890.5	893.1	893.4	895.3
Organic matter (g/kg dry matter)	937.7	944.1	936.5	943.2	937.9
Crude ash (g/kg dry matter)	62.3	55.9	63.5	56.8	62.1
Crude protein (g/kg dry matter)	188.5	185.8	194.6	189.4	193.1
Ether extract	41.9	37.8	42.9	38.5	41.4
Crude fibre	45.5	49.5	45.7	45.6	44.1
N-free-extractives	661.8	671.0	653.3	669.6	695.3
Metabolizable energy[2] (MJ/kg)	15.1	15.0	15.2	15.0	15.2

[1] Provided per kg diet: Ca 1.0 g, P 1.4 g, Na 0.9 g, Mg 0.1 g, Fe 75 mg, Cu 15 mg, Mn 40 mg, Zn 50 mg, I 1.0 mg, Se 0.2 mg, Co 0.4 mg, vitamin A 10000 IU, vitamin D3 1000 IU, vitamin E 50 mg, vitamin B1 1.0 mg, vitamin B2 3.1 mg, vitamin B6 2.5 mg, vitamin B12 20.0 µg, vitamin K3 2.0 mg, nicotinic acid 12.5 mg, pantothenic acid 7.5 mg, choline chloride 125 mg, biotine 50 µg, folic acid 0.5 ng, vitamin C 50 mg.
[2] Calculated using the equation by Kirchgessner and Roth (1983).

A diet consisting of barley, wheat, soybean extraction meal and maize was formulated to meet or exceed all nutritional requirements of weaning piglets as recommended by the Society of

Nutrition Physiology (GfE, 2006) (Table 1). One control and four mycotoxin-contaminated diets were formulated to contain 0, 1.25, 2.5, 5 and 10 % of the naturally-contaminated maize at the expense of control maize. The proportions of maize included were based on the toxin concentrations of the contaminated maize. An uneven distribution of toxins within the maize batch that might be possible was counteracted by homogenizing the ground maize prior to mixing into the diets.

2.2 Experimental design and procedures

The experiment and procedures were conducted according to the European Community regulations concerning the protection of experimental animals and the guidelines of the Regional Council of Braunschweig, Lower Saxony, Germany (File Number 33.14-42502.04.037/08).The study was carried out at the experimental station of the Friedrich-Loeffler-Institute in Braunschweig. A total of 120 female weaned crossbred Piétrain x (PIC x DanZucht) piglets were allocated to a pig-rearing house with 20 pens (six piglets per pen) with slatted floors. For one week, the piglets were fed a pre-experimental diet, corresponding to the diet containing only control maize, to adapt to *ad libitum* feeding. Piglets were then weighed and randomly assigned to one of the five feeding groups (24 piglets per group) with an initial mean body weight of 8.9 ± 1.6 kg. The experimental diets were introduced with the morning feeding prior to the first sampling. The animals had free access to feed from automatic feeders and water from drinking nipples. During the experiment, individual body weight and feed consumed per pen were determined once weekly. To evaluate external symptoms of hyperoestrogenism, the horizontal vulva width of the individual animals was measured with a common ruler at weighing times. After 1, 3, 8, 15, 22 and 29 days of feeding the experimental diets, each 20 piglets (1 piglet per pen, resulting in 4 piglets per group) were euthanized by intracardial injection of T61® (embutramide/mebezoniumiodide/tetracainhydrochloride, Intervet Unterschleißheim, Germany) after i.m. premedication with ketamin/azaperon (15 mg/kg Ursotamin® and 0.05 mg/kg Stresnil®, Janssen Animal Health, Neuss, Germany) and general inhalation anaesthesia using isoflurane. During anaesthesia, blood was collected by heart puncture for haematology, preparation of serum and isolation of peripheral blood mononuclear cells (PBMC). Following euthanasia of the piglets, organs including liver, kidney, heart, spleen, uterus and ovaries, were dissected, weighed and subjected to examination by a veterinarian. Samples of the mentioned organs and additional samples from duodenum, jejunum, ileum,

Peyer plaques, vulva, vagina and teat were fixed in a 4 % formalin solution for histopathological examinations.

2.3 Analyses

2.3.1 Chemical composition and mycotoxins

Feed samples were ground to pass through a 1 mm sieve prior to analysing the chemical composition. Dry matter, crude ash, crude fibre, crude protein, ether extract and N-free-extractives were analysed according to the suggestions of the VDLUFA (Naumann and Bassler, 1993).

The concentrations of DON in maize and feed were analysed by high-performance liquid chromatography (HPLC) with diode array detection after clean up with immuno-affinity columns (IAC) (DON-prep®, R-Biopharm AG, Darmstadt, Germany) as described by Oldenburg et al. (2007). The analysis of ZEA was carried out according to a slightly modified method of the VDLUFA (2006) by HPLC with fluorescence detection after clean up with IAC (ZearalaTest™ WB, Vicam, Milford, USA). Further trichothecene mycotoxins as well as precursors, derivates of ZEA, dipepsipeptides and other *Fusarium* and *Alternaria* metabolites were determined by the Institute of Agrobiotechnology (Tulln, Austria) applying a LC-MS/MS method as described by Vishwanath et al. (2009).

2.3.2 Isolation and proliferation of peripheral blood mononuclear cells

Both isolation and proliferation of porcine PBMC were carried out according to Goyarts *et al.* (2006a). Briefly, PBMC were separated from diluted, heparinised blood by density gradient centrifugation and then frozen and stored at -80°C in dimethyl sulfoxide until the beginning of the proliferation test. For the proliferation test 10 replications of thawed and washed PBMC were seeded into 96-well plates and 5 of them were stimulated with Concanavalin A (ConA, Sigma-Aldrich, Steinheim, Germany). A MTT-assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was used to test the metabolic activity of the proliferating cells. The optical density (OD) of the incubated PBMC was determined at wavelength of 570 nm and corrected by blank value. The ratio between OD of ConA stimulated cells and non-stimulated cells was calculated as stimulation index (SI).

2.3.3 Haematology and serum biochemical analyses

Total leukocytes were manually counted in stained whole blood samples using an improved Neubauer counting chamber (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) and Türk`s

solution (VWR International GmbH, Darmstadt, Germany). Stained whole blood smears were generated on microscope slides to perform manual 200-cell differential counts.

Activities of aspartate amino-transferase (AST), glutamate dehydrogenase (GLDH), γ-glutamyltransferase (γ-GT) as well as concentrations of total protein, albumin, glucose, bilirubin, cholesterol, triglycerides and urea in serum were determined photometrically by an automatic clinical chemistry analyser (Eurolyser, Qinlab Diagnostic GbR, Martinsried, Germany).

2.3.4 Histopathology

Organ specimens from group 1 and group 5 from day 29 of the experiment were chosen for histopathological examination, as effects of the treatment were expected to be most apparent between the groups exposed to the lowest and highest toxin concentration in the diet. The samples (spleen, liver, kidney, heart, intestine, uterus, vulva, vagina, ovaries and teat) were fixed in a 4 % formaldehyde solution (Roti®-Histofix 4 %, Carl Roth GmbH + Co KG, Karlsruhe, Germany) for at least 24 hours before being embedded in paraffin and sliced. After staining with haematoxylin and eosin the specimens were examined for tissue changes by two pathologists without knowledge of the feeding group of the individual animal. Additionally, the proportion of primordial, primary and secondary follicles in the periphery of the ovaries was determined.

2.3.5 Statistical analyses

All statistics were carried out using the software package SAS version 9.1 (SAS Institute, Cary, NC, USA). Only data from animals exposed to the experimental diets for at least one week were considered for performance and vulva analyses. Data were processed using the PROC MIXED procedure containing the treatment group, experimental day and their interaction as fixed factors. The individual piglet effects as well as the effects of the particular pen (concerning feed intake data) resulting from the frequent measurements in the course of the experiment were considered by the RANDOM statement.

Relative organ weights and normally distributed serum analyses were subjected to analysis of variance (ANOVA) according to a 2-factorial design, considering the treatment group, experimental day and their interaction as fixed factors. Histological findings in tissues were not evaluated statistically due to the limited number of animals and diagnoses. The distribution of follicular stages in the ovaries was subjected to ANOVA according to a one-factorial design, including the treatment group as fixed factor.

Statistically significant differences of means or LSmeans were detected using the Tukey's multiple range test at $p<0.05$ while differences at $p<0.1$ were considered as trends.

3 Results

3.1 Mycotoxin content in maize

Analysis of *Fusarium* toxins of the naturally contaminated maize revealed high concentrations of DON, deoxynivalnol-3-glucosid (DON-3G), 15-acetyl-deoxynivalnol (15-ADON), ZEA, butenolid, aurofusarin, culmorin, 15- and 5- hydroxy culmorin. In contrast, control maize was only slightly contaminated with these toxins (Table 2).

Table 2. Mycotoxin pattern of control and naturally contaminated maize (µg/kg; 88% dry matter)

	Control maize	Naturally contaminated maize
DON	379	37,540
DON-3-glucosid	26	4,426
3-Acetyl DON	10	545
15-Acetyl DON	199	3,657
Nivalenol	12	407
ZEA	14	4,269
ZEA-4-Sulfat	< 1	183
α-ZOL	< 1	33
β-ZOL	< 1	42
Enniatin B	< 2	179
Enniatin B1	< 2	113
Enniatin A1	< 1	17
Enniatin A	< 1	< 2
Beauvericin	82	14
Butenolid	125	2,714
Moniliformin	229	62
Apicidin	< 1	2
Equisetin	2	126
Fusaproliferin	129	183
Aurofusarin	97	25,904
Avenacein Y	246	580
Chlamydosporol	< 1	10
Culmorin	1,046	13,625
15-Hydroxy Culmorin	12	2,350
5-Hydroxy Culmorin	< 1	12,081
15-Hydroxy Culmoron	< 1	814
Alternariol	< 1	2
Alternariolmethylether	< 1	< 2
Tentoxin	< 1	< 1
Altertoxin I	< 1	< 1
Emodin	< 3	< 1
Chrysophanol	< 2	< 1

3.2 Animal performance

Table 3. Performance parameters and toxin intake of piglets exposed to increasing dietary *Fusarium* toxin concentrations in the course of the experiment (LSmeans; n = 4, except for BWG[3])

Experimental week	Diets[1]	Feed intake [g/d]	DON [µg/kg BW/d]	ZON [µg/kg BW/d]	BWG [g/d]	Feed-to-gain ratio [kg/kg]
1	1	451	1	0.2	323	
	2	451	24	2.1	323	
	3	435	50	3.2	325	
	4	435	81	6.8	317	
	5	360	156	10.0	255	
2	1	653	1	0.2	364	
	2	667	28	2.3	405	
	3	651	59	3.7	387	
	4	634	85	7.2	342	
	5	525	194	12.4	244	
3	1	820	1	0.2	463	
	2	806	27	2.3	451	
	3	755	59	3.7	354	
	4	801	93	7.8	487	
	5	618	197	12.7	285	
4	1	1,057	2	0.3	486^{ab}	
	2	995	28	1.8	732^{a}	
	3	979	61	3.9	736^{a}	
	4	986	98	8.3	632^{ab}	
	5	772	221	14.1	444^{b}	
…	1	745	1^{a}	0.2^{a}	409^{a}	1.76^{ab}
	2	730	27^{b}	2.1^{b}	478^{a}	1.60^{a}
	3	705	57^{c}	3.6^{c}	451^{a}	1.63^{ab}
	4	714	89^{d}	7.6^{d}	444^{a}	1.67^{ab}
	5	569	192^{e}	12.3^{e}	307^{b}	1.86^{b}
P-Value						
Diet		0.059	<0.001	<0.001	<0.001	0.033
Week		<0.001	<0.001	<0.001	<0.001	
Diet x Week		0.804	<0.001	<0.001	0.006	
PSEM[2]		19	2	0.1	8	0.06

[1] ZEA/DON concentrations in mg/kg diet: 1 - 0.01/0.03; 2 - 0.05/0.59; 3 - 0.08/1.27; 4 - 0.17/2.01; 5 - 0.29/4.52
… denotes that the effect was pooled
[2] Pooled standard error of means
[3] Body weight gain; Due to continuous slaughter of piglets for sampling, the number of animals decreased during the trial but the number of animals per treatment remained equal (n=16 week 1; n=12 week 2; n=8 week 3; n=4 week 4)
[a,b,c,d,e] different superscript letters indicate significant differences between feeding groups

As presented in Table 3, daily feed intake tended to be affected by diet ($p=0.059$) and week ($p<0.001$), and was lowest in group 5 receiving the highest proportion of contaminated maize. Accordingly, both diet ($p<0.001$) and week ($p<0.001$) had significant effects on body weight gain being the lowest in the group exposed to the highest dietary toxin concentration. In all

groups, increase in mean daily feed intake was approximately linear during the experiment. Feed intake of the group exposed to the highest dietary toxin concentration was found to be consistently at least 20 % lower than that of the other groups. The intake of ZEA and DON per kg body weight per day remained relatively constant during the experimental period.
The development of daily body weight gain was comparable between control (group 1) and groups 2 and 4. The daily weight gain in group 3, receiving 2.5 % contaminated maize, decreased temporarily in the third experimental week but returned to a high level in week 4. During the first 3 weeks, the body weight gain of group 5 remained below 300 g/d but reached 444 g/d in the last week. Significant differences in body weight gain were detected in the last week comparing group 5 to group 2 and 3. No significant differences in the cumulative feed-to-gain ratio between control and the toxin exposed groups were observed. However, a significant decrease in this ratio was found in group 2 compared to group 5.

3.3 Body weights at slaughter, visceral and reproductive Organs

Differences in mean body weights between the group exposed to the highest toxin concentration and the remaining groups increased in the course of the experiment as shown in Table 4. However, the interaction of diet and treatment time was not significant ($p=0.944$). The relative weights of the investigated organs were mostly not influenced by the dietary treatments. Though the relative weights of heart, kidney and spleen were affected by the experimental time ($p<0.05$), the numerical differences between sampling days were predominantly found to be minimal. In contrast, the relative liver weights were not altered during the experiment ($p=0.889$). Uterus weights tended to increase with rising mycotoxin exposure ($p=0.077$) and experimental time ($p=0.005$), however relative ovary weights did not differ between the groups ($p=0.609$). On day 15, the differences in uterus weights between groups 1 and 5 were reflected in a trend of a diet effect ($p=0.079$). In the further experimental days the highest relative uterus weights were observed in the group receiving the highest toxin concentration. However, at the first three samplings no differences in uterus weights between the groups were recorded. The occurrence of visible follicles at the ovaries was generally scarce throughout the experimental period and could therefore not be subjected to statistical evaluation (data not shown). Dietary treatment and experimental week did not interact significantly ($p>0.05$).

Table 4. Body weight (BW) at slaughter and relative visceral and reproductive organ weights [g/kg BW] of piglets exposed to increasing dietary *Fusarium* toxin concentrations in the course of the experiment (Means; n=4)

Experimental day[1]	Diet[2]	BW [kg]	Heart	Liver	Kidney	Spleen	Uterus	Ovaries
1	1	9.0	5.6	27	5.1	3.8	0.49	0.016
	2	9.1	6.4	30	4.6	3.3	0.41	0.013
	3	7.4	6.7	31	5.3	4.1	0.47	0.013
	4	9.0	5.9	28	5.1	3.4	0.40	0.012
	5	8.5	7.5	31	4.8	3.3	0.39	0.013
3	1	8.8	5.8	27	4.8	3.3	0.47	0.009
	2	10.0	5.3	30	4.7	3.6	0.47	0.013
	3	10.5	5.6	27	4.2	3.3	0.44	0.009
	4	10.0	6.1	28	4.7	3.6	0.47	0.012
	5	9.0	5.7	28	4.6	3.8	0.47	0.010
8	1	10.9	5.2	28	4.6	3.4	0.46	0.010
	2	10.5	5.4	29	4.4	3.8	0.47	0.009
	3	11.1	5.6	31	4.8	3.8	0.49	0.012
	4	10.3	6.0	29	4.6	3.9	0.42	0.009
	5	11.1	5.0	28	4.4	3.7	0.48	0.008
15	1	14.4	5.4	27	5.5	5.5	0.38	0.009
	2	14.2	5.2	32	5.3	4.6	0.49	0.015
	3	14.3	5.4	30	5.4	5.3	0.48	0.008
	4	13.5	5.3	31	4.7	4.3	0.48	0.010
	5	12.1	6.1	30	6.4	5.6	0.61	0.012
22	1	17.6	4.9	29	4.6	4.1	0.48	0.011
	2	18.0	4.9	28	5.0	5.7	0.48	0.009
	3	16.4	5.0	30	5.0	5.8	0.48	0.014
	4	18.1	5.6	32	5.0	5.4	0.58	0.012
	5	15.0	5.3	28	5.2	4.9	0.63	0.011
29	1	19.4	6.0	31	5.2	4.8	0.52	0.021
	2	21.0	6.1	28	4.9	4.1	0.53	0.025
	3	20.2	5.5	31	5.3	4.9	0.44	0.011
	4	20.2	5.5	28	5.1	5.0	0.52	0.012
	5	16.0	5.8	27	4.7	4.9	0.59	0.013
P-value								
Diet		0.120	0.729	0.652	0.838	0.582	0.077	0.609
Week		<0.001	0.020	0.889	0.001	<0.001	0.005	0.058
Diet x Week		0.944	0.923	0.883	0.578	0.593	0.136	0.767
PSEM[3]		1.3	0.6	2	0.3	0.5	0.04	0.004

[1] At days 1, 3, 8, 15, 22 and 29 a total of 20 piglets each (4 piglets per group) were slaughtered

[2] ZEA/DON concentrations in mg/kg diet: 1 - 0.01/0.03; 2 - 0.05/0.59; 3 - 0.08/1.27; 4 - 0.17/2.01; 5 - 0.29/4.52

[3] Pooled standard error of means

The histopathological examination of organs of both the control and highest exposed group on day 29 showed no mycotoxin-related abnormalities. Group-independent mild to moderate mononuclear infiltrates were observed in liver, uterus, vagina, vulva and teat of all animals. In addition, low numbers of eosinophils were observed in the intestine of control and treated

animals, indicative of mild parasitic infection. Lymphatic hyperplasia was found to be of minor and average severity in spleen tissue and Peyer plaques, respectively. Moreover, most animals showed mild nephritis and hyperplasia of bilary ducts. The examination of the ovaries revealed no differences in the distribution of follicle stages between the two groups. On average, 87.7±1.6% (mean ± SE) primordial, 7.1±1.2% primary and 5.2±1.2% secondary follicles were found.

The results of vulva measurements are shown in Figure 1. In the course of the experiment, the vulva width-to-body weight ratio decreased constantly in all feeding groups ($p<0.001$). However, a lower decrease was observed in the group exposed to the highest dietary toxin concentration than in the remaining groups, leading to increasing differences between the groups with progressing experimental time. Though the different slopes resulted in a significant interaction between diet and experimental time ($p=0.049$), a comparison of the group values on the separate experimental days did not reveal significant effects.

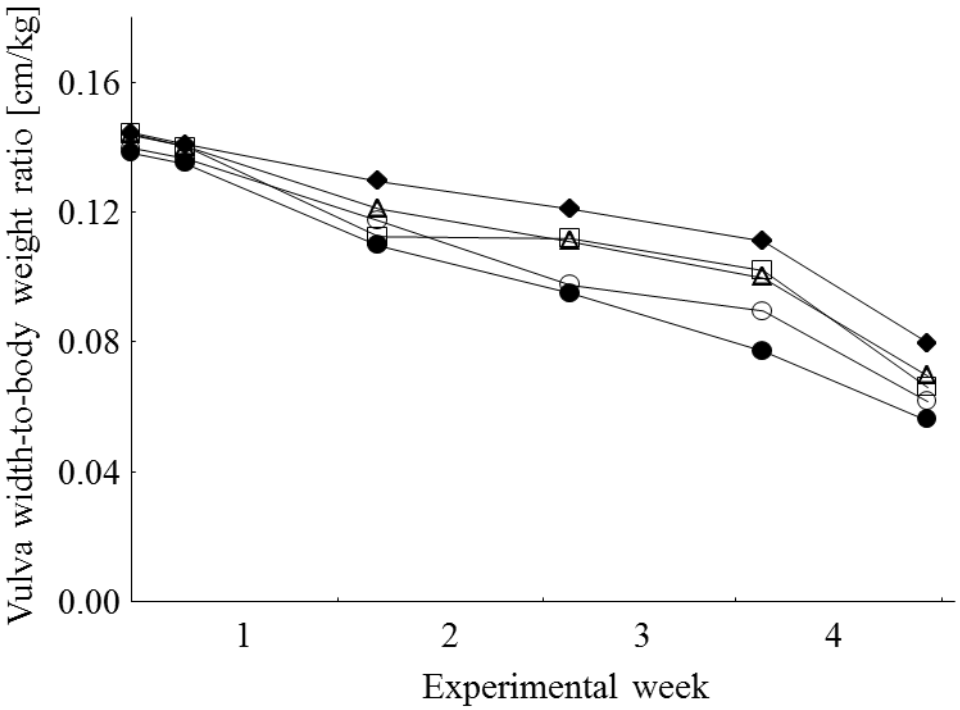

Figure 1. Vulva width-to-body weight ratio of piglets exposed to increasing dietary *Fusarium* toxin concentrations in the course of the experiment (LSmeans) (– • – group 1; – o – group 2; – Δ – group 3; – □ – group 4; – ♦– group 5). Due to continuous slaughter of piglets for sampling, the number of animals decreased during the trial but the number of animals per treatment remained equal (n=16 week 1; n=12 week 2; n=8 week 3; n=4 week 4) (PSEM= 0.01)

3.4 Haematological and biochemical analyses and proliferation of PBMC

The development of total leukocyte counts and their distribution was generally not affected by the experimental diets and interactions with time were found to be insignificant as shown in Table 5. Throughout the experiment basophils (0.38±0.04, Mean ± Standard error), eosinophils (0.55±0.05) and monocytes (0.27±0.04) were within the reference ranges given by Kraft and Dürr (2005), but were not statistically evaluated due to their low relative proportions of all 200 counted leukocytes (data not shown).

Figure 2 illustrates the development neutrophils, lymphocytes and stimulation index of PBMC during the 4 experimental weeks. The proportion of neutrophils prevalently exceeded the reference range in the first three days but decreased significantly in the further course of the experiment ($p<0.001$), whereas the percentage of lymphocytes increased to the same extent ($p<0.001$). Increasing dietary percentages of *Fusarium* toxin-contaminated maize did not influence the stimulation index of porcine PBMC. However, a significant effect of the sampling day was observed ($p<0.001$), resulting in a reduction of the index during the first two weeks of the trial.

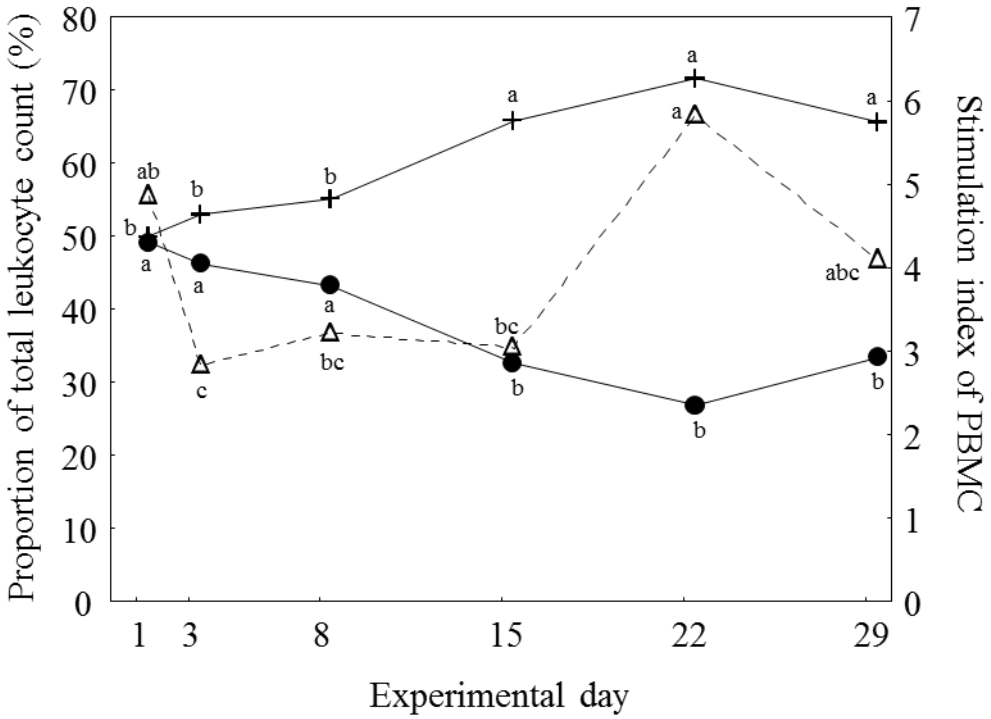

Figure 2. Proportion of leukocyte subpopulations and stimulation index of PBMC of piglets in the course of the experiment (Means; n=20) (– + – Lymphocytes; – • – Neutrophils; – Δ – Stimulation index of PBMC)

[a,b,c] different letters indicate significant differences between experimental days

Table 5. Differential blood count and stimulation index of PBMC of piglets exposed to increasing dietary *Fusarium* toxin concentrations in the course of the experiment (Means; n=4)

Experimental day[1]	Diet[2]	Leukocytes [10^7/L]	Lymphocytes [%]	Neutrophils [%]	Stimulation Index
		1 – 2.2[a]	49 – 85[a]	10 – 39[a]	
1	1	1.7	54.9	44.3	4.1
	2	1.3	46.4	52.8	5.8
	3	1.8	51.4	48.0	4.7
	4	1.6	51.4	47.6	5.0
	5	1.7	46.0	53.3	4.8
3	1	1.5	44.8	54.4	2.3
	2	1.8	59.9	39.6	3.6
	3	1.6	56.8	42.5	2.8
	4	1.6	54.1	44.8	2.4
	5	1.7	49.5	49.9	3.0
8	1	1.8	55.8	42.8	2.2
	2	1.8	56.8	41.8	2.1
	3	1.5	52.4	45.0	3.3
	4	1.7	56.0	42.9	3.7
	5	1.6	54.5	43.8	4.6
15	1	1.7	72.5	26.1	3.4
	2	1.7	68.8	30.4	2.6
	3	1.6	58.4	38.9	3.0
	4	1.5	68.7	30.2	2.5
	5	2.1	61.1	37.1	3.5
22	1	1.5	74.1	24.5	5.2
	2	1.5	73.3	25.5	4.9
	3	1.9	69.1	29.0	4.7
	4	1.7	70.4	28.1	7.4
	5	1.5	71.0	27.4	6.6
29	1	2.0	70.1	28.9	3.2
	2	1.7	64.9	34.3	4.4
	3	1.6	62.6	36.1	3.8
	4	1.2	66.4	32.5	4.9
	5	1.6	64.0	35.4	4.2
P-Value					
Diet		0.797	0.309	0.380	0.332
Day		0.944	<0.001	<0.001	<0.001
Diet x Day		0.681	0.720	0.787	0.960
PSEM[3]		0.2	4.4	4.4	0.9

[1] At days 1, 3, 8, 15, 22 and 29 a total of 20 piglets each (4 piglets per dietary treatment) were slaughtered

[2] ZON/DON concentrations in mg/kg diet: 1 - 0.01/0.03; 2 - 0.05/0.59; 3 - 0.08/1.27; 4 - 0.17/2.01; 5 - 0.29/4.52

[3] Pooled standard error of means

[a] Reference values according to Kraft and Dürr (2005)

The serum concentration of glucose decreased during the experiment (p=0.012) and showed a treatment related effect (p=0.019). The total content of protein in serum was not affected by diet. However a slight increase in the concentration was observed in the course of the experiment. While the concentrations of triglycerides and cholesterol in serum increased during the experiment, decreased concentrations of bilirubin were detected with progressing experimental time (Figure 3). The serum activities of aspartate-amino transferase and γ-glutamyl transferase as well as the concentrations of albumin and urea in serum were neither altered by the treatment nor affected by the experimental time (Table 6).

The activities of glutamate dehydrogenase in serum only rarely exceeded the limit of detection of 1.0 U/l and were therefore not evaluated statistically (data not shown).

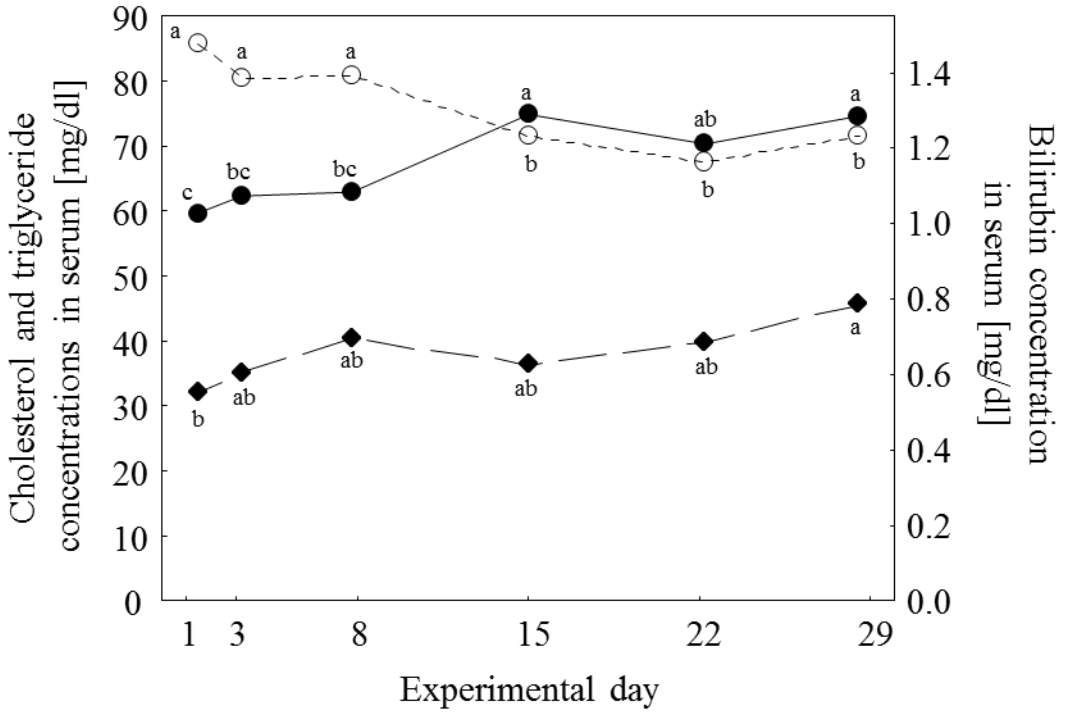

Figure 3. Cholesterol, triglycerides and bilirubin in serum of piglets in the course of the experiment (Means; n=20; – • – cholesterol; – ♦ – triglycerides; – o – bilirubin)

[a,b,c] different letters indicate significant differences between experimental days.

Table 6. Serum chemical parameters of piglets exposed to increasing dietary *Fusarium* toxin concentrations in the course of the experiment (Means; n=4)

Exp. day[1]	Diet[2]	Parameter[3]								
		Prot [g/l]	Alb [g/l]	AST [U/l]	γ-GT [U/l]	Bili [mg/dl]	Chol [mg/dl]	Gluc [mg/dl]	Tgl [mg/dl]	Urea [mg/dl]
		<86[4]	18-31[4]	<35[4]	<45[4]	<0.14[5]	77-128[6]	70-115[4]	<44[6]	20-50[4]
1	1	39.2	24.1	59.3	28.6	1.58	55.9	119.1	30.1	14.8
	2	37.7	23.7	37.8	28.6	1.47	59.9	130.5	31.5	19.2
	3	41.6	26.2	58.3	30.7	1.45	64.8	133.0	37.2	18.1
	4	39.4	24.1	34.0	22.9	1.47	57.6	116.1	29.0	17.6
	5	40.6	24.7	40.9	34.4	1.41	59.8	125.7	32.2	14.3
3	1	40.9	26.0	45.6	26.8	1.40	67.2	129.7	35.0	17.2
	2	38.6	22.4	39.0	30.7	1.37	60.7	153.4	36.2	12.1
	3	38.2	24.5	37.0	34.4	1.37	63.2	141.2	39.9	8.5
	4	36.8	23.1	40.6	28.5	1.37	57.6	116.5	32.4	14.2
	5	37.2	23.3	45.1	33.1	1.41	62.8	128.8	32.1	14.6
8	1	38.2	22.0	29.8	30.6	1.40	66.7	137.3	47.7	10.0
	2	38.8	24.3	40.3	24.7	1.34	68.7	123.3	45.6	12.7
	3	39.2	24.3	41.5	24.8	1.40	55.7	129.2	32.1	14.6
	4	38.5	24.7	40.1	30.2	1.39	62.3	123.9	39.6	15.9
	5	38.7	23.6	37.6	30.9	1.43	61.0	114.7	37.6	17.0
15	1	42.6	27.2	44.9	23.4	1.41	80.7	126.6	30.1	10.8
	2	43.0	25.2	37.6	24.2	1.23	78.4	126.4	41.9	16.4
	3	40.1	23.9	35.9	24.3	1.15	71.1	122.4	34.0	13.5
	4	42.2	22.0	40.9	28.4	1.19	73.9	115.1	35.8	12.1
	5	43.1	23.1	48.7	33.7	1.17	70.3	100.2	39.3	15.0
22	1	38.7	23.0	32.0	26.3	1.16	58.8	122.4	35.6	12.7
	2	41.8	24.7	30.4	29.9	1.12	70.3	119.4	34.9	10.0
	3	43.5	26.8	40.9	33.5	1.20	75.0	109.1	42.6	15.2
	4	40.9	25.2	36.8	24.3	1.17	71.8	108.9	50.4	10.8
	5	39.4	23.6	60.3	35.8	1.15	75.9	90.0	35.4	11.9
29	1	39.0	20.6	32.8	28.2	1.20	68.3	110.2	50.9	14.1
	2	38.9	24.5	46.7	29.7	1.28	74.9	128.3	50.4	12.2
	3	41.6	25.7	39.1	31.8	1.22	79.2	125.2	39.3	12.1
	4	40.6	26.0	34.4	21.8	1.24	77.2	114.3	37.3	13.9
	5	42.4	22.0	40.8	24.8	1.22	74.1	118.0	49.8	10.4
P-Value										
Diet		0.783	0.230	0.371	0.100	0.165	0.922	0.019	0.939	0.989
Day		0.001	0.855	0.566	0.650	<0.001	<0.001	0.012	0.012	0.097
Diet x Day		0.474	0.217	0.266	0.806	0.293	0.591	0.874	0.584	0.504
PSEM[7]		1.5	1.4	7.2	4.1	0.05	5.3	10.1	6.0	2.6

[1] At days 1, 3, 8, 15, 22 and 29 a total of 20 piglets each (4 piglets per dietary treatment) were slaughtered

[2] ZEA/DON concentrations in mg/kg diet: 1 - 0.01/0.03; 2 - 0.05/0.59; 3 - 0.08/1.27; 4 - 0.17/2.01; 5 - 0.29/4.52

[3] Prot, total protein; Alb, albumin; AST, aspartate amino-transferase; γ-GT, gamma-glutamyl-transferase; Bili, bilirubin; Chol, cholesterol; Gluc, glucose; Tgl, triglycerides

[4] Reference values according to Kraft and Dürr (2005)

[5] Reference value according to Kixmöller (2004)

[6] Reference values according to Bickhardt (1992)

[7] Pooled standard error of means

4 Discussion

Analysis of the naturally contaminated maize showed the occurrence of high concentrations of type B trichothecenes such as DON and its derivates 15-ADON and DON-3G. In pigs, high dietary concentrations of DON are well known to be related to decreases in feed intake and altered gut integrity (EFSA, 2004a; Pestka, 2010). Furthermore, the inhibition of protein synthesis and modification of the immune response have been demonstrated in several studies *in vitro* and *in vivo* in the last years (Döll and Dänicke, 2011). Since the acetylated form of DON, 15-ADON, has an equivalent toxicity to DON (Pestka, 2010; Dänicke et al., 2011), the effective DON exposure might have even been increased. Moreover, high amounts of ZEA were detected in the maize. Its presence is related to the occurrence of hyperoestrogenism and fertility disorders in pigs (EFSA, 2004b) due to its structural similarity with natural oestrogens. In contrast, no considerable effects in pigs are known for culmorin, which is similarly present in high concentrations in the maize (Rotter *et al.*, 1992) and a low toxicity was shown in *in-vitro* assays (Pedersen and Miller, 1999). Aurofusarin has been shown to contribute only to minor extents to the cytotoxic effect of the extracts of *Fusarium avenaceum* rice cultures in mammal cell lines (Uhlig et al., 2006). However, effects of toxins other than ZEA and DON may not be totally excluded and possible interactions have to be considered.

The reduced mean daily feed intake in group 5 in comparison to the other groups was in principle congruent with previous experiments. Dänicke et al. (2008a) reviewed the relationship between DON concentration in the diet and feed intake depression and suggested the voluntary feed intake of pigs fed DON-contaminated diets to decrease by 5.4 % per 1 mg DON/kg feed as compared to the respective control groups. That coincides with the present findings as feed intake was reduced by 20% in the first and by 27 % in the last experimental week in the group exposed to 4.52 mg DON/kg diet. Hence, the differences in daily feed intake between the control and the most highly contaminated group remained relatively constant throughout the complete experimental period as observed also by Döll et al. (2003). Decreases in mean daily body weight gain were generally attributed to the effects of a reduced feed intake (Goyarts et al., 2005).

Relative heart, liver and kidney weights were not affected by the investigated dietary treatments during the present experimental period. Accordingly, the weights of visceral organs of female piglets were not influenced by exposing the animals to a diet containing 0.42 mg ZEA and 3.9 mg DON per kg diet, respectively, for 5 weeks in a study of Döll et al.

(2003). Dietary *Fusarium* toxin concentrations in the range tested in the present experiment might therefore not induce deviations from physiological organ development. Effects on a cellular level however, cannot be totally excluded. The histological examination of organ specimens from piglets of the control and group 5, receiving the highest proportion of contaminated maize, did not reveal alterations related to toxin exposure. Though inconsistent effects of the duration of exposure were found for heart and kidney, the relative weight differences between the separate experimental days were negligible (heart: 5.2 – 6.4 g/kg BW; kidney: 4.6 – 5.5 g/kg BW). In contrast, relative liver weights were not influenced during the experimental period. Moreover, the present observations were in agreement with the results reported by Len et al. (2009), who did not observe considerable changes in relative visceral organ weights of piglets of a Landrace x Yorkshire origin within an age of 30 to 63 days.

ZEA with its oestrogenic properties is known to be involved in the development of hyperoestrogenism including enlarged uteri, increased numbers of visible follicles at the ovaries and ovarian atrophy. Furthermore, symptoms were generally noticed within 3 to 7 days of exposure (Etienne and Dourmad, 1994; Tiemann and Dänicke, 2007).

In contrast to these findings, on day 15 of the current experiment exclusively, uteri tended to be increased in group 5 as compared to the control group. The discrepancies among the studies may be caused by differences in toxin concentrations in the feed, origin of the contamination, age of the animals but also by the time of exposure and general experimental conditions. However, Döll et al. (2003) described considerably enlarged piglet uteri compared to the control group feeding similar toxin concentrations over a period of 5 weeks. In agreement with the present observations, the authors neither recorded differences in ovary weights nor in the number of visible follicles between the control and the group exposed to the highest dietary toxin concentration. The evaluation of the histological specimens of the ovaries with respect to the distribution of follicular stages consistently revealed no differences between the control and the group exposed to the almost 3-fold concentration of the guidance value for ZEA in the diet (EC, 2006) on day 29 of this experiment. Bauer et al. (1987) reported the occurrence of numerous tertiary follicles and follicular cysts at the ovaries after 11 days feeding diets containing 0.25 mg ZEA/kg to female piglets. However, that experiment started at an average body weight of approximately 20 kg and only two animals were included in the trial. Therefore, the age of the animals as well as strongly differing numbers of follicles between individuals (Dyck and Swierstra, 1983) could have contributed to this effect.

Bauer et al.(1987) also observed considerable and increasing reddening and swelling of the vulva after 7 days of treatment. The measured vulva width in relation to the body weight in

the present study differed strongly between the individuals and no clear diet effect was detected. However, differences between the groups, especially between the control and the highest exposed group seemed to increase with progressing experimental time leading to a significant interaction of diet and time. Similar to the present findings Teixeira et al. (2011) investigated female weaned piglets fed diets containing 0 and 0.75 mg ZEA/kg and observed increasing differences in the vulva area in the course of the trial which became significant after 21 days of exposure. As the concentrations fed in the present study were less than half of the concentrations fed in that experiment, a significant effect might have developed at longer times of exposure as well. Exposure to practically relevant *Fusarium* toxin concentrations in diets apparently causes inconsistent effects on the inner and outer reproductive tract. These effects might be assigned to the contribution of still unknown mechanisms, the overall health status of the animals or the presence of other known and unknown fungal metabolites and possible subsequent interactions.

In this study, the dietary treatments did not influence any of the investigated haematological or biochemical parameters. However, changes in the proportion of leukocytes were found in the course of the trial. The proportion of neutrophils decreased, while the percentage of lymphocytes increased. This development is in agreement with results obtained by Nerbas (2008), who analysed the leukocyte subsets in piglets from birth to their 90th day of life and observed a change from a neutrophilic to a lymphocytic hemogram within this interval. The increasing proportion of lymphocytes, however, seemed not to be related to their competence to react to stimuli, as the SI decreased at the beginning of the experiment. This transient immune suppression might reflect a general shift in the diets, which changed from the adaptation to the treatment diets. Nevertheless, variations of the SI in the course of the experiment were independent of the dietary treatment. This observation is in agreement with the results obtained by Dänicke et al. (2007) and Goyarts et al. (2006a) who fed diets containing 4.4 and 5.7 mg DON/kg diet to piglets for 5 and 4 weeks, respectively, and did not find any considerable alterations of the SI between the control and the toxin-exposed groups.

Bilirubin concentrations in serum were not affected by the toxin exposure but were found to reach the 10-fold level of the reference value, which is given for pigs in a body weight range of 25 to 30 kg (Kixmöller, 2004). This might on the hand be due to hepathopathies, increased disintegration of erythrocytes or cholestasis (Kraft and Dürr, 2005), but could as well be induced by development-related adaptations. However, cell and liver damage indicating enzymes such as AST or g-GT in serum remained unaffected during the entire experimental

period indicating a lack of considerable alterations in liver function or integrity. Kixmöller (2004) discussed that high serum bilirubin concentrations in young animals may be caused by an incompletely adapted liver metabolism. Consistently, bilirubin concentrations decreased in the course of the present experiment with progressing growth of the animals. A strong dependence on age of this parameter is similarly reported in previous studies by Seutter (1995) and Nerbas (2008).

AST activity exceeding the reference value might be related to effects of breed as Kixmöller (2004) found the highest activities of this enzyme (up to 65 U/l) in pigs of the breed Piétrain. Cholesterol levels were found to be below the reference range given by Bickhardt (1992) at the beginning of the study and increased in the course of the trial. Low levels in serum, which are related to liver diseases (Kraft and Dürr, 2005), are usually paralleled by higher concentrations of bilirubin. However, increasing concentrations with progressing animal growth coincided with the hypothesis that liver metabolism might still have been in a developing state and needed to adapt to the physiological status of the adult pig. The glucose concentration in serum was affected by diet but did not follow a clear dose-response relationship. During the experiment, glucose levels in serum prevalently exceeded the reference range. Increased concentrations in pigs are known to be related to agitation and stress (Kraft and Dürr, 2005) and may in this study have resulted from stressful situations prior to slaughter. Decreases in serum protein as reported by Döll et al. (2003) could not be confirmed in the present experiment. Both total serum protein and albumin remained unaffected by the dietary treatments. An influence of the tested DON concentrations on protein synthesis may however not be excluded, as Goyarts et al. (2006b) proved that the protein concentration in plasma is not an appropriate parameter for deriving DON effects on protein synthesis.

Overall, the development of serum biochemical parameters in the present experiment reveals a strong dependence on the age of the animals, which must be taken into account when evaluating their health status. Nevertheless, dietary *Fusarium* toxin concentration was not related to any effects on biochemical parameters.

5 Conclusion

In the present study, a diet containing *Fusarium* toxin concentrations at levels almost 3- and 5-fold higher than the EU guidance values in feed for ZEA and DON for piglets caused typical effects on performance parameters such as reduced feed intake and decreased body weight gains. Exposure to lower dietary concentrations, but even nearly 2-fold above the guidance values (group 4), was not related to marked deviations from the control group regarding the assessed parameters. Consequently, differences in body weight between the group exposed to the highest mycotoxin contamination and the other groups increased during the course of the experiment. ZEA did not induce considerable signs of hyperoestrogenism during 29 days of exposure. Uterus and ovary weights as well as the distribution of follicular stages were not influenced. However, increasing group differences in vulva width were observed during the experiment indicating that the time of exposure may play an important role in the expression of effects, especially at low doses. Further studies are therefore necessary in order to evaluate long-term effects of practice relevant mycotoxin concentrations, in particular on the development of hyperoestrogenism and future reproduction performance.

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References

Andretta, I., Lovatto, P.A., Lanferdini, E., Lehnen, C.R., Rossi, C.A.R., Hauschild, L., Fraga, B.N., Garcia, G.G. and Mallmann, C.A., 2010. Feeding of pre-pubertal gilts with diets containing aflatoxins or zearalenona. Archivos de Zootecnia 59: 123-130.

Bauer, J., Heinritzi, K., Gareis, M. and Gedek, B., 1987. Veränderungen am Genitaltrakt des weiblichen Schweines nach Verfütterung praxisrelevanter Zearalenonmengen. Tieraerztliche Praxis 15: 33-36.

Bickhardt, K., 1992. Kompendium der allgemeinen Inneren Medizin und Pathophysiologie für Tierärzte. Pareys Studientexte 69, Berlin, Hamburg, Germany, p. 231.

Biehl, M.L., Prelusky, D.B., Koritz, G.D., Hartin, K.E., Buck, W.B. and Trenholm, H.L., 1993. Biliary-Excretion and Enterohepatic Cycling of Zearalenone in Immature Pigs. Toxicology and Applied Pharmacology 121: 152-159.

Dänicke, S., Döll, S., Goyarts, T., Seeling, K., Valenta, H. and Ueberschar, K.H., 2008a. Metabolism, kinetics and occurrence of deoxynivalenol (DON) and zearalenone (ZON) in physiological samples of pigs, ruminants and poultry. In: Oswald, I.P. and Taranu, I. (ed.) Mycotoxins in Farm Animals. Transworld Research Network, Trivandrum, India, pp. 235-273.

Dänicke, S., Döll, S., Goyarts, T., Valenta, H., Ueberschar, K.H. and Flachowsky, G., 2008b. On the evaluation of the occurrence of the Fusarium-toxins deoxynivalenol (DON), zearalenone (ZON) and their metabolites in physiological substrates of the pig. Tieraerztliche Praxis Ausgabe Grosstiere Nutztiere 36: 35-47.

Dänicke, S., Goyarts, T. and Valenta, H., 2007. On the specific and unspecific effects of a polymeric glucomannan mycotoxin adsorbent on piglets when fed with uncontaminated or with Fusarium toxins contaminated diets. Archives of Animal Nutrition 61: 266-275.

Dänicke, S., Goyarts, T., Döll, S., Grove, N., Spolders, M. and Flachowsky, G., 2006. Effects of the Fusarium toxin deoxynivalenol on tissue protein synthesis in pigs. Toxicology Letters 165: 297-311.

Dänicke, S., Keese, C., Goyart, T., and Döll, S., 2011. Effects of deoxynivalenol (DON) and related compounds on bovine peripheral blood mononuclear cells (PBMC) in vitro and in vivo. Mycotoxin Research 27: 49-55.

Döll, S. and Dänicke, S., 2011. The Fusarium toxins deoxynivalenol (DON) and zearalenone (ZON) in animal feeding. Preventive Veterinary Medicine 102: 132-145.

Döll, S., Dänicke, S., Ueberschar, K.H., Valenta, H., Schnurrbusch, U., Ganter, M., Klobasa, F. and Flachowsky, G., 2003. Effects of graded levels of Fusarium toxin contaminated maize in diets for female weaned piglets. Archives of Animal Nutrition 57: 311-334.

D'Mello, J.P.F., Placinta, C.M. and Macdonald, A.M.C., 1999. Fusarium mycotoxins: a review of global implications for animal health, welfare and productivity. Animal Feed Science and Technology 80: 183-205.

Dyck, G.W. and Swierstra, E.E., 1983. Growth of the reproductive tract of the gilt from birth to puberty. Canadian Journal of Animal Science 63: 81-87.

Etienne, M. and Dourmad, J.Y., 1994. Effects of Zearalenone or Glucosinolates in the Diet on Reproduction in Sows - a Review. Livestock Production Science 40: 99-113.

European Commission (EC), 2006. Commission Recommendation of 17 August 2006 on the presence of deoxynivalenol, zearalenone, ochratoxin A, T-2 and HT-2 and fumonisins in products intended for animal feeding. Official Journal of the European Union L229: 7-9.

European Food Safety Authority, 2004a. Opinion of the Scientific Panel on contaminants in the food chain related to Deoxynivalenol (DON) as undesirable substance in animal feed. EFSA Journal 73: 1-42.

European Food Safety Authority, 2004b. Opinion of the Scientific Panel on contaminants in the food chain on a request from the Commission related to Zearalenone as undesirable substance in animal feed. EFSA Journal 89: 1-35.

Gesellschaft für Ernährungsphysiologie (GfE), 2006. Ausschuss für Bedarfsnormen der Gesellschaft für Ernährungsphysiologie: Empfehlungen zur Energie- und Nährstoffversorgung von Schweinen. DLG-Verlag, Frankfurt (Main), Germany.

Goyarts, T., Dänicke, S., Rothkötter, H.J., Spilke, J., Tiemann, U. and Schollenberger, M., 2005. On the effects of a chronic deoxynivalenol intoxication on performance, haematological and serum parameters of pigs when diets are offered either for ad libitum consumption or fed restrictively. Journal of Veterinary Medicine Series a-Physiology Pathology Clinical Medicine 52: 305-314.

Goyarts, T., Dänicke, S., Tiemann, U. and Rothkötter, H.J., 2006a. Effect of the Fusarium toxin deoxynivalenol (DON) on IgA, IgM and IgG concentrations and proliferation of porcine blood lymphocytes. Toxicology in Vitro 20: 858-867.

Goyarts, T., Grove, N. and Dänicke, S., 2006b. Effects of the Fusarium toxin deoxynivalenol from naturally contaminated wheat given subchronically or as one single dose on the in vivo protein synthesis of peripheral blood lymphocytes and plasma proteins in the pig. Food and Chemical Toxicology 44: 1953-1965.

Kirchgessner, M. and Roth, F.X., 1983. Schätzgleichungen zur Ermittlung des energetischen Futterwertes von Mischfuttermitteln für Schweine. Zeitschrift für Tierphysiologie Tierernährung und Futtermittelkunde 50: 270-275.

Kixmöller, M., 2004. Labordiagnostische Referenzbereiche bei unterschiedlichen Schweinerassen sowie histopathologische und immunhistochemische Untersuchung von Gehirnen älterer Sauen und Eber auf transmissible spongiforme Enzephalopathie im Rahmen der TSE-Studie. Dissertation, München, Germany. Available at: http://edoc.ub.uni-muenchen.de/2198/.

Kraft, W. and Dürr, U.M., 2005. Klinische Labordiagnostik in der Tiermedizin. 6. Auflage. Schattauer GmbH Stuttgart, Germany, p. 79-307.

Len, N.T., Hong, T.T.T., Ogle, B. and Lindberg, J.E., 2009. Comparison of total tract digestibility, development of visceral organs and digestive tract of Mong cai and Yorkshire x Landrace piglets fed diets with different fibre sources. Journal of Animal Physiology and Animal Nutrition 93: 181-191.

Naumann, C. and Bassler, R., 1993. Die chemische Untersuchung von Futtermitteln. VDLUFA-Verlag, Darmstadt, Germany.

Nerbas, E., 2008. Aktualisierung von Blutparametern beim Schwein. Dissertation, Hannover, Germany. Available at: http://d-nb.info/99235739X/34. Accessed 14 August 2012.

Oldenburg, E., Bramm, A. and Valenta, H., 2007. Influence of nitrogen fertilization on deoxynivalenol contamination of winter wheat - experimental field trials and evaluation of analytical methods. Mycotoxin Research 23: 7-12.

Pedersen, P.B. and Miller, J.D., 1999. The fungal metabolite culmorin and related compounds. Natural Toxins 7: 305-309.

Pestka, J.J., 2010. Deoxynivalenol: mechanisms of action, human exposure, and toxicological relevance. Archives of Toxicology 84: 663-679.

Powelljones, W., Raeford, S. and Lucier, G.W., 1981. Binding-Properties of Zearalenone Myco-Toxins to Hepatic Estrogen-Receptors. Molecular Pharmacology 20: 35-42.

Rotter, B.A., Prelusky, D.B. and Pestka, J.J., 1996. Toxicology of deoxynivalenol (vomitoxin). Journal of Toxicology and Environmental Health 48: 1-34.

Rotter, R.G., Thompson, B.K., Trenholm, H.L., Prelusky, D.B., Hartin, K.E. and Miller, J.D., 1992. A Preliminary Examination of Potential Interactions between Deoxynivalenol (Don) and Other Selected Fusarium Metabolites in Growing Pigs. Canadian Journal of Animal Science 72: 107-116.

Scientific Cooperation on Questions Relating to Food (SCOOP), 2003. Collection of occurrence data of Fusarium toxins in food and assessment of dietary intake by the population of EU member states. SCOOP Task 3.2.10 Final Report. Available at: http://ec.europa.eu/food/fs/scoop/task3210.pdf.

Seutter, U., 1995. Einfluß von Rasse, Haltung, Fütterung, Management, Alter und Reproduktionsstadium auf hämatologische und klinisch-chemische Parameter beim Schwein. Dissertation, München, Germany. Available at http://library.vetmed.fu-berlin.de/diss-abstracts/85646.html.

Teixeira, L.C., Montiani-Ferreira, F., Locatelli-Dittrich, R., Santin, E. and Alberton, G.C., 2011. Effects of zearalenone in prepubertal gilts. Pesquisa Veterinaria Brasileira 31: 656-662.

Tiemann, U. and Dänicke, S., 2007. In vivo and in vitro effects of the mycotoxins zearalenone and deoxynivalenol on different non-reproductive and reproductive organs in female pigs: A review. Food Additives and Contaminants 24: 306-314.

Uhlig, S., Jestoi, M., Knutsen, A. K. and Heier, B. T., 2006. Multiple regression analysis as a tool for the identification of extracts of relations between semi-quantitative LC-MS data and cytotoxicity of extracts of the fungus Fusarium avenaceum (syn. F. arthrosporioides). Toxicon 48: 567-579.

Verband Deutscher Landwirtschaftlicher Untersuchungs- und Forschungsanstalten (VDLUFA), 2006. Bestimmung von Zearalenon nach Immunaffinitätssäulenreinigung - HPLC-Verfahren. VDLUFA-Verlag, Darmstadt, Germany.

Vishwanath, V., Sulyok, M., Labuda, R., Bicker, W. and Krska, R., 2009. Simultaneous determination of 186 fungal and bacterial metabolites in indoor matrices by liquid chromatography/tandem mass spectrometry. Analytical and Bioanalytical Chemistry 395: 1355-1372.

PAPER II

Hydrothermal treatment of naturally contaminated maize in the presence of sodium metabisulfite, methylamine and calcium hydroxide, effects on the concentration of zearalenone and deoxynivalenol

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Abstract

Fusarium toxin contaminated ground maize was hydrothermally treated in the presence of different combinations of chemicals in order to simultaneously reduce zearalenone (ZEA) and deoxynivalenol (DON) concentrations. Treatments were carried out in a laboratory conditioner at 80 °C and 17 % moisture. Six different treatments were performed, consisting of 3 doses of methylamine (MMA; 2.5, 5 and 10 g/kg maize) at a constant dose of 5 g sodium metabisulfite (SBS)/kg, either with or without the addition of 20 g calcium hydroxide ($Ca(OH)_2$)/kg. The used maize was contaminated with approximately 45.99 mg DON/kg and 3.46 mg ZEA/kg. Without the addition of $Ca(OH)_2$, DON reductions reached approximately 82 % after 1-min treatment and the toxin disappeared nearly completely after 10 min when 2.5 or 5 g MMA were applied. ZEA concentrations were only marginally affected. In the presence of $Ca(OH)_2$, reductions in DON concentrations were lower, but were enhanced by increasing doses of MMA. ZEA concentrations were reduced by 72, 85 and 95 % within the first 5 min of the treatment at MMA dosages of 2.5, 5 and 10 g/kg maize, respectively. The application of SBS in combination with a strong alkaline during hydrothermal treatment seems to be a promising approach to simultaneously decontaminate even high amounts of DON and ZEA in ground maize and may contribute to reduce the toxin load of diets.

Keywords: Maize, Deoxynivalenol, Zearalenone, Hydrothermal treatment, Sodium metabisulfite, Methylamine

1 Introduction

Fusarium mycotoxins are frequent contaminations of cereals all over the world. Especially the occurrence of deoxynivalenol (DON) and zearalenone (ZEA) in toxicologically relevant concentrations is a challenging issue in animal feeds, as it is related to feed intake depressions, immune modulation, development of hyperoestrogenism and fertility problems (Döll and Dänicke 2011). Although several agronomic factors having high influence on the risk of toxin formation in cereals such as the tillage regime or the selection of cultivar or pre-crop are well known (Jouany 2007; Obst et al. 2000) toxin screenings keep revealing high concentrations of ZEA and DON, especially in maize (Abdellah et al. 2007; SCOOP 2003).

Different attempts have therefore been made to reduce toxin concentrations in harvested materials. Well-investigated physical approaches include, for example, dehulling, cleaning techniques or density segregation (House et al. 2003; Huff and Hagler 1985; Rios et al. 2009;

Rotter et al. 1995; Seitz et al. 1986). However, treatments often inefficient or are not economically viable (Jouany 2007).

Chemical approaches in contrast, seem to be more promising and have already demonstrated their potential to considerably reduce the concentrations of several individual mycotoxins. $Ca(OH)_2$-methylamine has successfully been applied during hydrothermal treatment in reducing ZEA concentrations in barley on a laboratory scale (Bauer et al. 1987). Sodium metabisulfite (SBS) is known from several studies to be effective in decontaminating DON during both hydrothermal treatment and wet preservation (Dänicke et al. 2005; Dänicke et al. 2009; Dänicke et al. 2010; Frobose et al. 2011; Young et al. 1987)

However, often measurements are only focusing on the reduction of an individual toxin while the concomitant reduction of DON and ZEA was only rarely investigated.

Therefore, the aim of the present study was to examine whether ZEA and DON concentrations in *Fusarium* toxin-contaminated maize may be simultaneously reduced when proven chemicals, such as SBS and MMA-$Ca(OH)_2$, are concurrently applied during hydrothermal treatment.

2 Materials and Methods

2.1 Maize treatments

All treatments were carried out at the Technical Institute of Amandus Kahl, Reinbek, Germany. A laboratory horizontal conditioner with an effective volume of approximately 100 L, surrounded by a heatable outer covering and equipped with a paddle mixing device, was used. To start with, the conditioner was filled with 30 kg of the contaminated ground maize (3 mm) whereupon sodium metabisulfite (SBS, $Na_2S_2O_5$, Merck, Darmstadt, Germany), methylamine solution ([40 %], CH_3NH_2, Sigma-Aldrich Chemie, Steinheim, Germany) and calcium hydroxide ($Ca(OH)_2$, VWR International, Darmstadt, Germany) were added during permanent mixing according to the plan.

Six different treatments were performed, consisting of 3 doses of methylamine solution (6.25, 12.5 and 25 g/kg maize) at a constant dose of 5 g SBS/kg, either with or without the addition of 20 g $Ca(OH)_2$/kg (Table 1). Considering the 60 % proportion of water in the methylamine solution, the effectively added doses of pure methylamine (MMA) amounted to 2.5, 5 and 10 g/kg maize. The moisture of the material was adjusted to 17 % by the supply of saturated steam at the beginning of the treatment and simultaneously heating it up to a conditioning

temperature of 80°C. In order to evaluate time-dependent reductions of the toxins in the course of the decontamination process, samples were drawn after 1-, 5-, 10-, 20- and 30-min treatments. Additionally, one sample was drawn prior to the treatments to measure the initial concentrations of DON and ZEA.

Table 1. Scheme of hydrothermal treatments of *Fusarium* toxin-contaminated maize (treatments were each applied for 1, 3, 5, 10, 20 and 30 minutes)

Variant	Treatment conditions	Supplement (g/kg maize)		
		SBS	MMA	$Ca(OH)_2$
I	17% moisture, 80°C temperature	5	2.5	-
II		5	5	-
III		5	10	-
IV		5	2.5	20
V		5	5	20
VI		5	10	20

2.2 Maize

Naturally contaminated maize, which was generated by inoculation, was used in the experiments. Briefly, maize (*Zea mays L.*, cultivar "Magister") was cultivated on an experimental field site of the Friedrich-Loeffler-Institute in Braunschweig, Germany, in 2010 according to local farm practices. At the lactic ripeness stage, the maize was infected with *Fusarium graminearum* by the applying of spore solution directly into the cob through the opening of the husk by using an automatic syringe. The mycotoxin-contaminated maize was harvested two months later.

Possible uneven distribution of the toxins within the maize batch was counteracted by homogenizing the ground maize prior to the experimental treatments.

2.3 Toxin analyses

The concentrations of DON in maize were analysed by high-performance liquid chromatography (HPLC) with diode array detection after clean up with immuno-affinity columns (IAC) (DON-prep®, R-Biopharm, Darmstadt, Germany) as described by Oldenburg et al. (2007). Since the antibodies against DON are pH sensitive, the pH of the extraction solution was measured and adjusted to 6.5 – 7.5 when it was not in the range of 6 – 8. The analysis of ZEA was carried out according to a slightly modified method of the VDLUFA (2006) by HPLC with fluorescence detection after clean up with IAC (ZearalaTest™ WB,

Vicam, Milford, USA). In the case of ZEA, the pH of the extract had to be in the range of 7.2-7.5 before applying it on the IAC. The limits of detection of DON and ZEA were 30 and 2 µg/kg, respectively.

2.4 Calculations and statistics

As only one sample per time point was drawn and analysed, the data of DON and ZEA concentrations were evaluated regressively.

The effects of the duration of the conditioning process on DON (all variants) and ZEA (only variants IV, V and VI; with $Ca(OH)_2$-addition) concentrations were described by multiple bi-exponential regressions:

$$\text{DON or ZEA (mg/kg dry matter [DM])} = A \cdot e^{-\alpha \cdot t} + B \cdot e^{-\beta \cdot t} + C \quad (1)$$

where the sum of A + B + C equals the initial concentration of ZEA or DON for time=0 min; C is the infinite DON or ZEA concentration; α and β are the respective rate constants and $t_{1/2\alpha}$ and $t_{1/2\beta}$ are the corresponding half-lives for the initial steep and the later prolonged decrease in concentration, respectively.

Minor time-dependent changes in ZEA concentrations (variants I, II and III; without the addition of $Ca(OH)_2$) were described by linear regressions:

$$\text{ZEA [mg/kg DM]} = -\alpha \cdot t + A \quad (2)$$

Where A equals the initial concentration of ZEA for time=0; α is the respective rate constant and $t_{1/2\alpha}$ the corresponding half-life for the decrease in concentration.

All statistics were carried out using STATISTICA for Windows (v.10.0, StatSoft 2011).

3 Results

Toxin analyses of the utilised maize revealed concentrations of 45.99 mg DON/kg and 3.46 mg ZEA/kg DM prior to the beginning of the experiments.

Overall, the performed hydrothermal treatments resulted in marked decreases in DON concentrations of 64-99 % after 30-min conditioning depending on the particular treatment variant. ZEA concentrations were only marginally affected in the absence of $Ca(OH)_2$, while reductions reached 89-98 % after 30 min when $Ca(OH)_2$ was added. Thereby, the majority of detectable toxins disappeared within the first 5 min of the treatments with a slower rate of

decrease thereafter. The kinetics of DON in all applied variants and ZEA in variants with $Ca(OH)_2$ addition were best characterised by a bi-exponential course (Eq. 1), while a linear regression model (Eq. 2) was fitted to the data of ZEA in variants without $Ca(OH)_2$. The targeted moisture content of 17 % during the treatment was not totally achieved, but amounted to 12.9±0.4 % after 1 min and reached a constant level of 15.5±0.5 % after 5 min.

DON concentrations decreased rapidly in the absence of $Ca(OH)_2$ (Figure 1a), reaching a decrease of approximately 82 % after 1-min treatment independent of the added dose of MMA. This also becomes obvious when considering the half-life times ($t_{1/2\alpha}$) that ranged from 0.26 to 0.35 min (Table 2). During the following treatment time, DON-concentrations in variant III (10 g MMA/kg) did not undergo further reductions, while treatment variants I and II (2.5and 5 g MMA/kg) led to a nearly complete decontamination of DON.

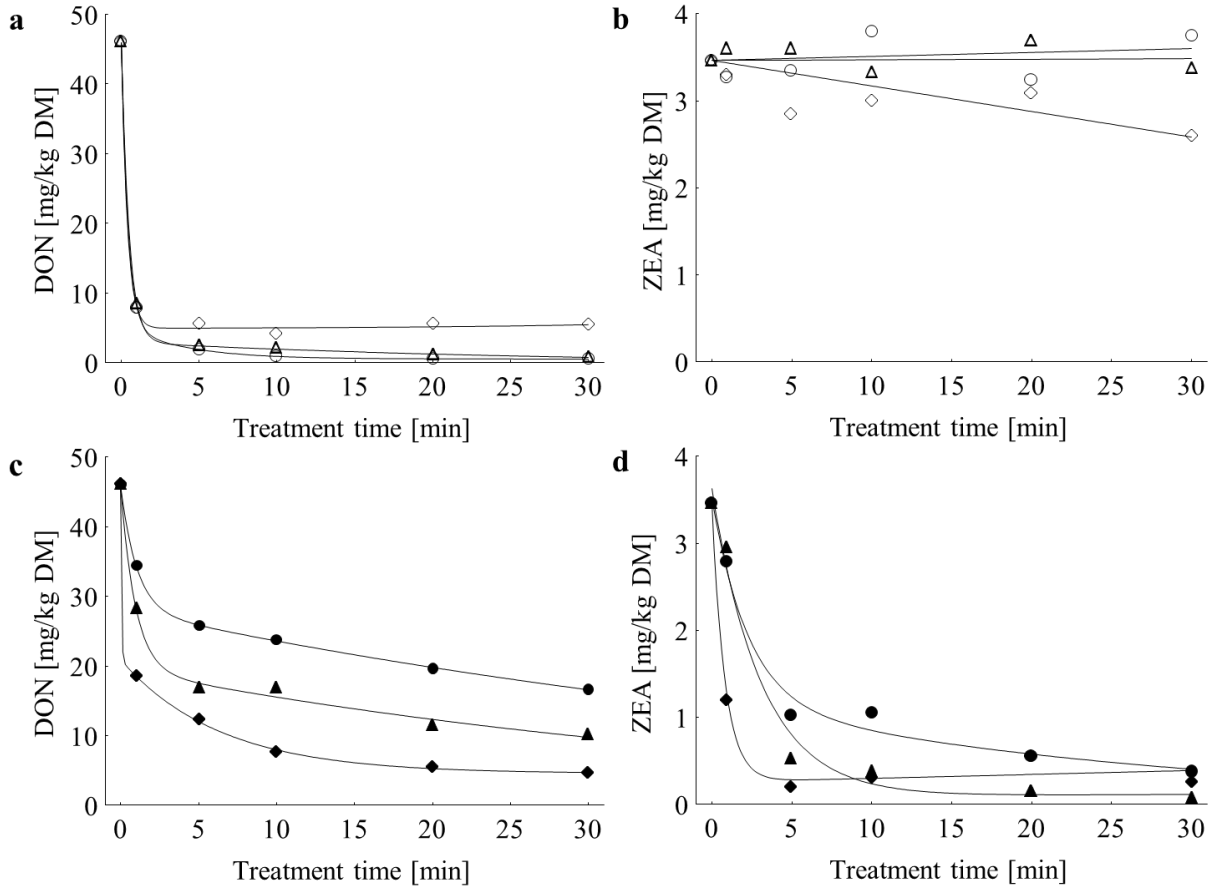

Figure 1. Time-dependent reduction of deoxynivalenol (DON) and zearalenone (ZEA) concentrations in maize hydrothermally treated in the presence of 5g sodium metabisulfite/kg and varying amounts of methylamine (2.5g MMA/kg o/●, 5g MMA/kg Δ/▲, 10g MMA/kg maize ◊/♦) and $Ca(OH)_2$ (0 g/kg **a**, **b**, variant: I o, II Δ , III ◊; 20 g/kg **c**, **d**, variant: IV ●, V ▲, VI ♦)

In the absence of $Ca(OH)_2$, no substantially directed decreases in ZEA-concentrations were achieved in variants I and II (2.5 and 5 g MMA/kg; Fig 1b), characterised by rate constants of 0 for the fitted linear regression lines (Table 2, parameter α). However, the addition of the highest dose of MMA (variant III) resulted in a 29 % reduction at the end of the conditioning process. Due to high variations of the treatment time-related ZEA concentration profile with regard to the calculated linear regression, the coefficient of determination was rather low.

In the presence of $Ca(OH)_2$, reductions in DON concentration were considerably weaker as compared to variants without $Ca(OH)_2$ but were enhanced by increasing doses of MMA resulting in estimated half-lives for the initial steep decrease ($t_{1/2\alpha}$) of 0.71, 0.65 and 0.04 min and for the further decrease ($t_{1/2\beta}$) of 40.9, 31.76 and 4.43 min at 2.5, 5 or 10 g MMA/kg, respectively (Figure 1c, Table 2). Similarly, ZEA concentrations were reduced by 72, 85 and 95 % within the first 5 min of the treatment (Figure 1d). During the following 25 min decreases in ZEA-concentration were only slight or stagnated completely, indicated by high values for the terminal half-life ($t_{1/2\beta}$) (Table 2). However, the reductions reached at least 89 % at the end of the conditioning in all three variants.

Although the values for the coefficient of determination of the fitted bi-exponential regression course r^2 was generally above 0.99 for both DON and ZEA, the calculated residual standard deviations were relatively high.

Table 2. Summary of regressions of time of the hydrothermal treatment (80°C, 17% moisture) (x) on deoxynivalenol (DON) and zearalenone (ZEA) concentration (y) of maize initially contaminated with 45.99 mg DON and 3.46mg ZEA/kg dry matter according to Eq. (1)[a] and (2)[b]

Variant – part of Fig. 1		y	A	α	B	β	C	$t_{1/2\,\alpha}$ (min)	$t_{1/2\beta}$ (min)	RSD[1] (mg/kg)	r^2
I	a	DON[1]	40.68	2.41	4.79	0.25	0.5	0.29	2.78	0.045	1.000
II	a	DON[1]	43.06	2.04	3.37	0.04	-0.4	0.34	19.71	0.089	1.000
III	a	DON[1]	41.04	2.63	0.07	-0.07	4.9	0.26	-9.39	1.123	1.000
IV	c	DON[1]	17.86	0.97	28.82	0.02	-0.7	0.71	40.90	0.326	1.000
V	c	DON[1]	26.44	1.07	20.35	0.02	-0.8	0.65	31.76	1.660	0.999
VI	c	DON[1]	25.06	18.74	16.34	0.16	4.6	0.04	4.43	0.507	1.000
I	b	ZEA[2]	3.46	<0.0						0.232	0.318
II	b	ZEA[2]	3.46	<0.0						0.150	0
III	b	ZEA[2]	3.46	0.03				59.23		0.248	0.604
IV	d	ZEA[1]	2.47	0.43	331.3	<0.01	-330.3	1.59	9935.75	0.327	0.993
V	d	ZEA[1]	3.55	0.32	6.60	<0.0	-6.5	2.16	-3115.36	0.466	0.991
VI	d	ZEA[1]	3.20	1.23	253.64	<0.0	-253.4	0.56	-39191.19	0.267	0.996

[1] Residual standard deviation

[a] DON or ZEA (mg/kg DM) = $A \cdot e^{-\alpha \cdot t} + B \cdot e^{-\beta \cdot t} + C$, where the sum of A+B+C equals the regressively estimated initial concentrations of DON and ZEA for time=0 minutes; C is the infinite DON or ZEA concentration; α and β are the respective rate constants and $t_{1/2\alpha}$ and $t_{1/2\beta}$ are the corresponding half-lives for the initial steep and the later prolonged decrease in concentration, respectively.

[b] ZEA (mg/kg DM) = $A + \alpha \cdot t$, where A equals the initial concentration of ZEA for time=0 minutes, α is the respective slope of the regression.

4 Discussion

The present study aimed to elucidate the kinetics of DON and ZEA-concentrations in naturally contaminated maize during hydrothermal treatments in the presence of SBS, MMA and $Ca(OH)_2$.

SBS was only applied at a dose of 5 g/kg maize, as previous experiments proved this amount to be necessary for a nearly complete reduction of DON in cereal grains (Dänicke et al. 2008; Dänicke et al. 2009). The supplementation doses for MMA and $Ca(OH)_2$ were adapted from Bauer et al. (1987), who successfully reduced high concentrations of ZEA in barley during a hydrothermal treatment in the presence of 5 g MMA/kg and 20 g $Ca(OH)_2$/kg. In order to derive possible dose-response relations, the supplemented doses of MMA were additionally adjusted to half and double the mentioned amount. Since the addition of a strong base ($Ca(OH)_2$) was suspected to influence the acid mediated decontamination of DON, the omitting of $Ca(OH)_2$ was also tested.

Best results with regard to a simultaneous reduction of both toxins were achieved in variants with 20 g $Ca(OH)_2$, while in the absence of $Ca(OH)_2$ marked decreases only in DON concentrations were observed. Reduction kinetics were characterised by an initial steep and a following slower decrease that were best described by a bi-exponential course.

Initially high concentrations of both chemicals and probably easily accessible mycotoxins from dust and superficial layers of the maize matrix might have caused the sharp decrease at the beginning of the treatment (1^{st} term in Eq. (1)) (Dänicke et al., 2010). Further but considerably slower reductions (2^{nd} term in Eq. (1)), presumably affecting toxins inside the ground maize particles, seem to require a time dependent soaking of the chemicals (Dänicke et al. 2010).

In the absence of $Ca(OH)_2$, DON reductions reached at least 82 % after the first minute of the treatment, reflected in half-lives ranging from 0.260.34 minutes. While the initial steep decrease seemed to be independent of the supplemented dosage of MMA, the terminal prolonged DON reductions were obviously influenced by different concentrations of the chemical. Nearly complete DON reductions were measured in variants I and II (2.5 g MMA/kg and 5 g MMA/kg, 0 g $Ca(OH)_2$/kg) at the end of the 30 minutes conditioning, while a dose of 10 g MMA/kg maize (variant III) impeded the further decrease of DON and led to stagnating concentrations of about 5 mg DON/kg after 5 minutes treatment. Accordingly, infinite DON concentrations estimated by the regression model amounted 0.5, -0.4 and 4.9 in variants I, II and III, respectively.

Since MMA is a strong alkaline compound, the addition of the highest dose might have resulted in a basic pH during the later treatment time and caused a shift in the predominating sulfite ions of SBS which are required for the decontamination of DON. At weak acidic or neutral conditions the prevailing form of SBS is the bisulfite ion (HSO_3^-) which forms together with DON its nontoxic derivate DONS (Young et al. 1987), while in an alkaline ambient the sulfite ion (SO_3^{2-}) predominates (Rose 1993). At the beginning of the treatment the effect of SBS may have predominated, which in turn resulted in an equally high availability of bisulfite ions for the reaction with DON in all three variants (I, II and III; without $Ca(OH)_2$). This view is supported when considering the small variation in half-lives of the three treatments. Due to a rapid transformation of DON and a high consumption of SBS equivalents during the first treatment minute, the alkaline effect of MMA in variant III (10 g MMA/kg) seemed to become more pronounced only thereafter. A possible increase in the ambient pH might have resulted in a reduced availability of bisulfite equivalents to react with DON and thereby decelerating the speed of the decontamination reaction. Unfortunately, pH data were not recorded during the treatments.

The applied ratio of SBS to toxin in the present experiment of 110:1 was relatively low as compared to previous studies using 900:1 to 5000:1 during hydrothermal treatment or wet preservation (Dänicke et al. 2005; Dänicke et al. 2009; Young et al. 1987). However, the dosage was in principle sufficient to almost completely decontaminate DON when considering its 98 % and 99 % reduction in variants II and I, respectively.

In the absence of $Ca(OH)_2$ inconsistent or no reductions in ZEA-concentration were observed in variants I and II, while in variant III a slight decrease in the course of the treatment was estimated by the regression model (Eq. (2)). This lack of effects might strongly be related to the depletion of an alkaline agent which has been shown to be essential for the hydrolysis of ZEA (Gora et al. 2004). Consistently, increased concentrations of MMA, which were applied in variant III (10 g MMA/kg), operating itself as base, led to a slight improvement in ZEA-reduction (30 %) as compared to variants containing 2.5 g MMA/kg and 5 g MMA/kg(0 and 8%). Nevertheless, the estimated linear decrease in concentration has to be interpreted with caution, as an r^2 of 0.604 is relatively poor.

A nearly complete reduction of DON in variants I and II (2.5 g MMA/kg and 5 g MMA/kg, 0 g $Ca(OH)_2$/kg) was possibly mediated by a weak acidic pH, while the reduction of ZEA was not enabled under these conditions. Accordingly, the addition of 10 g MMA/kg in variant III presumably led to a basic pH after the first minutes which stopped the decontamination of DON but promoted the hydrolysis of ZEA.

Interestingly, DON reductions seemed to be enhanced by increasing concentrations of MMA when $Ca(OH)_2$ was present during the conditioning process. Nevertheless, the initial steep

reduction was only half as effective as the reduction in the absence of $Ca(OH)_2$. Whether the reduction can be assigned to the conversion of DON to DONS is questionable since the conditions in the presence of $Ca(OH)_2$ and MMA were probably rather alkaline and might have reduced the availability of bisulfite ions as mentioned earlier. Furthermore, Young (1986) demonstrated that DONS in weak alkaline solutions was not stable and underwent reconversion to DON. The fate of detectable DON might therefore rather be associated with an alkaline mediated change in conformation (Young 1986). The disappearance of DON in the presence of alkali has also been observed during food preparation (Abbas et al. 1988; Ragab et al. 2007). Accordingly, higher doses of MMA that presumably resulted in a higher pH led to shorter half-lives for both the initial steep decrease (0.71 min, 0.65 min and 0.04 min) and the further prolonged reduction of DON (40.9 min, 31.76 min and 4.43 min). However, the stability of DON hydrolysis products in altered pH conditions, especially at passage through the digestive tract, is unknown so far.

In order to conclusively elucidate the nature of the decrease of DON in these variants analyses of the DONS concentration in the course of the conditioning process would probably be helpful.

ZEA reductions were considerably improved in the presence of $Ca(OH)_2$ during the conditioning process, which underlines the importance of an alkaline component to favour the hydrolysis of ZEA. As estimated by the first part of the regression model, the majority of the achieved reduction occurred during the initial steep decrease within the first treatment minutes, indicated by half-lives varying between 0.59 min and 2.16 min, while thereafter only minimal further reductions were measured. Increasing doses of MMA led to an increased efficiency of the reduction reaction. Maize in variant I, treated with 2.5 g MMA/kg, needed a 30-minute conditioning to reach a ZEA concentration below 0.4 mg ZEA/kg. In contrast maize in variant II (5 g MMA/kg) and variant III (10 g MMA/kg) required only 10 min and 5 min, respectively, to undergo an equal reduction. Even though differences between the reaction rates of the three variants existed, final reductions only varied between 89 % and 98 %.

Cereals treated in this way may be included into diets to reduce the general load of mycotoxins and thereby prevent adverse effects on performance or fertility. However, the nutritional quality of the material needs clear determination when fed to animals since chemicals and/or hydrothermal treatment might alter physiochemical properties or crude nutrients (Malumba et al. 2010, Betz 1993) and thus affecting their digestibility or gastro intestinal availability. Furthermore, palatability might suffer from the presence of the

chemicals. In particular high doses of $Ca(OH)_2$ in diets may result in decreases in feed intake as demonstrated in a study with pigs (Betz 1993). The present study may therefore rather be seen as a general approach for the simultaneous reduction of ZEA and DON in maize by the combination of previously tested chemicals, which still needs further development.

5 Conclusion

The present study demonstrates that in principle the combination of two chemical treatments which have been individually proven to be successful in reducing DON and ZEA in prior experiments may be a promising tool in attaining simultaneous and considerable reductions of both toxins. However, the underlying reduction mechanisms cannot totally be clarified on the basis of this experiment. The formation of DONS as well as alkaline hydrolysis of DON may have contributed to the obtained results. The dependence of the reduction of ZEA on the presence of an alkaline component becomes clear. Whether a chemical bond with the latter is formed remains unknown so far. Detailed pH- and dose-dependence, other required conditions for the reductions or the applicability in practically more relevant systems such as wet preservation should be evaluated in further studies. Generally, the inclusion of treated maize into diets might contribute to reducing adverse effects of mycotoxins when fed to animals, but feed stuffs need to be characterised in detail and proved with regard to their acceptance and decontaminating potential in feeding trials.

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Conflict of interest

None

References

Abdellah Z, Soriano JM, Molto JC, Manes J (2007) Review on the toxicity, occurrence, metabolism, detoxification, regulations and intake of zearalenone: An oestrogenic mycotoxin. Food Chem Toxicol 45:1-18

Abbas HK, Mirocha CJ, Rosiles R, Carvajal M (1988) Decomposition of zearalenone and deoxynivalenol in the process of making tortillas from corn. Cereal Chem 65:15-19

Bauer J, Gareis M, Detzler W, Gedek B, Heinritzi K, Kabilka G (1987) Zur Entgiftung von Mykotoxinen in Futtermitteln. Tierärztliche Umschau 42:70-77

Betz J (1993) Detoxikation von Ochratoxin A: Auswirkungen des diätetischen Einsatzes der Alkalien Calciumhydroxid und Monomethylamin auf Blut- und Harnparameter sowie Rückstandsbildung beim Schwein. Dissertation, Ludwig Maximilian University of Munich

Dänicke S, Valenta H, Gareis M, Lucht HW, von Reichenbach H (2005) On the effects of a hydrothermal treatment of deoxynivalenol (DON)-contaminated wheat in the presence of sodium metabisulphite (Na2S2O5)on DON reduction and on piglet performance. Anim Feed Sci Technol 118:93-108

Dänicke S, Beineke A, Goyarts T, Valenta H, Beyer M, Humpf HU (2008) Effects of a Fusarium toxin-contaminated triticale, either untreated or treated with sodium metabisulphite (Na2S2O5, SBS), on weaned piglets with a special focus on liver function as determined by the C-13-methacetin breath test. Arch Anim Nutr 62:263-286

Dänicke S, Pahlow G, Goyarts T, Rohweder D, Wilkerling K, Breves G, Valenta H, Döll S (2009) Effects of increasing concentrations of sodium metabisulphite (Na 2S 2O 5, SBS) on deoxynivalenol (DON) concentration and microbial spoilage of triticale kernels preserved without and with propionic acid at various moisture contents. Mycotoxin Research 25:215-223

Dänicke S, Pahlow G, Beyer M, Goyarts T, Breves G, Valenta H, Humpf HU (2010) Investigations on the kinetics of the concentration of deoxynivalenol (DON) and on spoilage by moulds and yeasts of wheat grain preserved with sodium metabisulfite (Na2S2O5, SBS) and propionic acid at various moisture contents. Arch Anim Nutr 64:190-203

Döll S, Dänicke S (2011) The Fusarium toxins deoxynivalenol (DON) and zearalenone (ZON) in animal feeding. Prev Vet Med 102:132-145

Frobose HL, Tokach MD, Hansen EL, McKinney LJ, DeRouchey JM, Dritz SS, Goodband RD, Nelssen JL (2011) Evaluating the effects of pelleting deoxynivalenol-contaminated dried distillers grains with solubles in the presence of sodium metabisulfite on analyzed DON levels. Kansas State University Swine Day 2011. Report of Progress 1056:90-95

Gora M, Luczynski MK, Smoczynski L, Obremski K, Polak M, Swist M, Zlelonka L, Gajecki M (2004) Modification of zearalenone structure in model and natural conditions. Polish Journal of Veterinary Sciences 7:181-185

House JD, Nyachoti CM, Abramson D (2003) Deoxynivalenol removal from barley intended as swine feed through the use of an abrasive pearling procedure. J Agric Food Chem 51:5172-5

Huff WE, Hagler WM (1985) Densitiy segregation of corn and wheat naturally contaminated with aflatoxin, deoxynivalnol and zearalenone. J Food Prot 48:416-420

Jouany JP (2007) Methods for preventing, decontaminating and minimizing the toxicity of mycotoxins in feeds. Anim Feed Sci Technol 137:342-362

Malumba P, Janas S, Roiseux O, Sinnaeve G, Masimango T, Sindic M, Deroanne C, Bera F (2010) Comparative study of the effect of drying temperatures and heat-moisture treatment on the physicochemical and functional properties of corn starch. Carbohyd Polym 79:633-641

Obst A, Lepschy J, Beck R, Bauer G, Bechtel A (2000) The risk of toxins by Fusarium graminearum in wheat — interactions between weather and agronomic factors. Mycotoxin Research 16:16-20

Oldenburg E, Bramm A, Valenta H (2007) Influence of nitrogen fertilization on deoxynivalenol contamination of winter wheat - experimental field trials and evaluation of analytical methods. Mycotoxin Research 23:7-12

Ragab WSM, Drusch S, Schnieder F, Beyer M (2007) Fate of deoxynivalenol in contaminated wheat grain during preparation of Egyptian 'balila'. Int J Food Sci Nutr 58:169-177

Rios G, Pinson-Gadais L, Abecassis J, Zakhia-Rozis N, Lullien-Pellerin V (2009) Assessment of dehulling efficiency to reduce deoxynivalenol and Fusarium level in durum wheat grains. J Cereal Sci 49:387-392

Rose AH (1993) Sulphur dioxide and other preservatives. Journal of Wine Research 4:43-47

Rotter RG, Rotter BA, Thompson BK, Prelusky DB, Trenholm HL (1995) Effectiveness of densitiy segregation and sodium-carbonate treatment on the detoxification of Fusarium-contaminated corn fed to growing pigs. J Sci Food Agric 68:331-336

Scientific Cooperation on Questions relating to Food (SCOOP) (2003) Collection of occurrence data of Fusarium toxins in food and assessment of dietary intake by the population of EU member states. SCOOP Task 3.2.10 Final Report. http://ec.europa.eu/food/fs/scoop/task3210.pdf

Seitz LM, Eustace WD, Mohr HE, Shogren MD, Yamazaki WT (1986) Cleaning, milling, and baking tests with hard red winter-wheat containing deoxynivalenol. Cereal Chem 63:146-150

VDLUFA (2006) Bestimmung von Zearalenon nach Immunaffinitätssäulenreinigung - HPLC-Verfahren. VDLUFA-Verlag, Darmstadt

Young JC (1986) Formation of sodium bisulfite addition-products with trichothecenones and alkaline-hydrolysis of deoxynivalenol and its sulfonate. J Agric Food Chem 34:919-923

Young JC, Trenholm HL, Friend DW, Prelusky DB (1987) Detoxification of deoxynivalenol with sodium bisulfite and evaluation of the effects when pure mycotoxins or contamminated corn was treated and given to pigs. J Agric Food Chem 35:259-261

PAPER III

Effects of a *Fusarium* toxin-contaminated maize treated with sodium metabisulfite, methylamine and calcium hydroxide in diets for female piglets

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Abstract

Deoxynivalenol (DON) and zearalenone (ZEN) contaminated maize was hydrothermally treated in the presence of sodium metabisulfite (SBS), methylamine (MMA) and calcium hydroxide ($Ca(OH)_2$) and included into diets for female piglets to evaluate effects on performance, organ weights, development of hyperestrogenism, serum biochemical parameters, stimulation of peripheral blood mononuclear cells and toxin residues in serum. For this purpose, both uncontaminated maize (CON) and *Fusarium* toxin-contaminated maize (FUS) were included into diets either untreated (-) or treated (+) according to a 2 by 2-factorial design. 100 female weaned piglets were assigned to one of the four treatment groups (n=25) CON-, CON+, FUS- and FUS+ with DON/ZEN-concentrations of 0.43/0.03, 0.04/0.0, 3.67/0.32 and 0.36/0.08 mg per kg diet, respectively. After a feeding period of 27 days, 20 piglets (n=5) were slaughtered. Performance parameters such as feed intake, live weight gain and feed-to-gain ratio remained unaffected by the treatments. Uterus weights were significantly reduced in group FUS+ compared to FUS- ($p=0.028$), while visceral organ weights were not influenced. Vulva width in relation to body weight was highest in group FUS- at the end of the trial, while hydrothermal treatment significantly reduced the parameter ($p<0.008$). The highest toxin and toxin metabolite concentrations in serum were detected in group FUS-, whereas ingestion of diet FUS+ reduced concentrations to the level of the control groups. Serum biochemical and haematological parameters were mainly within the given reference ranges und showed no treatment related alterations. Stimulation of peripheral blood mononuclear cells was not affected.

An effective detoxification of maize by hydrothermal treatment in the presence of SBS, MMA and $Ca(OH)_2$ could be demonstrated by means of serum toxin analyses. No undesired side-effects of the treated feed stuff or the chemicals themselves on the health of piglets were detected.

Keywords: Hydrothermal treatment, Sodium metabisulfite, Methylamine, Deoxynivalenol, Zearalenone, Piglets

1 Introduction

Contamination of cereals with *Fusarium* mycotoxins may hardly be avoided even at the best management of agricultural strategies (Jouany 2007) as weather conditions during flowering may play an important role in the formation of toxins (Oldenburg et al. 2000). Due to their

frequent occurrence in toxicologically relevant concentrations zearalenone (ZEN) and deoxynivalenol (DON) are two of the most important mycotoxins in animal nutrition and livestock, especially pigs, often react sensitively when these toxins are ingested. In the case of DON feed intake depression and consequently loss of performance are the predominating effects, while immunmodulating properties have also been observed. The ingestion of ZEN is known to be related to hyperestrogenism and reproductive disorders (for review see (Döll and Dänicke 2011)). Earlier studies showed that treatments of contaminated cereals with sodium metabisulfite (SBS) either applied during hydrothermal treatment or wet preservation may overcome the adverse effects of DON in piglets (Young et al. 1987; Dänicke et al. 2005b; Dänicke et al. 2008). In female piglets, the effects of the ingestion of highly ZEN-contaminated barley such as swollen and reddened vulva and uterus were reduced when barley was hydrothermally treated with calcium hydroxide ($Ca(OH)_2$) and methylamine (MMA) prior to feeding (Bauer et al. 1987a). Although naturally contaminated materials are often characterised by the presence of more than one mycotoxin, most previous studies focused on the reduction of individual toxins. However, a recent investigation on the simultaneous reduction of DON and ZEN demonstrated that a combined application of previously individually evaluated chemicals such as SBS, MMA and $Ca(OH)_2$ during hydrothermal treatment may achieve an approximately 90 %-reduction of both toxins (Rempe et al. 2013b).

Therefore, the aim of the present study was to evaluate if feeding so-treated *Fusarium* toxin-contaminated maize may also overcome the adverse effects of a toxin exposure on performance, development of hyperestrogenism and health of post-weaning female piglets. A special focus has been laid on the residues of toxins and their metabolites in blood serum as specific indicator for the success of the decontamination procedure.

2 Materials and methods

2.1 Experimental design and diets

A complete 2 by 2-factorial feeding experiment with piglets was designed including the mycotoxin contamination as one main effect (CON = uncontaminated control diet; FUS = *Fusarium* toxin contaminated diet, mainly containing DON and ZEN) and the hydrothermal treatment with sodium metabisulfite (SBS, $Na_2S_2O_5$, Merck KGaA, Darmstadt, Germany), methylamine (MMA, CH_3NH_2, Sigma-Aldrich Chemie GmbH, Steinheim,

Germany) and calcium hydroxide ($Ca(OH)_2$, VWR International GmbH, Darmstadt, Germany) of both diet types as the other main effect.

For the contaminated diets a naturally mycotoxin contaminated maize (43.4 mg DON and 2.48 mg ZEN/kg) was used and mixed into the respective diets at a proportion of 10 %, while for the control diets uncontaminated maize was incorporated (Table 1).

Table 1. Composition of the experimental diets

	CON-	CON+	FUS-	FUS+
Components [g/kg]				
Barley	350	350	350	350
Wheat	340	340	340	340
Maize, untreated	100	0.0	0.0	0.0
Maize, treated*	0.0	100	0.0	0.0
Contaminated maize, untreated	0.0	0.0	100	0.0
Contaminated maize, treated*	0.0	0.0	0.0	100
Soybean meal	150	150	150	150
Soya bean oil	10.0	10.0	10.0	10.0
Marker	10.0	10.0	10.0	10.0
Premix[1]	10.0	10.0	10.0	10.0
Calciumcarbonate	10.0	10.0	10.0	10.0
Sodium chloride	0.5	0.5	0.5	0.5
L-lysine-HCl	7.0	7.0	7.0	7.0
L-tryptophane	1.0	1.0	1.0	1.0
DL-methionine	2.9	2.9	2.9	2.9
L-threonine	2.3	2.3	2.3	2.3
Formic acid	6.0	6.0	6.0	6.0
Phytase	0.3	0.3	0.3	0.3
Analysed composition				
Zearalenone [mg/kg]	0.03	0	0.32	0.08
Deoxinivalenol [mg/kg]	0.43	0.04	3.67	0.36
Dry matter [g/kg]	890	895	893	895
Organic matter [g/kg DM]	942	935	941	938
Crude ash [g/kg DM]	57.9	65.3	59.5	62.4
Crude protein [g/kg DM]	184	191	192	192
Ether extract [g/kg DM]	35.7	39.1	37.5	37.9
Crude fibre [g/kg DM]	31.9	29.7	27.7	30.5
N-free-extractives [g/kg DM]	690	675	683	677
Metabolizable energy[§] [MJ/kg]	15.4	15.5	15.7	15.5

* Hydrothermal treatment in the presence of 5 g sodium metabisulfite, 10 g methylamine and 20 g $Ca(OH)_2$ at 17 % moisture and 80 °C for 30 min.

[1] Provided per kg diet: Ca 1.0 g, P 1.4 g, Na 0.9 g, Mg 0.1 g, Fe 75 mg, Cu 15 mg, Mn 40 mg, Zn 50 mg, I 1.0 mg, Se 0.2 mg, Co 0.4 mg, vitamin A 10000 IU, vitamin D3 1000 IU, vitamin E 50 mg, vitamin B1 1.0 mg, vitamin B2 3.1 mg, vitamin B6 2.5 mg, vitamin B12 20.0 µg, vitamin K3 2.0 mg, nicotinic acid 12.5 mg, pantothenic acid 7.5 mg, choline chloride 125 mg, biotine 50 µg, folic acid 0.5 ng, vitamin C 50 mg;

§ Calculated using the equation of the GfE (2008)

The hydrothermal treatment of the control (CON+) and the contaminated maize (FUS+) was performed in cooporation with Amandus Kahl GmbH & Co. KG in Reinbek, Germany. In a conditioner, ground maize was mixed with 5 g SBS, 10 g MMA and 20 g $Ca(OH)_2$ per kg maize and was adjusted to a total moisture content of 17 % by the supply of saturated steam. After 30 minutes treatment at a temperature of 80 °C, the material was processed in an annular gap expander, then cooled and ground. The kinetics of the toxins in the course of the conditioning process are described in detail by (Rempe et al. 2013b). Chemical characteristics of treated maize variants as compared to untreated maize are shown in Table 2.

Table 2. Characterisation of uncontaminated (CON) or *Fusarium* toxin-contaminated maize (FUS), either untreated (-) or hydrothermally treated in the presence of sodium metabisulfite, methylamine and calcium hydroxide (+)

	CON-	CON+	FUS-	FUS+
Mycotoxin concentration [mg/kg]				
Zearalenone	0.49	0.08	2.48	0.52
Deoxynivalenol	2.19	0.44	43.40	3.96
Chemical composition [g/kg dry matter]				
Dry matter [g/kg]	876	876	880	888
Organic matter	981	955	981	955
Crude ash	18.53	44.62	19.47	45.46
Crude protein	110	116	117	122
Ether extract	52.27	49.37	48.50	48.09
Crude fibre	26.0	18.44	23.33	18.81
N-free-extractives	793	772	791	766
Starch	694	692	701	694
Starch gelatinization degree [%]	11.4	27.7	11.8	32.3

2.2 Animal experiment and procedures

Experiment and procedures were conducted according to the European Community regulations concerning the protection of experimental animals and the guidelines of the Regional Council of Braunschweig, Lower Saxony, Germany.

The study was carried out at the experimental station of the Friedrich-Loeffler-Institute in Braunschweig. A total of 100 female weaned crossbred piglets from the Bundes Hybrid Zucht Programm (BHZP) were allocated to a pig-rearing house with 20 pens (five piglets per pen) with slatted floors. The piglets were fed a pre-experimental diet, corresponding to the untreated control diet (CON-), for 4 days for adaptation to *ad libitum* feeding. Piglets were then weighed and assigned to one of the four feeding groups (25 piglets per group) with an initial mean body weight (BW) of 8.8 ± 1.0 kg. The animals had free access to feed from

automatic feeders and water from drinking nipples. During the experiment individual BW and consumed feed per pen were determined once weekly. In order to evaluate external symptoms of hyperestrogenism, the horizontal vulva width of the individual animals was measured. After 4 weeks feeding the experimental diets, a total of 20 piglets (n = 5) were slaughtered and blood was collected for haematology, isolation of peripheral blood mononuclear cells (PBMC) and preparation of serum for biochemical and toxin analyses. At necropsy organs, including liver, kidney, heart, spleen, uterus and ovaries, were dissected, weighed and subjected to examination by a veterinarian.

2.3 Mycotoxin content and chemical composition of diets and maize

Feed samples were ground to pass through a 1 mm sieve prior to analysing the chemical composition. The concentrations of DON in maize and feed were analysed by high-performance liquid chromatography (HPLC) with diode array detection after clean up with immuno-affinity columns (IAC) (DON-prep®, R-Biopharm AG, Darmstadt, Germany) as described by Oldenburg et al. (2007). The analysis of ZEN was carried out according to a slightly modified method of the VDLUFA (2006) by HPLC with fluorescence detection after clean up with IAC (ZearalaTest™ WB, Vicam, Milford, USA). Dry matter, crude ash, crude fibre, crude protein, ether extract and N-free-extractives were analysed according to the official procedures of the VDLUFA (Naumann and Bassler 1993). Additionally, the proportion of gelatinized starch from total starch was determined at the Research Institute of Feed Technology (IFF, Braunschweig-Thune, Germany) according to a method of the VDLUFA (1976).

2.4 Toxin residues in serum

DON, de-epoxy-DON (de-DON), ZEN and α-zearalenol (α-ZEL) were determined in serum by LC-MS/MS after incubation with β-glucuronidase and extraction on an Oasis® HLB solid phase extraction column (Waters, Milford, USA). The method will be described in detail elsewhere (Brezina et al., in preparation). The obtained results were evaluated with internal standards. The limits of quantification for DON, de-DON, ZEN and α-ZEL were 0.45, 0.76, 0.08 and 0.78 ng/ml, respectively. The recoveries were in the range of 80 to 110 % with IS.

2.5 Haematology and biochemical serum analyses

Total leukocyte count was determined by an automated hematology analyzer (MEK-6450, Nihon Kohden Europe GmbH, Rosbach, Germany). Stained whole blood smears were generated on microscope slides to perform manual 200-cell differential counts. Activities of aspartate amino-tranferase (AST), glutamate dehydrogenase (GLDH), γ-glutamyltransferase (γ-GT) as well as total protein, albumin, glucose, bilirubin, cholesterol, triglycerides and urea in serum were determined photometrically by an Eurolyser CCA 180 VET (Greiner Diagnostic, Bahlingen, Germany).

2.6 Isolation and proliferation of peripheral blood mononuclear cells (PBMC)

Both isolation and proliferation of porcine PBMC were carried out according to Goyarts et al. (2006). Briefly, PBMC were separated from diluted, heparinised blood by density gradient centrifugation and then frozen and stored at -80 °C in dimethyl sulfoxide until the beginning of the proliferation test. For the proliferation test, 10 replications of thawed and washed PBMC were seeded into 96-well plates and 5 of them were stimulated with Concanavalin A (ConA, Sigma-Aldrich, Steinheim, Germany). A MTT-assay (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was used to test the metabolic activity of the proliferating cells. The optical density (OD) of the incubated PBMC was determined at wavelength of 570 nm and corrected by blank value. The ratio between OD of ConA stimulated cells and non-stimulated cells was calculated as stimulation index (SI).

2.7 Statistical analyses

Statistical analyses were carried out using the software package SAS, version 9.1 (SAS Institute, Cary, NC, USA). All data except for mycotoxin residues in serum and differential blood count were subjected to analysis of variance (ANOVA) according to a complete 2 by 2-factorial design, including the contamination of the maize, the hydrothermal treatment and their interaction as fixed factors. Statistically significant differences of means were detected using Tukey's multiple range test at $p<0.05$ while differences at $p<0.1$ were considered as trends. Not normally distributed parameters such as values for mycotoxin residues in serum and proportions of granulocytes in blood were evaluated by using the nonparametric Wilcoxon rank-sum test. Values below the limit of quantification were considered as zero.

3 Results

3.1 Maize and diets

Hydrothermal treatment of naturally contaminated maize (43.4 mg DON and 2.48 mg ZEN/kg) for 30 min at 80 °C in the presence of 5 g SBS, 10 g MMA and 20 g $Ca(OH)_2$ resulted in a reduction of 91 % and 79 % in DON and ZEN concentrations, respectively.
The mean content of crude fibre was decreased by 6 g by the hydrothermal treatment, but the variations within the four maize treatments also in the other crude nutrients (dry matter, crude protein, ether extract) were mostly in the range of 1 %. However, the addition of chemicals for the hydrothermal treatment increased the ash content by approximately 2.6 %, while the proportion of N-free-extractives was decreased by 2.3 % in treated variants (CON+, FUS+). The degree of starch gelatinization was increased by 16.3 and 20.5 % in treated control and contaminated maize.
In complete diets, the incorporation of untreated contaminated maize (FUS-) resulted in concentrations of 0.32 and 3.67 mg ZEN and DON/kg, respectively, while concentrations amounted to 0.08 and 0.36 mg/kg with treated contaminated maize (FUS+). Similarly, the treatment reduced ZEN and DON concentrations in the diet containing uncontaminated maize (CON+) to 0 and 0.04 mg/kg, respectively, which was approximately 90 % lower than concentrations in diet containing the untreated uncontaminated maize (CON-). The chemical analysis and calculation of metabolizable energy of the obtained diets revealed comparable nutrient and dry matter levels.

3.2 Performance

Generally, performance parameters did not differ between the four experimental treatment groups (Table 3). Considering the mean cumulated values for daily feed intake and live weight gain for the different periods of the trial variations between the groups, as indicated by the PSEM, were low. The daily feed intake ranged between 0.457 and 0.486 kg during the first two experimental weeks and increased to 0.769-0.872 kg in the last two weeks. The ingested amounts of ZEN and DON per kg bodyweight remained relatively constant over the experimental period and resulted in a mean exposure of 162 µg DON and 14µg ZEN per kg body weight (BW) in group FUS-, while in the remaining groups FUS+, CON- and CON+ the mean exposure amounted to 16, 18 and 2 µg DON and 4, 1 and 0 µg ZEN/kg BW, respectively. Live weight gain for the whole period ranged between 0.356-0.387 kg. However,

the feed to gain ratio tended to be increased in the groups receiving the contaminated diets (p=0.086), resulting in a 0.120 kg higher feed consumption per kg weight gain.

Table 3. Performance of female piglets fed diets containing uncontaminated (CON) or *Fusarium* toxin-contaminated maize (FUS), either untreated (-) or hydrothermally treated in the presence of sodium metabisulfite, methylamine and calcium hydroxide (+) (means)

Maize	Hydrothermal treatment	Feed intake [kg/d] (n=5)			Live weight gain [kg/d] (n=25)			Feed to gain ratio [kg/kg] (n=5)		
		Day 1-13	Day 14-27	Day 1-27	Day 1-13	Day 14-27	Day 1-27	Day 1-13	Day 14-27	Day 1-27
CON	-	0.484	0.769	0.630	0.245	0.519	0.386	1.982	1.536	1.674
CON	+	0.464	0.804	0.640	0.257	0.496	0.381	1.819	1.641	1.689
FUS	-	0.457	0.803	0.637	0.227	0.476	0.356	2.115	1.710	1.800
FUS	+	0.486	0.872	0.694	0.244	0.519	0.387	2.180	1.680	1.804
ANOVA (p-values)										
Maize		0.854	0.132	0.167	0.442	0.635	0.401	0.243	0.225	0.086
Treatment		0.766	0.143	0.138	0.459	0.635	0.372	0.814	0.665	0.885
Maize x treatment		0.107	0.609	0.286	0.898	0.112	0.203	0.583	0.438	0.928
PSEM*		0.014	0.032	0.021	0.019	0.021	0.014	0.204	0.085	0.066

* Pooled standard error of means

3.3 Visceral and reproductive organs

BWs at slaughter were comparable in all four feeding groups and remained unaffected by the contamination of the diets (CON and FUS), the hydrothermal treatment (+, -) or their interaction (Table 4). Relative liver weights were slightly lower in groups receiving the contaminated maize in the diet as compared to control groups, but differences were not significant. Heart and kidney weights ranged from 4.3 – 4.6 and 4.4 – 4.9 g/kg BW, respectively, and were not influenced by the experimental factors.

Table 4. Body weight (BW) and organ weights [g/kg BW] of female piglets fed diets containing uncontaminated (CON) or *Fusarium* toxin-contaminated maize (FUS), either untreated (-) or hydrothermally treated in the presence of sodium metabisulfite, methylamine and calcium hydroxide (+) (means; n=5)

Maize	Hydrothermal treatment	BW at slaughter [kg] (n=25)	Liver	Heart	Kidneys	Spleen
CON	-	19.3	26.1	4.6	4.9	2.0
CON	+	19.1	25.0	4.3	4.6	1.6
FUS	-	18.4	23.8	4.5	4.4	1.9
FUS	+	19.3	23.0	4.4	4.7	1.9
ANOVA (p-values)						
Maize		0.489	0.284	0.924	0.488	0.614
Treatment		0.461	0.635	0.271	0.922	0.563
Maize x treatment		0.270	0.938	0.725	0.413	0.411
PSEM*		0.5	2.0	0.2	0.4	0.2

* Pooled standard error of means

However, the hydrothermal treatment significantly reduced uterus weights (p=0.009), while ovaries weights tended to be decreased (p=0.062) (Table 5). Though uterus weights in group FUS- were significantly higher as compared to group FUS+, differences to the control groups were insignificant. Vulva width in relation to BW was affected by the hydrothermal treatment (p=0.002) and tended to be influenced by the contamination of the diets (p=0.058). The highest value was detected in group FUS-, which was significant when compared to groups receiving treated maize (FUS+ and CON+) (p<0.008).

Table 5. Weight of reproductive organs and vulva width of female piglets fed diets containing uncontaminated (CON) or *Fusarium* toxin-contaminated maize (FUS), either untreated (-) or hydrothermally treated in the presence of sodium metabisulfite, methylamine and calcium hydroxide (+) (means; n=5)

Maize	Hydrothermal treatment	Uterus [g/kg body weight]	Ovaries [g/kg body weight]	Vulva width [cm/kg body weight] (n=25)
CON	-	0.55ab	0.013	0.092ab
CON	+	0.48ab	0.012	0.087b
FUS	-	0.66a	0.016	0.103a
FUS	+	0.41b	0.011	0.088b
ANOVA (p-values)				
Maize		0.758	0.457	0.058
Treatment		0.009	0.062	0.002
Maize x treatment		0.123	0.119	0.136
PSEM*		0.06	0.002	0.003

* Pooled standard error of means

3.4 Mycotoxin residues in serum

Serum toxin analyses revealed the highest median concentrations of ZEN, DON and their main metabolites α-ZEL and de-DON (0.4/ 21/ 0.7 and 6 ng/ml, respectively) in serum of piglets that received the contaminated maize in the diet (FUS-), while median toxin concentrations in the other three treatment groups were significantly lower and often below the detection limit (Figure 1). The ingestion of the contaminated and hydrothermally treated maize (FUS+) reduced serum toxin concentrations to the level of the control groups.

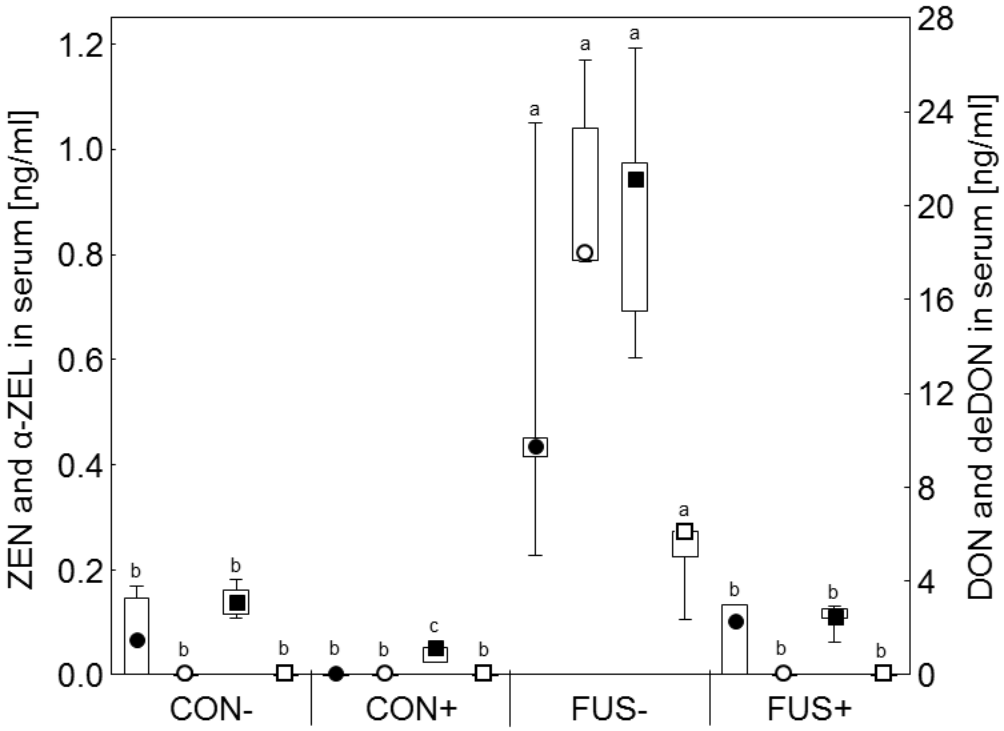

Figure 1. Serum concentrations of zearalenone (ZEN; ●), α-zearalenol (α-ZEL; ○), deoxynivalenol (DON; ■) and de-epoxy-DON (de-DON;□) in piglets fed diets containing uncontaminated or *Fusarium* toxin-contaminated maize either untreated (CON-, FUS-) or treated with sodium metabisulfite, methylamine and calcium hydroxide(CON+, FUS+) (n=5)

Boxes represent the 25–75th percentiles and symbols within each box represent the median. Within identical symbol, values with different letters (a, b) are significantly different ($p< 0.05$).

3.5 Differential blood count and stimulation index of PBMC

Generally, the different treatments did not have significant effects on haematological parameters or the stimulation index of PBMC and were mainly within the given reference ranges by Kraft and Dürr (2005) (Table 6). However, feeding diet FUS- slightly increased the

number of total leukocytes as compared to piglets fed the control or treated contaminated diets (FUS+). The proportion of neutrophils was found to be above the reference range of 10-39 % in groups CON-, FUS- and FUS+. The proportion of monocytes (0.19±0.08; mean± standard error), eosinophils (1.34±0.43) and basophils (0.59±0.18) remained unaffected by the contamination of the diet, the hydrothermal treatment or their interaction (data not shown).

Table 6. Differential blood count and stimulation index of peripheral blood mononuclear cells of female piglets fed diets containing uncontaminated (CON) or *Fusarium* toxin-contaminated (FUS) maize, either untreated (-) or hydrothermally treated in the presence of sodium metabisulfite, methylamine and calcium hydroxide (+) (means; n=5)

Maize	Hydrothermal treatment	Leukocytes [10^9/L] 10-22[§]	Lymphocytes [%] 49-85[§]	Neutrophils [%] 10-39[§]	Stimulation index
CON	-	16.8	56.3	45.5	6.3
CON	+	16.1	67.1	31.0	4.1
FUS	-	20.3	50.2	47.7	6.0
FUS	+	16.0	56.0	41.0	5.9
ANOVA (p-values)					
Maize		0.455	0.170	0.410	0.291
Treatment		0.286	0.182	0.164	0.106
Maize x treatment		0.420	0.675	0.595	0.147
PSEM[*]		2.6	6.3	8.0	0.7

[*] Pooled standard error of means

[§] Reference values according to (Kraft and Dürr 2005)

3.6 Biochemical serum analyses

Serum biochemical parameters mainly remained uninfluenced by the two experimental factors or their interaction (Table 7). Activities of GLDH were detectable in none of the groups. However, AST activities were found to be above the reference range of 35 U/l (Kraft and Dürr 2005) in all groups except for group FUS+. Similarly, high bilirubin values were determined in all feeding groups. In contrast, analyses of cholesterol and urea revealed concentration below given values (Bickhardt 1992; Kixmöller 2004). Urea concentration in group CON+ was about 1.8 mmol/L higher than the lowest value of 2.4 in group FUS+, resulting in a trend (p=0.079).

Table 7. Serum biochemical analyses of female piglets fed diets containing uncontaminated (CON) or *Fusarium* toxin-contaminated (FUS) maize, either untreated (-) or hydrothermally treated in the presence of sodium metabisulfite, methylamine and calcium hydroxide (+) (means; n=5)

Maize	Hydrothermal treatment	Parameter[§]								
		Prot [g/L]	Alb [µmol/L]	AST [U/L]	γ-GT [U/L]	Bili [µmol/L]	Chol [mmol/L]	Gluc [mmol/L]	Tgl [mmol/L]	Urea [mmol/L]
		<86[&]	261-449[&]	<35[&]	<45[&]	<2.4[$]	2.0-3.3[◊]	3.9-6.4[&]	<0.50[◊]	3.3-8.3[&]
CON	-	34.5	333	55.8	31.6	23.6	1.7	6	0.43	2.7
CON	+	30.5	312	53.8	26.5	23.3	1.5	6.7	0.43	4.2
FUS	-	37.4	368	52.8	29.1	24.5	1.5	5.6	0.46	3.2
FUS	+	27.4	295	26.6	24.8	23.1	1.3	4.8	0.34	2.4
ANOVA (p-values)										
Maize		0.985	0.875	0.196	0.794	0.807	0.609	0.153	0.721	0.177
Treatment		0.302	0.414	0.228	0.555	0.505	0.497	0.947	0.476	0.488
Maize x treatment		0.653	0.653	0.294	0.962	0.631	0.993	0.323	0.498	0.029
PSEM[*]		6.67	3.95	11.38	7.99	0.08	10.51	13.7	7.82	2.87

[*] Pooled standard error of means

[§] Prot, total protein; Alb, albumin; AST, aspartate amino-transferase; γ-GT, gamma-glutamyl-transferase; Bili, bilirubin; Chol, cholesterol; Gluc, glucose; Tgl, triglycerides

[&] Reference values according to (Kraft and Dürr 2005)

[$] Reference value according to (Kixmöller 2004)

[◊] Reference values according to (Bickhardt 1992)

4 Discussion

4.1 Maize and diets

The applied chemicals (SBS, MMA, $Ca(OH)_2$) for the hydrothermal treatment were frequently tested for decontamination but were usually used separately for the reduction of individual toxins. Previous studies have shown that the use of SBS is a potent method to reduce DON-concentrations in different cereals by the formation of DON-sulfonate (review by Dänicke et al. (2012)). However, ZEN-concentrations mostly remain unaffected by the presence of this chemical (Dänicke et al. 2008). Considerable reductions in ZEN-concentrations were achieved in the presence of alkaline compounds such as sodium carbonate (Rotter et al. 1995; Polak et al. 2009) or $Ca(OH)_2$-MMA (Bauer et al. 1987a). With regard to a simultaneous reduction of both toxins a combination of chemicals therefore seemed promising and was confirmed by the results of the present trial. The contamination of the maize with 43.4 mg DON/kg and 2.48 mg ZEN/kg was reduced by 91 and 79%, respectively, by the hydrothermal treatment in the presence of 5 g SBS, 10 g MMA and 20 g $Ca(OH)_2$. As expected, the content of crude ash increased by the amount of added chemicals. However, when mixed into diets the difference in ash content between treated and untreated diets amounted only to 0.5 % of the total diet. Similarly, the increase in the proportion of gelatinized starch to approximately 30 % in treated maize variants is within the range for conditioning and expanding procedures reported in literature (Thomas et al. 1999; Ie et al. 2012).

4.2 Performance

Though the concentrations of DON and ZEN in group FUS- (3.67 mg DON/kg and 0.32 mg ZEN/kg) were almost 4- and 3-fold higher than the guidance values introduced by the (The Commission of the European Communities 2006), no differences in feed intake or live weight gain compared to the control fed groups (CON-, CON+) were observed. In contrast, Döll et al. (2003) found significantly affected performance when feeding diets contaminated with 3.9 mg DON and 0.42 mg ZEN/kg to piglets. Similarly, the exposure of piglets to diets containing 4.52 mg DON and 0.29 mg ZEN/kg resulted in a minimum reduction in feed intake of 20 % (Rempe et al. 2013a). These varying effects on performance parameters in different studies may be attributed to a number of factors such as the experimental design or general

conditions, the overall health status of the animals and the presence of other mycotoxins or undesired substances.

The hydrothermal treatment and consequential presence of chemicals in the diets did not affect performance parameters. However, it has to be taken into account that diets were composed of only 10 % treated maize. The actual amounts of SBS, MMA and $Ca(OH)_2$ per kg diet thereby resulted in approximately 0.5, 1 and 2 g, respectively, which increased the content of pure sodium and calcium of the complete diet by 0.06 and 0.11 % in comparison to diets containing untreated maize. Nevertheless, the total contents of calcium and sodium were still in the range recommended for piglet diets (GfE 2006). Consistently, Til et al. (1972) observed decreases in performance parameters in pigs only at concentrations above 8.3 g SBS/kg diet. Similarly, the inclusion of a 10-fold higher amount of MMA and $Ca(OH)_2$ into pig diets caused a significantly reduced feed intake (Betz 1993). The dietary concentrations of chemicals that were fed in the present study may thus be considered on a no-effect level with regard to performance of piglets.

4.3 Visceral and reproductive organs

High doses of ZEN and DON may influence growth of visceral and reproductive organs. 3 mg ZEN/kg in a diet for female piglets increased liver and kidney weights, while spleen weights were reduced in a study by Jiang et al. (2011). Increased liver weights were also observed when growing pigs were fed oats containing 3.5 mg DON/kg (Bergsjø et al. 1993). Feeding 4.5 or 3.9 mg DON/kg, however, did not affect growth of liver, kidney or heart in other studies (Döll et al. 2003; Rempe et al. 2013a). These results seem contradictory but differences between studies might result from the time of exposure and the age of the animals (Döll et al. 2003). Accordingly, in the present study exposure to 3.67 mg DON/kg and 0.32 mg ZEN did not cause alterations in visceral organ growth. Weights of visceral organs were neither influenced by the treatment of the maize. However, it has to be considered that the concentrations of the chemicals actually fed were rather low. In a study of Betz (1993), the inclusion of 0.5 % MMA and 2 % $Ca(OH)_2$ in a diet for pigs seemed to increase liver and kidney weights, while $Ca(OH)_2$ alone did no influence organs. With regard to the presence of SBS in diets, Til et al. (1972) observed increased liver, kidney, heart and spleen weights of SBS-exposed piglets only at concentrations >8.3 g SBS/kg diet. Below this dose the authors did not detect any alterations from the control group. Therefore, the concentrations fed in the present study did not seem to affect physiological organ growth of female piglets.

Reproductive organs such as uterus and vulva often show considerable response to lower dietary ZEN-concentrations. A diet for female piglets containing 0.42 mg ZEN/kg increased uterus weights by almost 100 % (Döll et al. 2003), while in a different study 0.2-0.3 mg ZEN/kg had the same effect (Gutzwiller et al. 2007). Swelling of the vulvae may occur at concentrations below 1 mg ZEN/kg (Bauer et al. 1987b; Teixeira et al. 2011). In the present study, highest relative uterus weights were observed in group FUS- (0.32 mg ZEN/kg) and vulva width was similarly increased in this group. The hydrothermal treatment seemed to reduce the oestrogenic effect of the diet as decreased uterus weights and vulva width were observed in the group receiving the treated contaminated diet (FUS+). The detectable reduction of ZEN might therefore have been accompanied by a treatment-mediated change in conformation of ZEN, which implied a loss of its oestrogenic potential.

4.4 Mycotoxin residues in serum

Although a 4-week exposure to diet FUS- did not affect performance parameters, serum concentrations of DON and ZEN were higher in this group (21 and 0.45 ng/ml) as compared to the groups receiving the treated FUS or control diets which ranged below 4 and 0.1 ng DON and ZEN per ml, respectively. The magnitude of detectable concentration is mainly influenced by the amount of toxin ingested (Döll et al. 2003) and the time point of sampling after the toxin-containing meal. The maximum levels of ZEN and DON in serum seem to occur approximately 3 and 4 hours after oral exposure, respectively (Dänicke et al. 2004; Dänicke et al. 2005a). In the present study, piglets in group FUS- were exposed to approximately 31±11 µg DON and 3±1 µg ZEN/kg BW 4 hours prior to slaughter. The resulting serum concentrations are however slightly higher than concentrations reported in previous studies with similar experimental conditions (Döll et al. 2003; Dänicke et al. 2004). The determined toxin values, particularly in group FUS- are characterised by a high variation between individuals, which might contribute to this effect. Serum concentrations of α-ZEL and de-DON, which are among the main metabolites of ZEN and DON that are detectable in serum, were similarly reduced to the level of the control group in piglets fed the treated FUS diet. According to Dänicke et al. (2005b), these findings can be seen as a true indicator of a successful detoxification.

4.5 Haematological analyses and stimulation index of PBMC

Leukocytes are known to be a main target of trichothecenes such as DON which may cause inhibitory or stimulatory reactions in the immune cells (Pestka et al. 2004). Neither the stimulation index of PBMC nor the differential blood count were affected by the different experimental factors in this study. The count of total leukocytes and lymphocytes were in the range reported in (Kraft and Dürr 2005), while neutrophils were slightly above the mentioned value. Since the subset of leukocytes changes during growth of piglets from a neutrophilic to lymphocytic hemogram (Rempe et al. 2013a), this observation rather seems to be a snapshot of a still not completed development. Moreover, neither the presence of *Fusarium*-toxins nor chemicals impaired the ability of PBMC to react to stimuli and the stimulation index was in the range reported in other studies, where similar toxin concentrations did not influence this parameter (Dänicke et al. 2007; Grenier et al. 2011; Rempe et al. 2013a).

4.6 Biochemical serum analyses

Serum biochemical parameters remained unaltered by the presence of contaminated maize or chemicals in the diet but were generally characterised by a high variation between individuals. Total protein and albumin in serum of piglets were within the reference ranges (Kraft and Dürr 2005). Others studies however, found reduced protein and albumin levels in pigs fed similar DON-concentrations (Bergsjø et al. 1993; Grenier et al. 2011). In accordance with the present results, a diet contaminated with 4.52 mg DON and 0.29 mg ZEN/kg did not induce this effect in a recent study (Rempe et al. 2013a). Possible reasons for differences between studies might arise from differences in the age and sex of the animals but as well the time of exposure which was about 2 weeks longer in studies by Grenier et al. (2011) and Bergsjø et al. (1993). Bilirubin levels were almost 10-fold above the reference value introduced by Kixmöller (2004). This might hint at liver damage or cholestasis (Kraft and Dürr 2005) but may as well be induced by development-related adaptations. High levels of bilirubin are usually accompanied by low levels of cholesterol as in the present study, which may similarly be related to liver diseases. However, specific liver enzymes such as GLDH and γ-GT showed no difference between the treatment groups, indicating that neither the contamination of the diet nor the presence of the chemicals considerably affected liver function or integrity. Kixmöller (2004) discussed that high values of bilirubin might rather be caused by an incompletely adapted liver metabolism of young animals. Accordingly, Rempe et al. (2013a) observed that bilirubin in serum decreased during a feeding trial with weaned piglets, while

cholesterol levels increased. Relatively high activities of AST in serum were independent of the dietary treatment and might be influenced by the breed of the animals, since Kixmöller (2004) found the highest AST-values in pigs of the breed Piétrain. However, a possible effect of the slaughtering procedure cannot be excluded.

Taken together, no treatment-related effects that might hint at a severe impairment of health of the animals were detected.

5 Conclusion

The hydrothermal treatment of a mainly DON and ZEN-contaminated maize with SBS and MMA-$Ca(OH)_2$ achieved a simultaneous and considerable reduction of both toxins. The inclusion of treated feed in a diet for female piglets reduced the occurrence of hyperestrogenism. Moreover, significantly decreased concentrations of DON, ZEN and their metabolites in serum indicated a successful decontamination of the maize. Nevertheless, unaltered growth of organs and haematological and serum biochemical parameters indicated that no adverse effects on the health of the animals emanate from the treated maize or the chemicals in the presently applied doses.

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References

Bauer J, Gareis M, Detzler W, Gedek B, Heinritzi K, Kabilka G. 1987a. Detoxification of mycotoxins in feedstuffs. Tierärztl Um. 42:70-77.

Bauer J, Heinritzi K, Gareis M, Gedek B. 1987b. Changes in the genital tract of female pigs after feeding practive-relevant amounts of zearalenone. Tierärztl Prax. 15:33-36.

Bergsjø B, Langseth W, Nafstad I, Jansen JH, Larsen HJS. 1993. The effects of naturally deoxynivalenol-contaminated oats on the clinical condition, blood parameters, performance and carcass composition of growing pigs. Vet Res Commun. 17:283-294.

Betz J. 1993. Detoxification of ochratoxin A: effects of dietary alkali sources calcium hydroxide and monomethylamine on blood and urine variables and residues in pigs [dissertation]. München: Ludwig-Maximilians-Universität München.

Bickhardt K. 1992. Kompendium der allgemeinen Inneren Medizin und Pathophysiologie für Tierärzte: Studientexte 69. Berlin, Hamburg: Parey.

Dänicke S, Beineke A, Goyarts T, Valenta H, Beyer M, Humpf HU. 2008. Effects of a Fusarium toxin-contaminated triticale, either untreated or treated with sodium metabisulphite (Na2S2O5, SBS), on weaned piglets with a special focus on liver function as determined by the C-13-methacetin breath test. Arch Anim Nutr. 62:263-286.

Dänicke S, Goyarts T, Valenta H. 2007. On the specific and unspecific effects of a polymeric glucomannan mycotoxin adsorbent on piglets when fed with uncontaminated or with Fusarium toxins contaminated diets. Arch Anim Nutr. 61:266-275.

Dänicke S, Kersten S, Valenta H, Breves G. 2012. Inactivation of deoxynivalenol-contaminated cereal grains with sodium metabisulfite: a review of procedures and toxicological aspects. Mycotoxin Research. 28:199-218.

Dänicke S, Swiech E, Buraczewska L, Ueberschär KH. 2005a. Kinetics and metabolism of zearalenone in young female pigs. J Anim Physiol Nutr. 89:268-276.

Dänicke S, Valenta H, Döll S. 2004. On the toxicokinetics and the metabolism of deoxynivalenol (DON) in the pig. Arch Anim Nutr. 58:169-180.

Dänicke S, Valenta H, Gareis M, Lucht HW, von Reichenbach H. 2005b. On the effects of a hydrothermal treatment of deoxynivalenol (DON)-contaminated wheat in the presence of sodium metabisulphite (Na2S2O5)on DON reduction and on piglet performance. Anim Feed Sci Technol. 118:93-108.

Döll S, Dänicke S. 2011. The Fusarium toxins deoxynivalenol (DON) and zearalenone (ZON) in animal feeding. Prev Vet Med. 102:132-145.

Döll S, Dänicke S, Ueberschär KH, Valenta H, Schnurrbusch U, Ganter M, Klobasa F, Flachowsky G. 2003. Effects of graded levels of Fusarium toxin contaminated maize in diets for female weaned piglets. Arch Anim Nutr. 57:311-334.

GfE. 2006. Ausschuss für Bedarfsnormen der Gesellschaft für Ernährungsphysiologie: Empfehlungen zur Energie- und Nährstoffversorgung von Schweinen [Recommendations on the energy and nutrient supply of pigs]. Frankfurt am Main: DLG-Verlag.

GfE. 2008. Prediction of Metabolisable Energie in compound feed for pigs. Proc Soc Nutr Physiol. 17.

Goyarts T, Dänicke S, Tiemann U, Rothkötter HJ. 2006. Effect of the Fusarium toxin deoxynivalenol (DON) on IgA, IgM and IgG concentrations and proliferation of porcine blood lymphocytes. Toxicol in Vitro. 20:858-867.

Grenier B, Loureiro-Bracarense A-P, Lucioli J, Pacheco GD, Cossalter A-M, Moll W-D, Schatzmayr G, Oswald IP. 2011. Individual and combined effects of subclinical doses of deoxynivalenol and fumonisins in piglets. Mol Nutr Food Res. 55:761-771.

Gutzwiller A, Czegledi L, Stoll P, Bruckner L. 2007. Effects of Fusarium toxins on growth, humoral immune response and internal organs in weaner pigs, and the efficacy of apple pomace as an antidote. J Anim Physiol Nutr. 91:432-438.

Ie PS, Petros D, Stinner DH, Phelan PL, Hamaker B, Koelling KW, Vodovotz Y. 2012. Comparison of the gelatinization behavior of organic and conventional spelt starches assessed by thermal and rheological analyses. J Agric Food Chem. 60:9229-9235.

Jiang SZ, Yang ZB, Yang WR, Gao J, Liu FX, Broomhead J, Chi F. 2011. Effects of purified zearalenone on growth performance, organ size, serum metabolites, and oxidative stress in postweaning gilts. J Anim Sci. 89:3008-3015.

Jouany JP. 2007. Methods for preventing, decontaminating and minimizing the toxicity of mycotoxins in feeds. Anim Feed Sci Technol. 137:342-362.

Kixmöller M. 2004. Labordiagnostische Referenzbereiche bei unterschiedlichen Schweinerassen sowie histopathologische und immunhistochemische Untersuchung von Gehirnen älterer Sauen und Eber auf transmissible spongiforme Enzephalopathie im Rahmen der TSE-Studie [dissertation]. München: Ludwig-Maximilians-Universität München. Available at: http://edoc.ub.uni-muenchen.de/2198/

Kraft W, Dürr UM. 2005. Klinische Labordiagnostik in der Tiermedizin [Clinical laboratory diagnostics in veterinary medicine]. Stuttgart: Schattauer GmbH

Naumann C, Bassler R. 1993. Die chemische Untersuchung von Futtermitteln [The chemical analysis of animal feed]. Darmstadt VDLUFA-Verlag.

Oldenburg E, Bramm A, Valenta H. 2007. Influence of nitrogen fertilization on deoxynivalenol contamination of winter wheat - experimental field trials and evaluation of analytical methods. Mycotoxin Res. 23:7-12.

Oldenburg E, Valenta H, Sator C. 2000. Risikoabschätzung und Vermeidungsstrategien bei der Futtermittelerzeugung. In: Dänicke S, Oldenburg E, Editors. Risikofaktoren für die Fusariumtoxinbildung und Vermeidungsstrategien bei der Futtermittelerzeugung und Fütterung. SH 216:5-34.

Pestka JJ, Zhou HR, Moon Y, Chung YJ. 2004. Cellular and molecular mechanisms for immune modulation by deoxynivalenol and other trichothecenes: unraveling a paradox. Toxicol Lett. 153:61-73.

Polak M, Gajecki M, Kulik T, Luczynski MK, Obremski K, Gora M, Gajecka M, Jakimiuk E, Zielonka L. 2009. The evaluation of the efficacy of sodium carbonate as zearalenone destructor in feeding stuffs. Pol J Vet Sci. 12:103-111.

Rempe I, Kersten S, Brezina U, Hermeyer K, Beineke A, Dänicke S. 2013a. Time-dependent effects of graded levels of Fusarium toxin-contaminated maize in diets for female piglets. World Mycotoxin J. 6:51-63.

Rempe I, Kersten S, Valenta H, Dänicke S. 2013b. Hydrothermal treatment of naturally contaminated maize in the presence of sodium metabisulfite, methylamine and calcium hydroxide; effects on the concentration of zearalenone and deoxynivalenol. Mycotoxin Res.1-7.

Rotter RG, Rotter BA, Thompson BK, Prelusky DB, Trenholm HL. 1995. Effectiveness of density segregation and sodium.carbonate treatment on the detoxification of Fusarium-contaminated corn fed to growing pigs. J Sci Food Agric. 68:331-336.

Teixeira LC, Montiani-Ferreira F, Locatelli-Dittrich R, Santin E, Alberton GC. 2011. Effects of zearalenone in prepubertal gilts. Pesqui Vet Bras. 31:656-662.

The Commission of the European Communities. 2006. Commission Recommendation of 17 August 2006 on the presence of deoxynivalenol, zearalenone, ochratoxin A, T-2 and HT-2 and fumonisins in products intended for animal feeding. L229:7-9.

Thomas M, Huijnen P, van Vliet T, van Zuilichem DJ, van der Poel AFB. 1999. Effects of process conditions during expander processing and pelleting on starch modification and pellet quality of tapioca. J Sci Food Agric. 79:1481-1494.

Til HP, Vanderwa.P, Degroot AP, Feron VJ. 1972. Toxicity of Sulfite .2. Short-Term and Long-Term Feeding Studies in Pigs. Food Cosmet Toxicol. 10:463-&.

VDLUFA. 1976. Die chemische Untersuchung von Futtermitteln. Band III, Kapitel 7.2.6 Stärkeaufschlussgrad. Darmstadt: VDLUFA-Verlag.

VDLUFA. 2006. Bestimmung von Zearalenon nach Immunaffinitätssäulenreinigung - HPLC-Verfahren. Verbandsmethode, 6. Erg. Darmstadt: VDLUFA-Verlag.

Young JC, Trenholm HL, Friend DW, Prelusky DB. 1987. Detoxification of deoxynivalenol with sodium bisulfite and evaluation of the effects when pure mycotoxin or contaminated corn was treated and given to pigs J Agric Food Chem. 35:259-261.

GENERAL DISCUSSION

The global occurrence of different mycotoxins in cereals is a challenging issue in animal nutrition that can considerably impair animal health and productivity. In particular ZEN and DON are often detected in concentrations high enough to cause adverse effects in pigs, which are regarded as most sensitive among livestock. Typical effects are reduced feed intake and growth performance, immune-modulation as well as fertility disorders and hyperoestrogenism (EFSA, 2004a, b). The intensity of these effects is thereby influenced by a wide range of both experimental and physiological conditions. These include the toxin concentrations in the feed, the source of the toxins (natural or crystalline), age, sex, breed and health status of the animals but as well the time of exposure. Especially the influence of the duration of experiments with regard to the development of effects has received little attention in past literature, as most parameters were evaluated exclusively at the end of experiments. However, this may be a crucial point in particular concerning ZEN-effects, since ZEN and metabolites are retarded in the organism by an intensive enterohepatic cycling (Biehl et al., 1993) and may thus be accumulated during a continuous toxin intake. Especially for the evaluation of decontamination procedures it is therefore important to determine the onset of significant effects during a continuous exposure to the toxin.

In the first experiment of this thesis, thus, detailed information on the time- and dose-dependence of effects of a *Fusarium* toxin-contaminated diet fed to female piglets were aimed to be gained (**Paper I**). Female piglets are characterized by their sensitivity to DON and ZEN and may therefore reliably show adverse effects on health or performance parameters. A special focus has been laid on the surveillance of a physiological organ growth and the development of clinical chemical and immunological parameters during a continuous toxin exposure.

For all experiments in this thesis naturally contaminated maize was chosen, as effects in animals were shown to differ between crystalline toxins and naturally contaminated feed (Forsyth et al., 1977). This may likely be due to the parallel presence of several toxins. Plants are often infected by different fungi, which in turn may build a range of different substances (Oldenburg et al., 2000). *F. graminearum* for example is able to form beside DON and ZEN also nivalenol and diacetoxyscirpenol. Though possible interactions of toxins might require a more complex and cautious observation and interpretation of the obtained data, naturally contaminated feed reflects the conditions in practice and thus ensures a better transferability and relevance of the results.

The further development of methods to reduce mycotoxins in feed and thus prevent adverse effects on health and performance of livestock is inevitable in terms of intensified and growing animal production in times of a growing human population and subsequent growing demands for high-quality feed and food. Different products and applications were evaluated previously for their abilities to bind or degrade mycotoxins from contaminated feed but the results and efficiencies partially remain questionable (Friend et al., 1984; Döll et al., 2005; Dänicke and Döll, 2010; Dänicke et al., 2012a). In contrast, pre-feeding reduction of toxins seems more likely to be accompanied by a successful reduction of adverse effects. However, most approaches only focused on the reduction of individual toxins, which might be inappropriate in consideration of the co-occurrence of several toxins in contaminated cereals. Half of the more than 4000 feed samples analyzed in a survey 2012 were contaminated by more than one toxin (Biomin, 2012). Therefore, the second study performed in this thesis aimed to evaluate a simultaneous reduction of ZEN and DON by hydrothermal treatment in the presence of a combination of chemicals that individually proved to be effective in reducing either ZEN or DON (**Paper II**). Whether the reduction of detectable toxins in the feed also implied the amelioration of adverse effects when fed to piglets was also investigated in this thesis (**Paper III**). Therefore, the variant with the best simultaneous reduction results in the hydrothermal treatment screening was chosen and included into a diet for female piglets. During a 4-week feeding trial effects on performance, development of hyperoestrogenism, various health parameters were evaluated. A special focus has been laid on the toxin residues in serum, as specific indicator for the success of the decontamination.

1 Time-dependency of effects of an exposure to dietary *Fusarium* toxins

1.1 Toxin residues in physiological samples

The serum concentrations of ZEN, DON and their metabolites de-DON and α-ZEL in dependence on both dietary treatment and experimental day are given in Figure 7. ZEN concentrations increased during the first days of the experiment in the high exposed groups, while concentrations remained on a constantly lower level in groups exposed to dietary concentrations below 0.08 mg ZEN/kg throughout the trial. α-ZEL concentrations in the investigated serum samples were generally found to follow a similar progression, but were observed to be on a higher level than the mother compound. In an experiment of Biehl et al. (1993), plasma ZEN and α-ZEL were observed to have a biological half-life of more than 3

days in female Yorkshire piglets. The animals were fed on a diet based on corn and soybean and received high single oral doses of 10 mg /kg BW crystalline ZEN. The extended terminal half-life was suggested to result from an intensive entero hepatic cycling which allows the reabsorption of bilary excreted metabolites into the systemic blood. As the main accumulation site of ZEN and metabolites in pigs was shown to be the bile fluid (Biehl et al., 1993; Döll et al., 2003) a constant excretion of bile containing ZEN and metabolites into the intestine and subsequent reabsorption into the systemic circulation may have led to the increasing blood concentrations of the toxin during the first experimental days in the high exposed groups in the present study. Thereafter, the concentrations seem to reach a steady-state, while in the low exposed groups blood concentrations are subjected to only slight fluctuations from the beginning of the trial.

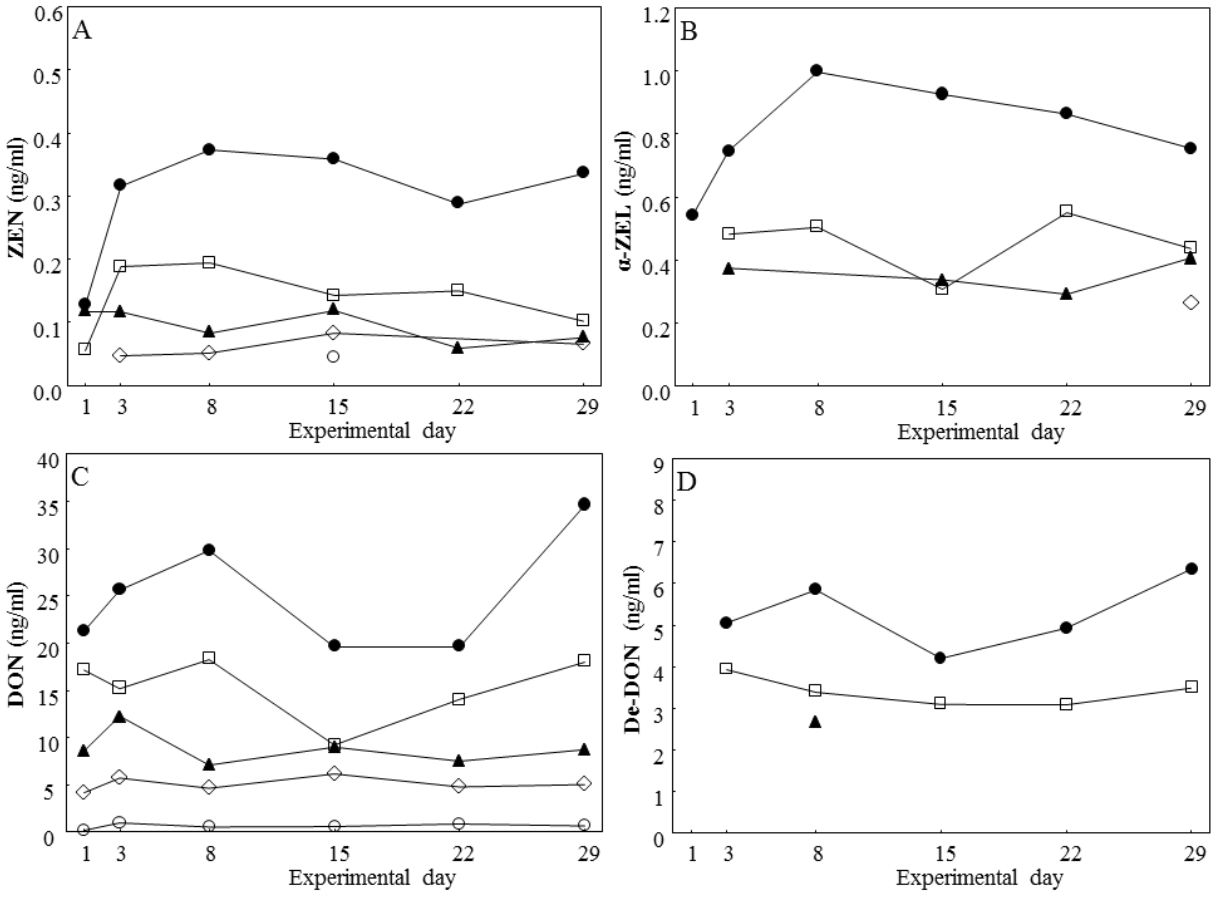


Figure 7. Mean concentrations of zearalenone (ZEN [A]), α-zearalenol (α-ZEL [B]), deoxynivalenol (DON [C]) and de-epoxy-DON (de-DON [D]) in serum of piglets exposed to increasing dietary *Fusarium* toxin concentrations (unpublished data based on **Paper I**. Shown values are above limit of detection). Treatments:

	○	◊	▲	□	●
ZEN (mg/kg diet)	0.005	0.05	0.08	0.17	0.29
DON (mg/kg diet)	0.03	0.59	1.27	2.01	4.52

From day three, the elimination of ZEN and metabolites appears to reach the necessary quantity of toxin dissipation to eliminate the constantly flooding amount of ingested ZEN in the high exposed groups which may have prevented a further toxin accumulation in the blood. In the low exposed groups the physiological elimination mechanisms seem to assure a relatively constant and sufficient excretion of the toxins as indicated by unaltered serum levels of ZEN and α-ZEL during the whole 29 experimental days as illustrated in Figure 7 (A, B). This hypothesis is substantiated by results obtained in a study by Olsen et al. (1985), who observed increasing ZEN and α-ZEL plasma concentrations during the first days of an oral exposure to ZEN with a maximum circulating amount of both compounds on day 4 of the treatment.

Serum concentrations of DON and partly de-DON were generally characterized by a higher variability in the groups exposed to dietary concentrations above 1.27 mg DON/kg than found for lower exposed groups (Figure 6, [C,D]). As described in **Paper I**, serum samples were obtained 4 hours after the intake of the toxin containing meal and thus likely reflect the maximum DON concentration in serum, which typically occurs 4.1 hours after the DON intake as previously observed by Dänicke et al. (2004a). As, first, the same author stated that the blood half-life of the substance is reached approximately 5.8 hours postprandial and, secondly, the animals in the present experiment were fasted for 16 hours prior to the last feed intake, DON was likely to be almost completely eliminated from the blood before the last feeding. The relatively high variation of serum DON in high exposed groups in the present study may thus be attributable to differences in the amount of ingested toxin caused by potential differences in individual feed intake. In particular, in these high exposed groups small alterations in feed intake can lead to relatively high absolute differences in the amount of ingested DON. The current observations are in line with previous literature, as DON is considered not to be accumulated in the organism (Prelusky and Trenholm, 1991) but the concentration of DON in blood is primarily related to the total amount of consumed toxin and the time of blood sampling after this consumption (Döll et al., 2003; Dänicke et al., 2004a).

1.2 Reproductive organs

Relative uterus weights of the piglets investigated only at the end of the present experiments (day 29 in **Paper I** and day 27 in **Paper III**) were found to be positively correlated to the concentrations of ZEN in serum at this time point of sampling ($r=0.629$, $p<0.05$, Figure 8), although variations between individuals were relatively high. In both trials the highest relative uterus weights were measured in the groups with the highest ZEN concentration in the diet

and subsequently serum (up to 1.1 ng ZEN/ml serum). In contrast, correlating all uterus weights and ZEN serum concentration values obtained during the whole experimental periods from the first to the last slaughtering of both trials resulted in a considerably lower correlation coefficient of r=0.457 (not graphically shown). This less close correlation reflects the dependence of the development of ZEN-effects on the time of exposure. Constantly high serum concentrations of ZEN were measurable after a few days of exposure in the group receiving the highest ZEN concentrations in the diet as illustrated in Figure 6, but did not necessarily result in the simultaneous development of clinical signs of hyperoestrogenism as shown in **Paper I** (Table 4**).** Only after approximately 2 weeks of *Fusarium* toxin exposure, the highest relative uterus weights were observed in the group exposed to the highest dietary toxin concentration of 0.29 mg ZEN and 4.52 mg DON/kg. In past literature, increased weights of uteri were observed after 21 days of exposure to 2 mg ZEN/kg feed (Wang et al., 2012)and after approximately 35 days of exposure to 0.2-0.3 mg ZEN/kg diet (Gutzwiller et al., 2007), respectively.

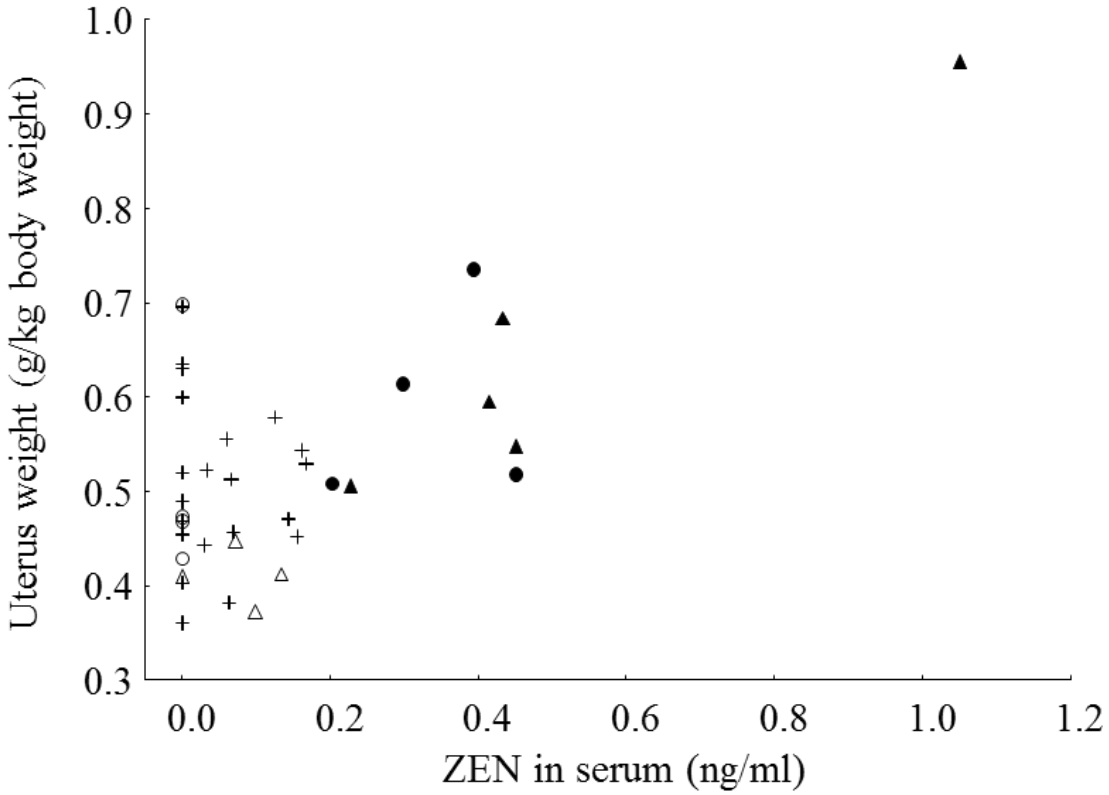


Figure 8. Relative uterus weight of piglets in relation to the concentrations of ZEN in serum measured at the last day of the experiments (day 29 in **Paper I** and day 27 in **Paper III**) (● 0.29 mg ZEN and 4.52 mg DON/kg diet, ○ 0.005 mg ZEN and 0.03 mg DON/kg diet, **Paper I**; ▲ FUS-: 0.32 mg ZEN and 3.67 mg DON/kg diet, Δ FUS+: 0.08 mg ZEN and 0.36 mg DON/kg diet, **Paper III**; + remaining feedings groups of experiments of **Paper I** and **Paper III**, concentrations were in the range of 0 – 0.17 mg ZEN and 0.04 – 2.01 mg DON/kg diet)

Similarly, the relative vulva width was found to be subjected to a lower decrease during the trial in the group with the highest toxin exposure (Figure 1, **Paper I**), indicating the relevance of the time of exposure for the development and intensity of oestrogenic effects. A time dependent increase of vulva features was also observed by other authors, feeding higher (0.75 mg/kg) or comparable (about 0.25 mg/kg) ZEN concentrations to female pigs (Bauer et al., 1987b; Cheng et al., 2006; Teixeira et al., 2011). Whether a macroscopically visible alteration in uterus and vulva morphology is also related to altered fertility or influences on the reproductive performance in the further development of the animals remains to be elucidated in more comprehensive experiments.

1.3 Feed intake and weight gain

The most obvious effect of a DON exposure in pigs is well known to be a reduction in voluntary feed intake und a subsequent loss of zootechnical performance (Rotter et al., 1996; D'Mello et al., 1999). Figure 9 presents an updated version of a literature compilation previously published by Dänicke et al. (2008b) on the effect of the dietary DON-concentration on the feed intake of pigs expressed as relative deviation from pigs fed DON-free diets. The evaluation revealed a close negative correlation between these factors (r=-0.77) resulting in a coefficient of determination of r^2=0.597 for the respective regression line. However, other experimental factors such as the age, sex and breed of the animals or the DON source or duration of the trial were not taken into account. According to the linear regression, feed intake decreases by 5.5% per 1 mg increase in the DON concentration of a diet compared to the control. The cumulated feed intakes that were measured during the first trial of this thesis coincide almost perfectly with the predicted values of the calculated regression. While a concentration of 4.52 mg DON in diet 5, resulted in a reduction in feed intake of 24 %, an exposure to 1.27 mg DON/kg diet led to a 5% feed intake depression when compared to the control group (Table 3**, Paper I**). In contrast, the feed intake of the piglets that received a diet containing 3.67 mg DON/kg in the second trial remained uninfluenced by the contamination of the diet. Although the hydrothermal treatment even seemed to enhance the voluntary feed intake, the numerical but not significant difference amounted to approximately 60 g.

Feed intake data are mostly given as cumulated values over the total experimental period. However, considering the development of feed intake during a DON exposure it becomes clear that differences between the groups may increase in the course of an exposure as illustrated in Figure 10. While the reduction of feed intake in the highest exposed group in

comparison to the control amounted to approximately 20% during the first 2 weeks, the difference increased to 25% and 27% in weeks 3 and 4, respectively. This development is considered to be a natural consequence of an initially reduced feed intake as it implies a reduction in weight gain (Goyarts et al., 2005) and subsequent lower body weights, which in turn further restrict the feed intake capacities of the animals. This underlines the importance of the time of exposure as well to DON-containing diets with regard to the intensity of the measurable decrease in performance.

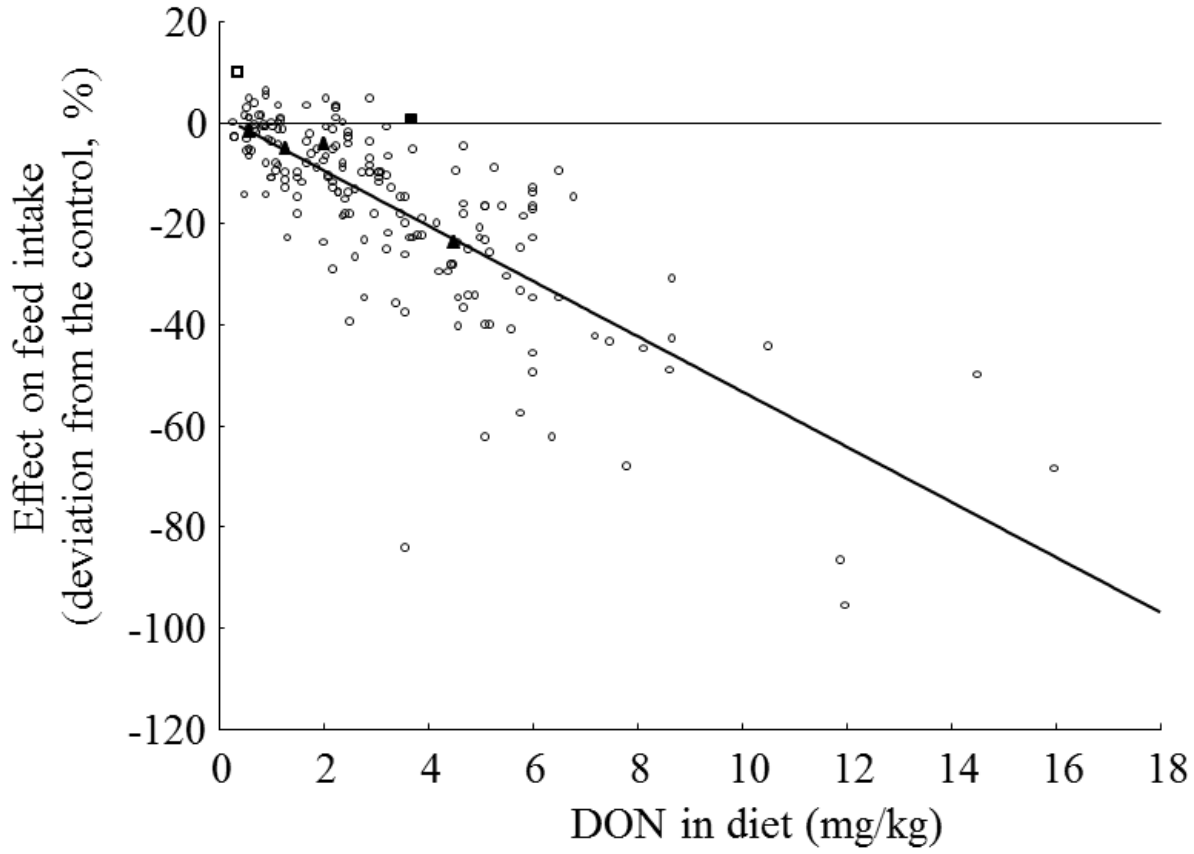


Figure 9. Effect of dietary DON-concentration on feed intake of pigs expressed as relative change compared to control fed groups (y=1.4-5.5·x, r^2=0.597; ○ literature data, ▲ dose and time experiment, **Paper I**, ■ untreated diet, **Paper III**, □ hydrothermally treated diet, **Paper III**) (literature compilation by Dänicke et al. (2008b), updated with data from Dänicke et al. (2007; 2008a; 2012a), Döll et al. (2007), Gutzwiller et al. (2007), Dänicke and Döll (2010))

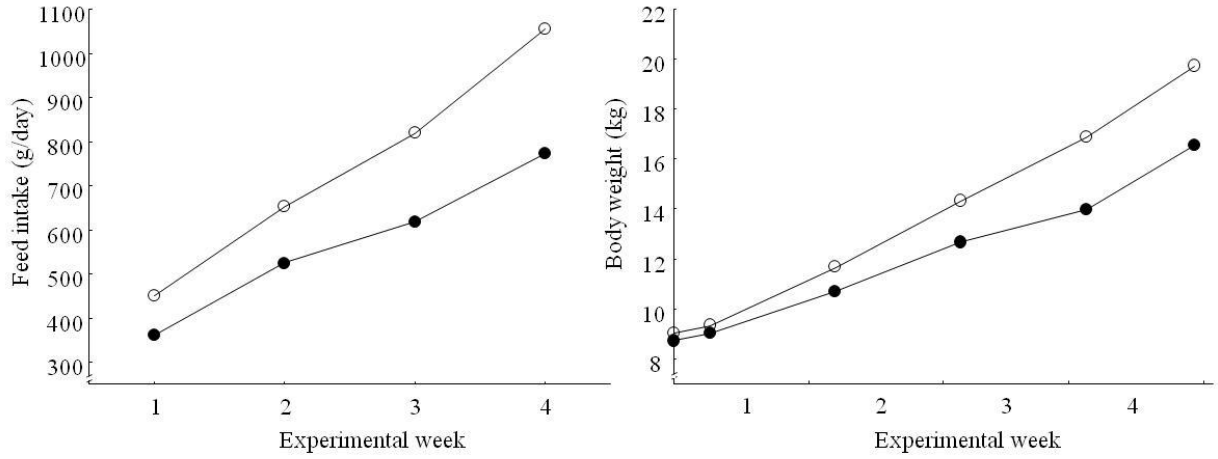


Figure 10. Progression of feed intake (left) and body weight (right) of piglets exposed to a diet containing 4.52 mg DON and 0.29 mg ZEN/kg (●) compared to the control group (○) (data from Table 3, **Paper I**; and unpublished data based on **Paper I**)

1.4 Visceral organs

Alterations in the physiological growth of visceral organs such as liver or spleen were also related to an exposure to *Fusarium* toxins. Increased relative liver and kidney weight were detected in a study by Jiang et al. (2012) who fed a diet containing 1.05 mg ZEN/kg to piglets for 22 days. Accordingly, a diet contaminated with 3.5 mg DON and 0.21 mg ZEN/kg resulted in increased liver weights of piglets after 95 days in a study by Bergsjø et al. (1993). In the present piglet experiments, dietary concentrations of 4.52 and 0.29 mg DON and ZEN/kg and 3.67 and 0.32 mg DON and ZEN/kg, respectively, were fed for 29 and 27 days. These toxin concentrations did not induce alterations in the relative weights of liver, kidney or spleen were observed (Table 4, **Paper I**; Table 4, **Paper III**). Furthermore, this lack of effects on the macroscopic level was accompanied by the absence of mycotoxin-related abnormalities on the histopathological level during the examination of the respective organ specimens. Döll et al. (2003) could neither find effects on the weight of organs of female piglets after a 35-day exposure to dietary concentrations of 3.9 and 0.42 mg DON and ZEN/kg, respectively. Observations from the present studies and the literature let suggest, that the presently investigated dietary mycotoxin concentrations might lead to measurable alterations in the physiological organ growth only at longer exposures. Consistently, higher concentrations as fed in the study by Jiang et al. (2012) caused effects already at relatively short exposure times. However, it cannot be excluded that age and sex of the experimental animals or the presence of other not analyzed mycotoxins may partly contribute to the observed effects.

2 Chemical decontamination of feed

Different chemical treatments of mycotoxin-contaminated feed were previously shown to be effective in reducing the concentration of toxins (Young et al., 1986; Bauer et al., 1987a; Rotter et al., 1995; Ragab et al., 2007), Table 3, **Background**). Young et al. (1986), for example, realized a 98% reduction of the DON-concentration in wheat in the presence of SBS already 26 years ago, while Bauer et al. (1987a) detoxified a ZEN-contaminated barley in the presence of MMA and $Ca(OH)_2$.

Some of the earlier investigated chemical treatments are already developed to the point to be applicable at the farm level without the technical equipment of a feed mill such as wet preservation. This is of central importance as about 47% of the cereal grains in Germany that are intended for animal feed are included into diets directly at the farm without being processed by commercial feed mills and this small scale feed formulation needs efficient decontamination solutions as well (Deutscher Verband Tiernahrung, 2009). Though, studies investigating the success of decontamination by means of wet preservation are of practical relevance with regard to a later application on farms, experiments are often very time- and labor-intensive. In contrast, results of hydrothermal treatment experiments may be obtained and evaluated relatively quick and provide general information on factors influencing the effectiveness of the investigated decontamination.

As an earlier experiment indicated that a combination of SBS and MMA was able to reduce the concentrations of DON and ZEN in an aqueous solution (Dänicke et al., 2012b) a first insight in the possibility of the simultaneous reduction of ZEN and DON in naturally contaminated cereals was aimed to be gained by the present investigations. For this purpose and for the above mentioned reasons, hydrothermal treatments were chosen in this thesis. During the treatments, knowledge on the optimal combination of the individual chemical compounds with regard to a simultaneous reduction of DON and ZEN and the time necessary for a maximum reduction of both toxins was aimed to be generated.

The results obtained in this thesis revealed that the combination of 5 g SBS, 10 g MMA and 20 g $Ca(OH)_2$ led to the highest reduction of the examined toxins (Figure 1, c, d, **Paper II**). Both ZEN and DON concentrations decreased rapidly during the first minutes of the treatment followed by a slower decrease until the end of the process. After 30 min only approximately 7 and 11% of the initial ZEN and DON concentrations, respectively, were detected in the samples.

2.1 Decontamination of DON by the formation of DONS

Measureable reductions in the concentration of DON that are achieved in experiments with SBS were shown to involve the formation of DONS, the sulfonated and non-toxic derivate of DON (Young, 1986a; Dänicke et al., 2005b). Different studies demonstrated that adverse effects of a DON exposure such as emesis or reduced feed intake were overcome when SBS-treated feed was used (Young et al., 1987; Dänicke et al., 2005b). Cell culture tests with different cell lines and porcine PBMC further proved the considerably lower cytotoxicity of DONS compared to DON (Beyer, 2009; Dänicke et al., 2010a). The decrease in toxicity is mediated by the addition of the sulfonate to the C10 and thereby loss of the 9,10-double bond (Figure 11). Though a second reaction at the keto group was also identified, this product was found to be formed only to a minor extent (Young, 1986a; Beyer et al., 2010). In these studies the epoxide-ring of the developed structures remained uninfluenced by SBS, while newer studies reveal that it may as well be involved in the reaction with SBS, as will be discussed later.

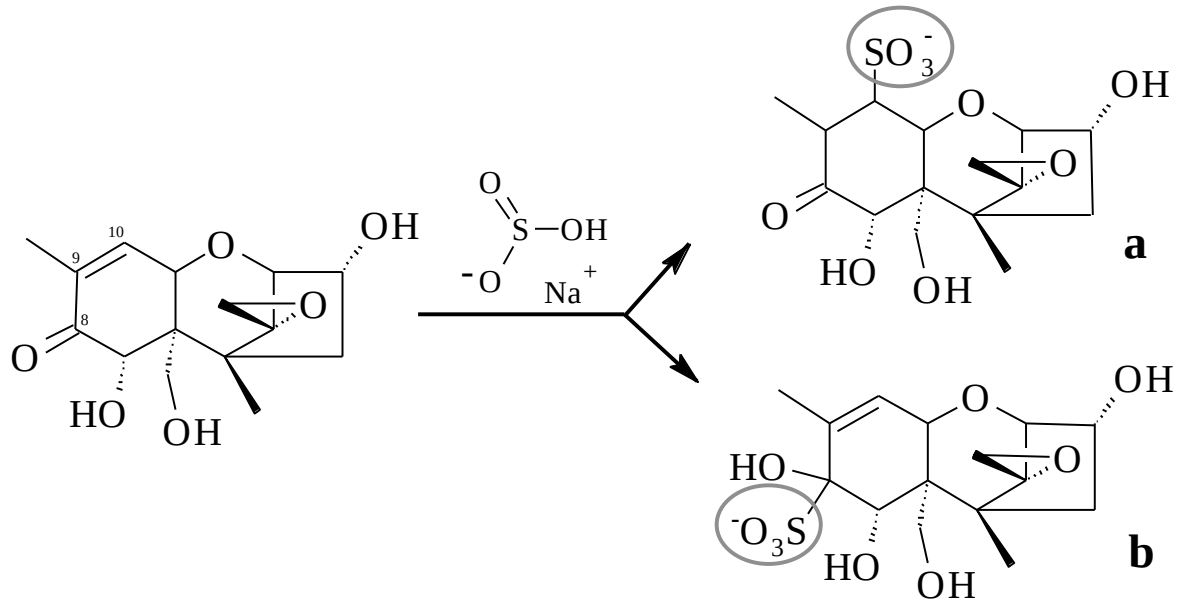


Figure 11. Reaction of SBS with DON results in two reaction products named as 10-DONS (a) and 8-DONS (b) (according to Beyer et al., 2010)

The extent of formation of DONS is also influenced by the ambient pH. The proportions of the three chemical forms in which metabisulfite exists in solution (SO_2, HSO_3^- and SO_3^{2-}) vary in dependence on pH (Rose, 1993) as shown in Figure 12. At neutral conditions the prevailing form is the bisulfite ion, while at acidic or alkaline pH SO_2 or sulfite ions

predominate, respectively. This is of special importance for the formation of DONS, as the predominance of sulfite in the form of SO_2 results in a loss of sulfur through volatilization and subsequent loss of equivalents that can react with DON (Dänicke et al., 2012b). Accordingly, the amount of SBS needed for a 50%-reduction of the initial DON concentration in triticale was 1.5 to 2-fold higher when an acidic compound was present during the preservation as compared to triticale with no additional supplement (Dänicke et al., 2009).

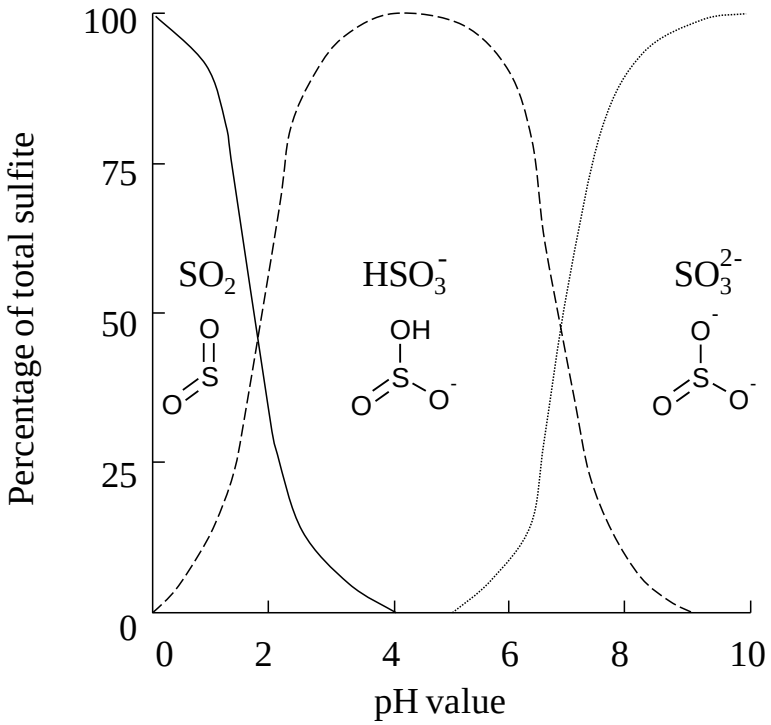
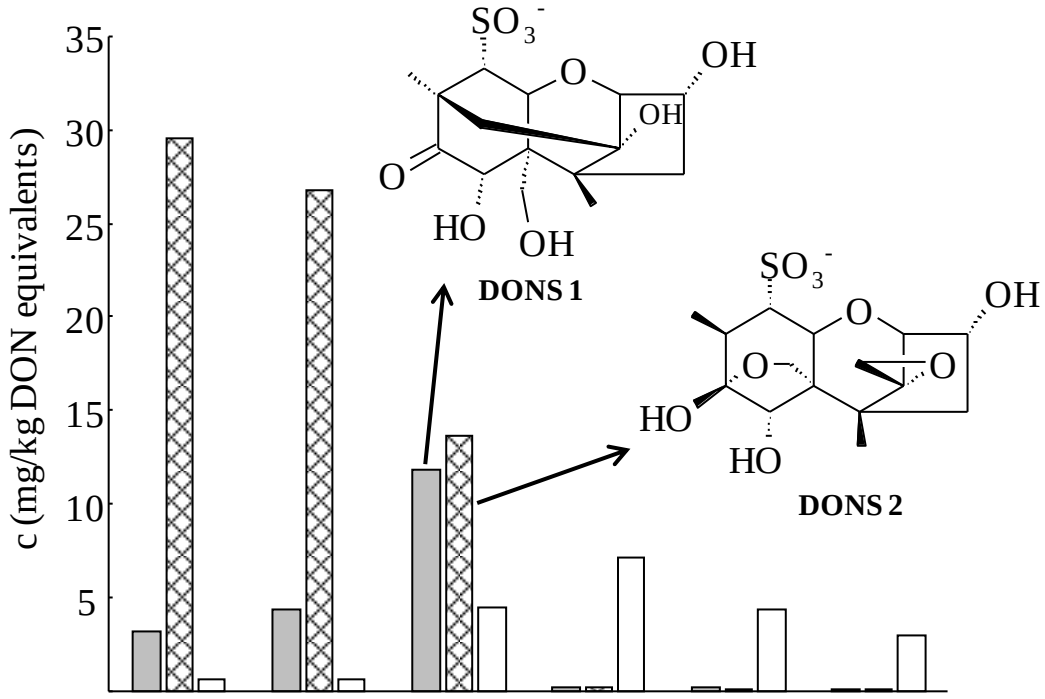


Figure 12. Proportions of SO_2, HSO_3^- and SO_3^{2-} chemical forms in which sulfite exists in solution, generated as a function of pH value (adapted from Rose, 1993)

DONS contents in variants that were hydrothermally treated in the presence of SBS and MMA without $Ca(OH)_2$ (variants I-III, **Paper II**)) revealed that most of the DON had reacted with SBS to form DONS (Figure 13). A detailed analysis of the reaction products, however, demonstrated that most of the formed DONS had a structure that differed from that discovered by Young (1986a). The structure named DONS 2 was characterized by the formation of a hemiketal (Schwartz et al., 2013). A second form of DONS (DONS 1, detected by the same authors), which lacked the epoxide group, occurred nearly to the same extent as DONS2 in variant III. Schwartz et al. (2013) proved that increasing amounts of these two compounds are formed of DON and SBS in buffer solution at increasing pH-values (pH ≥ 6).

Interestingly, in the present study in variants with $Ca(OH)_2$, which were presumably even more alkaline, none of the new or already known DONS forms was detectable.



Variant	I	II	III	IV	V	VI
SBS (mg/kg)	5	5	5	5	5	5
MMA (mg/kg)	2.5	5	10	2.5	5	10
$Ca(OH)_2$ (mg/kg)	0	0	0	20	20	20

Figure 13. Concentrations of DON (□) and new compounds DONS 1 (■) and DONS 2 (◪; Schwartz et al., 2013) in *Fusarium* toxin contaminated maize hydrothermally treated in the presence of different combinations of chemicals (unpublished data based on **Paper II**)

2.2 Degradation of DON via alkaline hydrolysis

DON is also known to undergo alkaline hydrolysis as shown by Young (1986a) and Bretz et al. (2006). After incubation of DON in alkaline solutions at approximately 70°C, 9 degradation products were detected and identified by gas chromatography and high-performance liquid chromatography coupled with mass spectrometry (Figure 14). IsoDON, norDON A, norDON B, norDON C were already previously characterized by Young (1986), while 9-hydroxymethyl DON lactone, norDON D, norDON E and norDON D were first described by Bretz et al. (2006)a few years ago. The authors also proved that the degradation products norDON A, B and C did not exert any cytotoxic effects in cell culture tests with

human kidney cells at concentrations more than 100-fold higher than the lowest concentration of DON at which a first decrease in the viability of the cells was measurable.

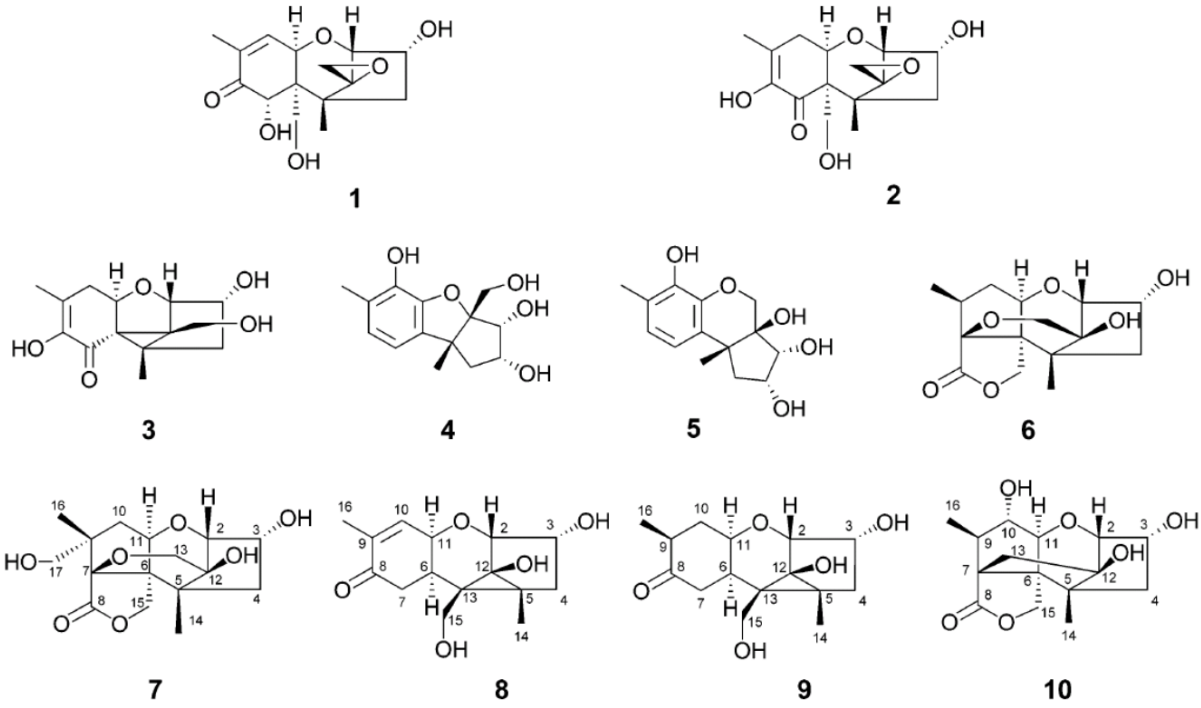


Figure 14. Structures of DON (1) and its known degradation products isoDON (2), norDON A (3), norDON B (4), nor DON C (5) and DON lactone (6) and the new compounds 9-hydroxymethyl DON lactone (7), norDON D (8), norDON E (9) and norDON F (10) (Bretz et al., 2006)

Considering the wide range of possible degradations products of DON in an alkaline ambient, it can be suggested that alkaline hydrolysis of DON in variants IV-VI might account for the loss of DON that was not detectable in any of the forms of DONS. However, comprehensive analyses of the maize samples are necessary to elucidate the nature of the reduction of DON in these variants in the course of the treatment. Furthermore, the formation of new products with regard to the presence of MMA cannot be excluded.

2.3 Degradation and decontamination of ZEN

ZEN concentrations decreased rapidly in the first treatment minutes when $Ca(OH)_2$ was present during the conditioning process, while in the absence of the alkaline no considerable reductions were measurable (Figure 1, b, d, **Paper II**). Accordingly, the presence of $Ca(OH)_2$ and MMA during a hydrothermal treatment of ZEN-contaminated barley led to a 85% reduction of the toxin concentration in a study by Bauer et al. (1987a). Although the authors

did not identify the products arising from this reaction, the oestrogenic activity of feed containing the treated barley was reduced in an experiment with female piglets. Gora et al. (2004) investigated the effect of an alkaline, sodium carbonate, on ZEN concentrations in solution at laboratory conditions and observed a complete disappearance of the toxin after 1.5 h. The analysis of the decontaminated solution revealed that the main products of the reaction included the open-chain form of ZEN and an equilibrium mixture of the decarboxylated and dehydrated form of the product (Urry et al., 1966) as illustrated in Figure 15.

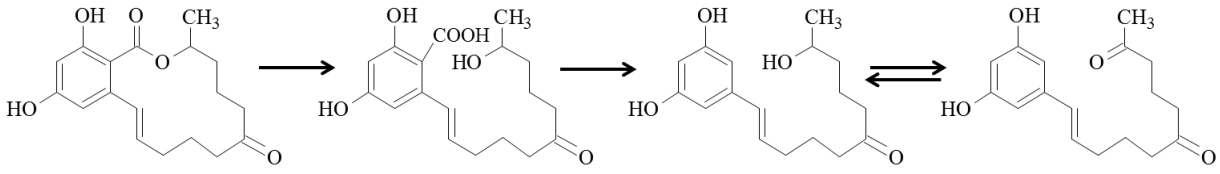


Figure 15. Reaction of ZEN in alkaline solution (according to Urry et al., 1966)

The opening of the lactone ring of ZEN, which is mediated by the presence of a strong alkaline compound might account for the reduction in detectable ZEN in this thesis. However, reactions with the chemicals that were present during the hydrothermal treatment are also possible. Due to its structure the ZEN molecule offers 4 reactive sites which might allow a potential reaction with SBS, $Ca(OH)_2$ and/or MMA (Figure 16).

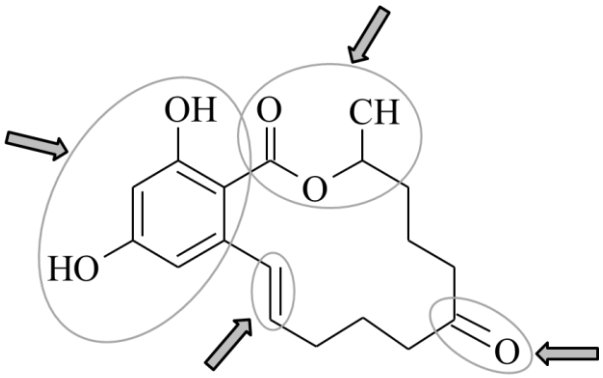


Figure 16. Potential reaction sites of ZEN

As the hydrothermal treatment was only a first approach for the simultaneous reduction of DON and ZEN, procedures of practical relevance that are applicable at a farm level should also be proved for their effectiveness in decontamination of these toxins when the presently investigated chemical mixture is used.

3 Evaluation of the decontamination success *in vivo*

During the hydrothermal treatment screening the maize of variant VI showed the best results with regard to a simultaneous reduction of ZEN and DON. However, it was questionable if the formed products would be stable under the conditions of the gastrointestinal tract when consumed by animals. High variations in pH in different segments of the intestine and the presence of a variety of digestive enzymes might lead to a reconversion into the parent compounds. In order to evaluate the success of the decontamination *in vivo* the treated maize (FUS+) was therefore included into a diet for female piglets. By the formulation of 3 additional diets containing untreated contaminated maize (FUS-) and control maize, either untreated (CON-) or treated (CON+), a complete 2 by 2-factorial design was realized, which also allowed the evaluation of possible unspecific effects of the treatment itself.

Although performance parameters of the piglets remained almost uninfluenced by the different diets, concentrations of the toxins in serum that were measured after 4 weeks of exposure allowed drawing clear conclusions. In the group receiving diet FUS- significantly higher concentrations of ZEN, DON and their metabolites were detectable in serum compared to the remaining groups (Figure 1, **Paper III**). The reduction of serum toxin residues in the group receiving diet FUS+ to the level of the control groups clearly demonstrated the success of the decontamination also *in vivo* (Dänicke et al., 2005b). Furthermore, the obtained results suggest that the products formed during the hydrothermal treatment were irreversibly altered with regard to a reconversion to the parent compounds by the influences emanating from the digestive tract. Though a lower toxicity of the products formed from DON in comparison to the parent compound could not definitely be confirmed by the present results, a ZEN detoxification is obvious. Figure 17 illustrates the effect of the different diets on the weight of uteri and morphology of the vulvae of the exposed piglets, which are considered as indicator organs for the expression of oestrogenic effects (Fink-Gremmels and Malekinejad, 2007). The treatment mediated loss of the oestrogenic potential of the maize in diet FUS+ is reflected in significantly reduced relative uterus weights in comparison to diet FUS-. Similarly, the swelling of the vulvae were reduced when the treated diet was fed.

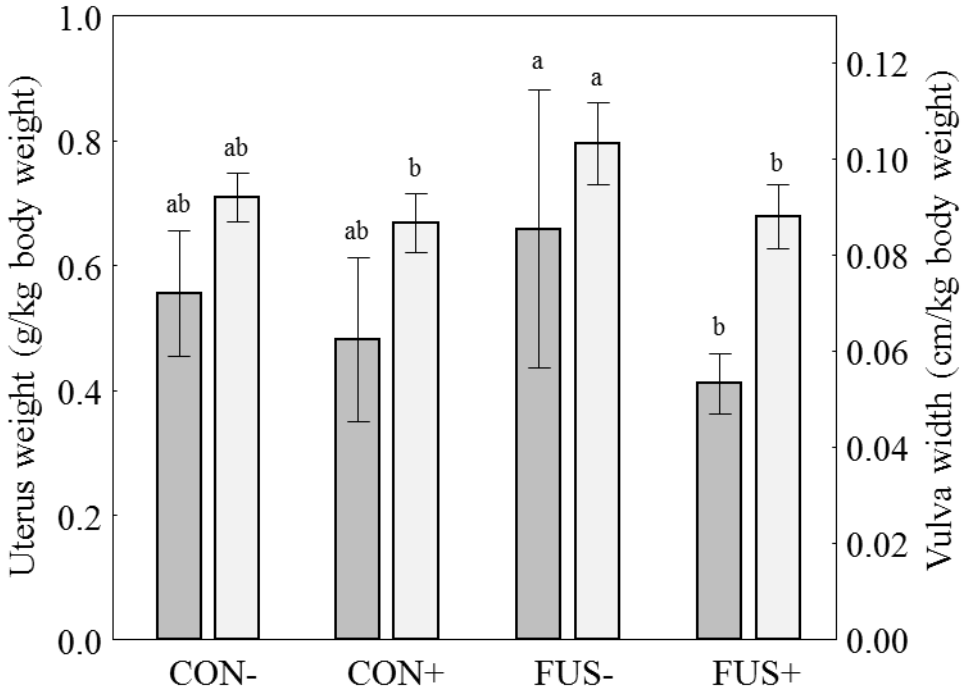


Figure 17. Relative uterus weight (■; n=5) and vulva width (□; n=25) of piglets exposed to *Fusarium* toxin-contaminated (FUS) or control maize (CON) either untreated (-) or hydrothermally treated in the presence of a combination of chemicals (+) (**Paper III**)

At first sight and with regard to the results obtained by serum biochemical and hematological analyses as well as proliferation tests of PBMC, it does neither seem that the presence of the chemicals in the diets led to any adverse effects in the exposed piglets nor that the decontamination resulted in new toxic products from DON or ZEN. However, further experiments are necessary to elucidate the structure and detailed characteristics of the formed products with regard to their harmlessness for animal health.

CONCLUSIONS

In piglets, the time of exposure to *Fusarium* toxin-contaminated diets mainly containing ZEN and DON plays an important role for the intensity of the observable effects related to animal health and performance.

In the present investigation, decreases in performance became obvious at dietary concentrations of 0.29 mg ZEN and 4.52 mg DON/kg diet already in the first week of mycotoxin exposure. Subsequently, differences in feed intake and body weight gain between the highest exposed and the other groups increased in the course of the trial.

Exposure to 0.29 mg ZEN and 4.52 mg DON/kg diet did not induce alterations in uterus or ovary weights nor in the distribution of follicular stages during the 29 days of exposure. However, group differences in the swelling of vulvae increased in the course of the experiment, indicating that even low dietary contamination may have an influence in dependence on the time of exposure. Future long-term studies are necessary to investigate the effects of low toxin doses on the later reproductive performance of female pigs.

Within the given experimental conditions, the applied toxin concentrations did not affect the physiological organ growth or the overall health of the piglets exposed to the contaminated diets during 4 weeks.

The combined application of sodium metabisulfite, methylamine and calcium hydroxide is a promising approach for the simultaneous reduction even of high concentrations of ZEN and DON in cereals. Further investigations are needed to define the products built during the decontamination process and to prove the efficiency of the chemicals also under the conditions of wet preservation.

The intake of chemically decontaminated maize was clearly demonstrated to result in reduced serum mycotoxin levels and a subsequent lower exposure of target tissues to these toxins. Signs of hyperoestrogenism were significantly reduced, while effects on performance data cannot be evaluated conclusively. The lack of alterations in serum biochemical parameters, physiological organ growth or the proliferation ability of PBMC indicated that health or performance of the piglets are likely to be not adversely affected by consumption of the treated diet.

SUMMARY

Zearalenone (ZEN) and deoxynivalenol (DON) are frequently occurring *Fusarium* mycotoxins in cereals all over the world. Agricultural strategies during growth of the plants may not completely prevent the fungal infection and formation of toxins as weather conditions during flowering have a great influence on these processes. Especially pigs are regarded sensitive to these toxins and react with feed intake depressions and reduced performance in the case of DON and with fertility disorders and hyperoestrogenism to exposure to the oestrogen-like ZEN. Guidance values for critical concentrations of these toxins in feed were therefore introduced by the European commission. However, data on the possible progression of effects during a continuous consumption of *Fusarium* toxin-contaminated diets are rare, as most investigations were carried out at the end of feeding trials.

The world-wide presence of the toxins in concentrations high enough to cause adverse effects especially in pigs is continuously promoting attempts to reduce toxin concentrations of contaminated feed. Earlier studies indicated that DON can be detoxified in the presence of sodium metabisulfite (SBS) by the formation of DON-sulfonate (DONS), while ZEN was observed to lose its oestrogenic properties after alkaline treatment. However, a simultaneous reduction of these two important compounds was only rarely the focus of investigation.

Thus, the present study aimed to (1) investigate the impact of the time of exposure to *Fusarium* toxin contaminated diets on the progression of effects in female piglets, (2) develop an approach for the simultaneous reduction of ZEN and DON in maize and (3) to evaluate the success of the decontamination *in vivo*.

In the first trial, 120 female weaned piglets were randomly assigned to feeding groups 1 -5 (n=24) receiving diets containing 0.01, 0.05, 0.08, 0.17, 0.29 mg ZEN/kg and 0.03, 0.59, 1.27, 2.01, 4.52 mg DON/kg feed, respectively. The contamination of the diets was realized by the graded inclusion of naturally *Fusarium* toxin-contaminated maize at the expense of control maize. During the trial, performance data and the individual vulva width with regard to the detection of hyperoestrogenism were regularly recorded. After periods of 1, 3, 8, 15, 22 and 29 days, each a total of 20 piglets (four piglets per group) were slaughtered for the dissection of organs and collection of blood for different analyses. The evaluation of performance data revealed that animals of group 5, receiving the highest toxin concentration in the diet, consumed about 20% less feed than animals of the other groups in the first week of the trial. Consequently, body weight gains were reduced which in turn resulted in a further restriction of the feed intake capacity and led to increasing differences in body weight and

feed intake between group 5 and the remaining groups in the course of the experiment. Relative visceral organ weights were not affected by the treatment but mostly showed minimal time dependant alterations. Uterus weights tended to be decreased in group 5 on day 15 of the experiment. Though not significant, signs of hyperoestrogenism such as swelling of the vulva became more pronounced with increasing time of exposure. The stimulation index of peripheral blood mononuclear cells as well as haematological and biochemical serum analyses were not affected by the treatment but revealed some time-dependent alterations which were partly attributed to the growth and physiological development of the animals.

For the evaluation of a simultaneous reduction of ZEN and DON, previously investigated chemicals such as SBS, methyl amine (MMA) and calcium hydroxide ($Ca(OH)_2$)were applied during hydrothermal treatments with naturally contaminated maize (45.99 mg DON and 3.46 mg ZEN/kg). In order to find the best dose and combination of the substances, 6 treatments were carried out: SBS was adjusted to 5 g/kg in all of the variants, while MMA was applied at 3 different doses (2.5, 5 and 10 g/kg maize) either with or without the addition of 20 g $Ca(OH)_2$/kg. Ground maize and chemicals were therefore filled into a laboratory conditioner and the temperature and moisture content were adjusted to 80°C and 17%, respectively, by the supply of saturated steam. Samples for mycotoxin analyses were frequently drawn during the 30-min conditioning process to observe the kinetics of the toxins during the treatment. In the 3 variants without $Ca(OH)_2$, DON-reductions reached approximately 82 % after 1-minute treatment and the toxin disappeared nearly completely after 10 minutes when 2.5 or 5 g MMA were applied. The disappearance of DON was accompanied by the formation of two newly described DONS-forms, DONS 1 and DONS 2, which were stated to be built in an alkaline ambient. ZEN-concentrations were only marginally affected in these variants. In the presence of $Ca(OH)_2$, reductions in DON-concentrations were lower but were enhanced by increasing doses of the alkaline MMA. As none of the DONS-form was detectable in these variants, the degradation of DON by alkaline hydrolysis was suggested. ZEN-concentrations were reduced by 72, 85 and 95 % within the first 5 minutes of the treatment in the presence of $Ca(OH)_2$ and MMA dosages of 2.5, 5 and 10 g/kg maize, respectively, indicating the dependence of the reduction on the presence of an alkaline. However, detailed analyses are necessary to elucidate the structure of the products formed during the treatment that account for the reductions in ZEN and DON concentrations.

To prove the effectiveness of the toxin reduction and acceptance of the treated maize as well as the effect of the treatment itself *in vivo* an experiment with piglets was carried out according to a 2 by 2-factorial design. Naturally *Fusarium* toxin-contaminated (FUS) and

control maize (CON) were included at a proportion of 10% into a diet either untreated (-) or hydrothermally treated in the presence of SBS, MMA and $Ca(OH)_2$ (+) according to the variant with the highest reduction of both ZEN and DON in the previous experiment. 100 female weaned piglets were assigned to one of the four treatment groups (n=25) CON-, CON+, FUS- and FUS+ with DON/ZEN-concentrations of 0.43/0.03, 0.04/0.0, 3.67/0.32 and 0.36/0.08 mg per kg diet, respectively. After a feeding period of 27 days, 5 animals per group were slaughtered for the dissection of organs and collection of blood for different analyses. Uterus weights were significantly reduced in group FUS+ compared to FUS- ($p=0.028$), while visceral organ weights were not influenced. Vulva width in relation to body weight was highest in group FUS- at the end of the trial, while hydrothermal treatment significantly reduced the parameter ($p<0.008$). Although performance parameters such as feed intake, live weight gain and feed-to-gain ratio remained unaffected by the treatments, serum toxin analyses clearly showed the success of the decontamination. The highest median concentrations of ZEN, DON and their main metabolites α-ZEL and de-DON (0.4/ 21/ 0.7 and 6 ng/ml, respectively) were detected in the serum of piglets that received diet FUS-, while median toxin concentrations in the other three treatment groups were significantly lower and often below the detection limit. The lack of effects on serum biochemical and haematological parameters as well as on the proliferation ability of peripheral blood mononuclear cells indicated the absence of undesired side-effects of the treated feed or the chemicals themselves on the health of the exposed piglets.

It can be concluded that the dose and time of exposure are essential factors influencing the intensity of the observable specific effects of an exposure to *Fusarium* toxin-contaminated diets. Therefore, long-term studies are needed to investigate the effect of exposure to low toxin doses especially with regard to the further reproductive performance of pigs. The combination of SBS, MMA and $Ca(OH)_2$ seems to be a promising approach for the simultaneous reduction of DON and ZEN in cereals and the alleviation of adverse effects *in vivo*. However, the treatment-mediated reduction of the toxins requires further investigations with regard to the characterisation of the formed products and their definite harmlessness for animal health. Further studies should also focus on the efficiency of the chemical mixture during wet preservation in order to develop the procedure for an application at the farm level.

ZUSAMMENFASSUNG

Zearalenon (ZEN) und Deoxynivalenol (DON) sind *Fusarium*-Mykotoxine, die häufig in Getreide auf der ganzen Welt zu finden sind. Präventive pflanzenbauliche Maßnahmen können die Infektion mit dem Pilz und die Bildung der Toxine jedoch nicht komplett verhindern, da die Wetterbedingungen während der Blütezeit der Pflanzen eine entscheidende Rolle spielen. Schweine gelten als besonders empfindlich bezüglich dieser Toxine und reagieren mit reduzierter Futteraufnahme und Leistung bei DON und mit Fruchtbarkeitstörungen und Hyperöstrogenismus bei einer Belastung mit dem östrogen-ähnlichen ZEN. Es wurden daher sogenannte Richtwerte für die Konzentration dieser Toxine im Futter von der Europäischen Kommission veröffentlicht, bei deren Unterschreitung nicht mit Beeinträchtigungen für Tiere zu rechnen ist. Daten zur möglichen Entwicklung von Effekten während einer kontinuierlichen Belastung mit *Fusarium*-Toxinen liegen jedoch nicht vor, da Untersuchungen meistens am Ende eines Versuchs stattfinden.

Das weltweite Vorkommen der Toxine in toxikologisch relevanten Konzentrationen besonders für Schweine treibt die Entwicklung von Maßnahmen zur Reduzierung dieser Komponenten im Futter an. Frühere Studien zeigten, dass DON in Gegenwart von Natriumbisulfit (SBS) durch die Bildung von DON-Sulfonat (DONS) entgiftet werden konnte, während ZEN sein östrogenes Potential nach Behandlung mit Alkalien verlor. Die gleichzeitige Reduktion dieser beiden wichtigen Substanzen wurde jedoch kaum fokussiert.

Die vorliegende Arbeit beschäftigt sich daher mit (1) dem Einfluss der Belastungszeit mit *Fusarium*-Toxin kontaminiertem Futter auf die Entwicklung von Effekten bei weiblichen Ferkeln, (2) der Entwicklung einer Methode für die gleichzeitige Reduktion von DON und ZEN in Mais sowie (3) der Überprüfung der Dekontamination *in vivo*.

Im ersten Versuch wurden 120 weibliche Absetzferkel auf 5 Gruppen aufgeteilt, die Rationen mit 0.01, 0.05, 0.08, 0.17, 0.29 mg ZEN/kg und 0.03, 0.59, 1.27, 2.01, 4.52 mg DON/kg erhielten. Die Kontamination der Rationen wurde über die schrittweise Einmischung von natürlich mit *Fusarium*-Toxin belasteten Mais anstelle von Kontrollmais erreicht. Während des Versuchs wurden Leistungsdaten erhoben und die Vulvabreite der Tiere im Hinblick auf die Detektion von Hyperöstrogenismus gemessen. Nach Fütterungszeiten von 1, 3, 8, 15, 22 und 29 Tagen wurden jeweils 4 Tiere pro Gruppe zur Untersuchung der Organe und Gewinnung von Blut für Analysen geschlachtet. Bei der Auswertung der Daten zeigte sich, dass die Tiere der Gruppe 5, mit der höchsten Toxinbelastung, in der ersten Versuchswoche rund 20% weniger Futter aufnahmen als die Tiere der anderen Gruppen. Dementsprechend waren auch die Zunahmen reduziert, was in einer weiteren Reduzierung der

Futteraufnahmekapazität resultierte und im Versuchsverlauf zu einer Vergrößerung der Differenzen in Futteraufnahme und Körpergewicht zwischen Gruppe 5 und den anderen Gruppen führte. Die Gewichte der viszeralen Organe waren durch die Fütterung nicht beeinflusst, während die Uterusgewichte am 15. Versuchstag in Gruppe 5 tendenziell erhöht waren. Obwohl nicht signifikant, wurde die Schwellung der Vulven, als Zeichen des Hyperöstrogenismus, mit steigender Expositionsdauer deutlicher. Die Proliferationsaktivität der peripheren mononukleären Blutzellen (PBMC) sowie hämatologische und klinisch-chemische Parameter blieben unbeeinflusst von der Versuchsfütterung zeigten aber einige zeitabhängige Veränderungen, die auf das Wachstum und die physiologische Entwicklung der Tiere zurückzuführen waren.

Zur Untersuchung der gleichzeitigen Reduktion von ZEN und DON wurden in früheren Experimenten eingesetzte Chemikalien wie SBS, Methylamin (MMA) und Calciumhydroxid ($Ca(OH)_2$) in hydrothermischen Behandlungen von natürlich-kontaminiertem Mais (45.99 mg DON und 3.46 mg ZEN/kg) verwendet. Zur Ermittlung der erfolgreichsten Dosis und Kombination der Substanzen wurden 6 Behandlungen getestet: SBS wurde zu 5 g/kg in allen Varianten eingesetzt, während MMA in 3 Dosen (2.5, 5 und 10 g/kg Mais) jeweils mit oder ohne die Zugabe von 20 g $Ca(OH)_2$/kg zum Einsatz kam. Gemahlener Mais und die Chemikalien wurden dazu in einen Konditionierer gefüllt und die Temperatur sowie der Feuchtegehalt auf 80°C bzw. 17% über die Zugabe von Wasserdampf eingestellt. Zur Ermittlung der Toxinkinetik im Verlauf der 30-minütigen Behandlung wurden bestimmten Zeitpunkten Proben zur Mykotoxinanalytik gezogen. In den 3 Varianten ohne $Ca(OH)_2$ erreichte die DON-Reduktion ca. 82% nach der ersten Minute und das Toxin verschwand nahezu vollständig nach 10 Minuten in den Varianten mit 2.5 und 5 g MMA. Das Verschwinden von DON resultierte in der Bildung 2 neu entdeckter Formen des DONS, DONS 1 und DONS 2, deren Bildung im Alkalischen gezeigt wurde. Die ZEN-Konzentration wurde dadurch indes kaum beeinflusst. In Gegenwart von $Ca(OH)_2$ fiel die Reduktion von DON zwar insgesamt schwächer aus, wurde aber durch steigende Dosen der Alkalie MMA verstärkt. Da in diesen Varianten keine Form des DONS zu messen war, wurde der Abbau von DON durch alkalische Hydrolyse angenommen. Die ZEN-Konzentration wurde in der Gegenwart von $Ca(OH)_2$ und 2.5, 5 und 10 g/kg um 72, 85 bzw. 95% innerhalb der ersten 5 Minuten reduziert und verdeutlichte damit die Abhängigkeit der Reaktion von alkalischen Komponenten.

Trotzdem sind noch Analysen notwendig, die die Strukturen der gebildeten Produkte charakterisieren, die für den Verlust an messbarem DON und ZEN verantwortlich sind.

Zur Überprüfung der Effektivität der Toxinreduktion und Akzeptanz des behandelten Futters sowie des Effekts der Behandlung selbst *in vivo* wurde ein Ferkelversuch nach einem 2-faktoriellen Design durchgeführt. Zu einem Anteil von 10% wurden folgende Varianten in eine Ration gemischt: natürlich mit *Fusarium*-Toxin kontaminierter (FUS) oder Kontrollmais (CON) entweder unbehandelt (-) oder hydrothermisch behandelt mit SBS, MMA und $Ca(OH)_2$ entsprechend der Variante mit der höchsten DON und ZEN-Reduktion im vorherigen Versuch. 100 weibliche Absetzferkel wurden auf eine der 4 Fütterungsgruppen (n=25) CON-, CON+, FUS- und FUS+ mit DON/ZEN-Konzentrationen von 0.43/0.03, 0.04/0.0, 3.67/0.32 bzw. 0.36/0.08 mg/kg Futter aufgeteilt. Nach 27 Fütterungstagen wurden 5 Tiere je Gruppe zur Untersuchung der Organe und Gewinnung von Blut für Analysen geschlachtet. Die Uterusgewichte in Gruppe FUS+ waren im Vergleich zu Gruppe FUS- signifikant geringer ($p=0.028$), während die viszeralen Organe unbeeinflusst blieben. Das Verhältnis von Vulvabreite zu Körpergewicht war in der Gruppe FUS- am Ende der Versuch am höchsten und wurde durch die Futterbehandlung signifikant verringert ($p<0.008$). Obwohl es keinen Effekt auf die Leistungsparameter gab, konnte der Erfolg der hydrothermischen Behandlung deutlich an den Toxinrückständen im Serum der Tiere beobachtet werden. Die höchsten medianen Konzentrationen von ZEN, DON und deren Hauptmetaboliten α-ZEL und de-DON (0.4/ 21/ 0.7 bzw. 6 ng/ml) wurden in der Fütterungsgruppe FUS- gemessen. Die medianen Konzentrationen dieser Substanzen in den anderen Gruppen lagen signifikant niedriger. Das Ausbleiben von Effekten auf die klinisch-chemischen und Blutparameter sowie auf die Aktivität der PBMC deutete auf die Abwesenheit unerwünschter Nebenwirkungen des behandelten Futters sowie der Chemikalien für die Gesundheit der exponierten Ferkel hin.

Insgesamt lässt sich schlussfolgern, dass die Dosis und Zeit der Exposition mit *Fusarium*-Toxin belastetem Futter entscheidenden Einfluss auf die Intensität der messbaren spezifischen Effekte hat. Daher sollten Langzeitstudien die Effekte von Belastungen mit geringen Toxinkonzentrationen besonders im Hinblick auf die Fruchtbarkeit von Schweinen weiter untersuchen. Die Kombination von SBS, MMA und $Ca(OH)_2$ erwies sich als vielversprechende Möglichkeit zur gleichzeitigen Reduktion von DON und ZEN in Getreide und zur Verminderung der Beeinträchtigungen durch diese Toxine *in vivo.* In weiteren Studien sollten die Struktur und Eigenschaften der gebildeten Produkte näher bestimmt und ihre Unbedenklichkeit für die Tiergesundheit geprüft werden. Außerdem sind Untersuchungen zur Effektivität der Chemikalienmischung bei der Getreide-Feuchtkonservierung sinnvoll.

REFERENCES (cited in Introduction, Background and General Discussion)

Abramson D, House JD, Nyachoti CM. 2005. Reduction of deoxynivalenol in barley by treatment with aqueous sodium carbonate and heat. Mycopathologia. 160: 297-301.

Alm H, Brussow KP, Torner H, Vanselow J, Tomek W, Danicke S, Tiemann U. 2006. Influence of *Fusarium*-toxin contaminated feed on initial quality and meiotic competence of gilt oocytes. Reproductive Toxicology. 22: 44-50.

Andretta I, Lovatto PA, Hauschild L, Dilkin P, Garcia GG, Lanferdini E, Cavazini NC, Mallmann CA. 2008. Feeding of pre-pubertal gilts with diets containing zearalenone. Arquivo Brasileiro De Medicina Veterinaria E Zootecnia. 60: 1227-1233.

Andretta I, Lovatto PA, Lanferdini E, Lehnen CR, Rossi CAR, Hauschild L, Fraga BN, Garcia GG, Mallmann CA. 2010. Feeding of pre-pubertal gilts with diets containing aflatoxins or zearalenona. Archivos de Zootecnia. 59: 123-130.

Babadoost M, Tyczkowska K, Hagler WM, Bowman DS, Huff WE. 1983. Zearalenone in sorghum fractionated by density segregation. Phytopathology. 73: 823-824.

Bauer J, Gareis M, Detzler W, Gedek B, Heinritzi K, Kabilka G. 1987a. Detoxification of mycotoxins in feedstuffs. Tieraerztliche Umschau. 42: 70-77.

Bauer J, Heinritzi K, Gareis M, Gedek B. 1987b. Changes in the genital tract of female pigs after feeding practive-relevant amounts of zearalenone. Tieraerztliche Praxis. 15: 33-36.

Bergsjø B, Langseth W, Nafstad I, Jansen JH, Larsen HJS. 1993. The effects of naturally deoxynivalenol-contaminated oats on the clinical condition, blood parameters, performance and carcass composition of growing pigs. Veterinary Research Communications. 17: 283-294.

Beyer M. 2009. Produktion, Analytik und Abbau bedeutender Trichothecen-Mykotoxine [Dissertation]. Wilhelms-Universität Münster.

Beyer M, Danicke S, Rohweder D, Humpf HU. 2010. Determination of deoxynivalenol-sulfonate (DONS) in cereals by hydrophilic interaction chromatography coupled to tandem mass spectrometry. Mycotoxin Research. 26: 109-117.

Biehl ML, Prelusky DB, Koritz GD, Hartin KE, Buck WB, Trenholm HL. 1993. Biliary-Excretion and Enterohepatic Cycling of Zearalenone in Immature Pigs. Toxicology and Applied Pharmacology. 121: 152-159.

Binder EM, Binder J, Ellend N, Schaffer E, Krska R, Braun R. 1998. Microbiological degradation of deoxynivalenol and 3-acetyl-deoxynivalenol. In: Mycotoxins and Phycotoxins - Developments in Chemistry, Toxicology and Food Safety. Proceedings of the IX International IUPAC Symposium on Mycotoxins and Phycotoxins, Rome. 279-285.

Biomin. 2012. BIOMIN Mycotoxin Survey - Global Mycotoxin Occurrence, Rose in 2012.

Boyens B. 2001. Zu den Effekten einer Zearalenonbelastung bei präpubertären Schweinen auf Uterusgewicht und renale Zearalenonausscheidung sowie protektiven Kapazitäten eines Zeolithzusatzes [Dissertation]. Tierärztliche Hochschule Hannover.

Bracarense A-PFL, Lucioli J, Grenier B, Pacheco GD, Moll W-D, Schatzmayr G, Oswald IP. 2012. Chronic ingestion of deoxynivalenol and fumonisin, alone or in interaction, induces morphological and immunological changes in the intestine of piglets. British Journal of Nutrition. 107: 1776-1786.

Bretz M, Beyer M, Cramer B, Knecht A, Humpf H-U. 2006. Thermal degradation of the *Fusarium* mycotoxin deoxynivalenol. Journal of Agricultural and Food Chemistry. 54: 6445-6451.

Chelkowski J. 1998. Distribution of *Fusarium* species and their mycotoxins in cereal grains. In: Sinha KK, Bhatnagar D, Editors. Mycotoxins in agriculture and food safety. Marcel Dekker Inc., New York-Basel-Hong Kong.

Chen F, Ma Y, Xue C, Ma J, Xie Q, Bi Y, Cao Y. 2008. The combination of deoxynivalenol and zearalenone at permitted feed concentrations causes serious physiological effects in young pigs. Journal of Veterinary Science. 9: 39-44.

Cheng Y-H, Weng C-F, Chen B-J, Chang M-H. 2006. Toxicity of different *Fusarium* mycotoxins on growth performance, immune responses and efficacy of a mycotoxin degrading enzyme in pigs. Animal Research. 55: 579-590.

Conte F, Baricco G. 2010. Managing mycotoxins in porcine nutrition. A practical approach. Suis. 69: 12-25.

Côté LM, Buck W, Jeffery E. 1987. Lack of hepatic microsomal metabolism of deoxynivalenol and its metabolite, DOM-1. Food and Chemical Toxicology. 25: 291-295.

D'Mello JPF, Placinta CM, Macdonald AMC. 1999. *Fusarium* mycotoxins: a review of global implications for animal health, welfare and productivity. Animal Feed Science and Technology. 80: 183-205.

Dänicke S, Beineke A, Goyarts T, Valenta H, Beyer M, Humpf HU. 2008a. Effects of a *Fusarium* toxin-contaminated triticale, either untreated or treated with sodium metabisulphite (Na2S2O5, SBS), on weaned piglets with a special focus on liver function as determined by the C-13-methacetin breath test. Archives of Animal Nutrition. 62: 263-286.

Dänicke S, Brezina U. 2013. Kinetics and metabolism of the *Fusarium* toxin deoxynivalenol in farm animals: Consequences for diagnosis of exposure and intoxication and carry over. Food and Chemical Toxicology. 60: 58-75.

Dänicke S, Brosig B, Klunker L, Kahlert S, Kluess J, Döll S, Valenta H, Rothkötter H-J. 2012a. Systemic and local effects of the *Fusarium* toxin deoxynivalenol (DON) are not alleviated by dietary supplementation of humic substances (HS). Food and Chemical Toxicology. 50: 979-988.

Dänicke S, Brüssow KP, Valenta H, Ueberschär KH, Tiemann U, Schollenberger M. 2005a. On the effects of graded levels of *Fusarium* toxin contaminated wheat in diets for gilts on feed intake, growth performance and metabolism of deoxynivalenol and zearalenone. Molecular Nutrition & Food Research. 49: 932-943.

Dänicke S, Döll S. 2010. A probiotic feed additive containing spores of *Bacillus subtilis* and *B. licheniformis* does not prevent absorption and toxic effects of the *Fusarium* toxin deoxynivalenol in piglets. Food and Chemical Toxicology. 48: 152-158.

Dänicke S, Döll S, T. G, Seeling K, Valenta H, Ueberschar KH. 2008b. Metabolism, kinetics and occurrence of deoxynivalenol (DON) and zearalenone (ZON) in physiological samples of pigs, ruminants and poultry. Oswald IP, Taranu I, Editors. Mycotoxins in Farm Animals. Transworld Research Network, Trivandrum, India. 235-273.

Dänicke S, Goyarts T, Döll S, Grove N, Spolders M, Flachowsky G. 2006. Effects of the *Fusarium* toxin deoxynivalenol on tissue protein synthesis in pigs. Toxicology Letters. 165: 297-311.

Dänicke S, Goyarts T, Valenta H. 2007. On the specific and unspecific effects of a polymeric glucomannan mycotoxin adsorbent on piglets when fed with uncontaminated or with *Fusarium* toxins contaminated diets. Archives of Animal Nutrition. 61: 266-275.

Dänicke S, Hegewald AK, Kahlert S, Kluess J, Rothkötter HJ, Breves G, Döll S. 2010a. Studies on the toxicity of deoxynivalenol (DON), sodium metabisulfite, DON-sulfonate (DONS) and de-epoxy-DON for porcine peripheral blood mononuclear cells and the Intestinal Porcine Epithelial Cell lines IPEC-1 and IPEC-J2, and on effects of DON and DONS on piglets. Food and Chemical Toxicology. 48: 2154-2162.

Dänicke S, Keese C, Goyarts T, Döll S. 2011. Effects of deoxynivalenol (DON) and related compounds on bovine peripheral blood mononuclear cells (PBMC) *in vitro* and *in vivo.* Mycotoxin Research. 27: 49-55.

Dänicke S, Kersten S, Valenta H, Breves G. 2012b. Inactivation of deoxynivalenol-contaminated cereal grains with sodium metabisulfite: a review of procedures and toxicological aspects. Mycotoxin Research. 28: 199-218.

Dänicke S, Pahlow G, Beyer M, Goyarts T, Breves G, Valenta H, Humpf HU. 2010b. Investigations on the kinetics of the concentration of deoxynivalenol (DON) and on spoilage by moulds and yeasts of wheat grain preserved with sodium metabisulfite (Na2S2O5, SBS) and propionic acid at various moisture contents. Archives of Animal Nutrition. 64: 190-203.

Dänicke S, Pahlow G, Goyarts T, Rohweder D, Wilkerling K, Breves G, Valenta H, Döll S. 2009. Effects of increasing concentrations of sodium metabisulphite (Na 2S 2O 5, SBS) on deoxynivalenol (DON) concentration and microbial spoilage of triticale kernels preserved without and with propionic acid at various moisture contents. Mycotoxin Research. 25: 215-223.

Dänicke S, Valenta H, Döll S. 2004a. On the toxicokinetics and the metabolism of deoxynivalenol (DON) in the pig. Archives of Animal Nutrition. 58: 169-180.

Dänicke S, Valenta H, Döll S, Ganter A, Flachowsky G. 2004b. On the effectiveness of a detoxifying agent in preventing fusario-toxicosis in fattening pigs. Animal Feed Science and Technology. 114: 141-157.

Dänicke S, Valenta H, Gareis M, Lucht HW, von Reichenbach H. 2005b. On the effects of a hydrothermal treatment of deoxynivalenol (DON)-contaminated wheat in the presence of sodium metabisulphite (Na2S2O5)on DON reduction and on piglet performance. Animal Feed Science and Technology. 118: 93-108.

Deutscher Verband Tiernahrung. 2009. Mischfutter-Tabellarium Ausgabe 2009.

Döll S, Dänicke S. 2011. The *Fusarium* toxins deoxynivalenol (DON) and zearalenone (ZON) in animal feeding. Preventive Veterinary Medicine. 102: 132-145.

Döll S, Dänicke S, Ueberschär KH, Valenta H, Schnurrbusch U, Ganter M, Klobasa F, Flachowsky G. 2003. Effects of graded levels of *Fusarium* toxin contaminated maize in diets for female weaned piglets. Archives of Animal Nutrition. 57: 311-334.

Döll S, Gericke S, Dänicke S, Raila J, Ueberschär KH, Valenta H, Schnurrbusch U, Schweigert FJ, Flachowsky G. 2005. The efficacy of a modified aluminosilicate as a

detoxifying agent in *Fusarium* toxin contaminated maize containing diets for piglets. Journal of Animal Physiology and Animal Nutrition. 89: 342-358.

Döll S, Goyarts T, Tiemann U, Dänicke S. 2007. Practically relevant concentrations of deoxynivalenol in diets for growing-finishing pigs offered as mash or pellets. Archives of Animal Nutrition. 61: 247-265.

EFSA. 2004a. Opinion of the Scientific Panel on contaminants in the food chain [CONTAM] related to Deoxynivalenol (DON) as undesirable substance in animal feed. EFSA Journal. 73: 1-42.

EFSA. 2004b. Opinion of the Scientific Panel on contaminants in the food chain [CONTAM] related to Zearalenone as undesirable substance in animal feed. EFSA Journal. 89: 1-35.

EFSA. 2011. Scientific Opinion on the risks for public health related to the presence of zearalenone in food. EFSA Journal. 9: 2197.

Ehrlich KC, Daigle KW. 1987. Protein-synthesis inhibition by 8-oxo-12,13-epoxytrichothecenes. Biochimica Et Biophysica Acta. 923: 206-213.

Fink-Gremmels J, Malekinejad H. 2007. Clinical effects and biochemical mechanisms associated with exposure to the mycoestrogen zearalenone. Animal Feed Science and Technology. 137: 326-341.

Forsyth DM, Yoshizawa T, Morooka N, Tuite J. 1977. Emetic and refusal activity of deoxynivalenol to swine. Applied and Environmental Microbiology. 34: 547-552.

Friend DW, Trenholm HL, Young JC, Thompson BK, Hartin KE. 1984. Effect of adding potential vomitoxin (deoxynivalenol) detoxificants or a F. graminearum-inoculated corn supplement to wheat diets fed to pigs. Canadian Journal of Animal Science. 64: 733-741.

Gajecka M, Rybarczyk L, Zwierzchowski W, Jakimiuk E, Zielonka L, Obremski K, Gajecki M. 2011. The effect of experimental, long-term exposure to low-dose zearalenone mycotoxicosis on the histological condition of ovaries in sexually immature gilts. Theriogenology. 75: 1085-1094.

Galtier P. 1999. Biotransformation and fate of mycotoxins. Journal of Toxicology-Toxin Reviews. 18: 295-312.

Gora M, Luczynski MK, Smoczynski L, Obremski K, Polak M, Swist M, Zlelonka L, Gajecki M. 2004. Modification of zearalenone structure in model and natural conditions. Polish Journal of Veterinary Sciences. 7: 181-185.

Goyarts T, Dänicke S. 2006. Bioavailability of the Fusarium toxin deoxynivalenol (DON) from naturally contaminated wheat for the pig. Toxicology Letters. 163: 171-182.

Goyarts T, Dänicke S, Rothkötter HJ, Spilke J, Tiemann U, Schollenberger M. 2005. On the effects of a chronic deoxynivalenol intoxication on performance, haematological and serum parameters of pigs when diets are offered either for ad libitum consumption or fed restrictively. Journal of Veterinary Medicine Series a-Physiology Pathology Clinical Medicine. 52: 305-314.

Goyarts T, Dänicke S, Tiemann U, Rothkötter HJ. 2006a. Effect of the Fusarium toxin deoxynivalenol (DON) on IgA, IgM and IgG concentrations and proliferation of porcine blood lymphocytes. Toxicology in Vitro. 20: 858-867.

Goyarts T, Grove N, Dänicke S. 2006b. Effects of the Fusarium toxin deoxynivalenol from naturally contaminated wheat given subchronically or as one single dose on the *in vivo* protein synthesis of peripheral blood lymphocytes and plasma proteins in the pig. Food and Chemical Toxicology. 44: 1953-1965.

Grenier B, Loureiro-Bracarense A-P, Lucioli J, Pacheco GD, Cossalter A-M, Moll W-D, Schatzmayr G, Oswald IP. 2011. Individual and combined effects of subclinical doses of deoxynivalenol and fumonisins in piglets. Molecular Nutrition & Food Research. 55: 761-771.

Gutzwiller A, Czegledi L, Stoll P, Bruckner L. 2007. Effects of Fusarium toxins on growth, humoral immune response and internal organs in weaner pigs, and the efficacy of apple pomace as an antidote. Journal of Animal Physiology and Animal Nutrition. 91: 432-438.

Hedman R, Pettersson H. 1997. Transformation of nivalenol by gastrointestinal microbes. Archives of Animal Nutrition. 50: 321-329.

House JD, Nyachoti CM, Abramson D. 2003. Deoxynivalenol removal from barley intended as swine feed through the use of an abrasive pearling procedure. Journal of Agricultural and Food Chemistry. 51: 5172-5.

Huff WE, Hagler WM. 1985. Density segregation of corn and wheat naturally contaminated with aflatoxin, deoxynivalenol and zearalenone. Journal of Food Protection. 48: 416-420.

Hussein HS, Brasel JM. 2001. Toxicity, metabolism, and impact of mycotoxins on humans and animals. Toxicology. 167: 101-134.

Jiang SZ, Yang ZB, Yang WR, Gao J, Liu FX, Broomhead J, Chi F. 2011. Effects of purified zearalenone on growth performance, organ size, serum metabolites, and oxidative stress in postweaning gilts. Journal of Animal Science. 89: 3008-3015.

Jiang SZ, Yang ZB, Yang WR, Wang SJ, Wang Y, Broomhead J, Johnston SL, Chi F. 2012. Effect on hepatonephric organs, serum metabolites and oxidative stress in post-weaning piglets fed purified zearalenone-contaminated diets with or without Calibrin-Z. Journal of Animal Physiology and Animal Nutrition. 96: 1147-1156.

Kiessling KH, Pettersson H, Sandholm K, Olsen M. 1984. Metabolism of aflatoxin, ochratoxin, zearalenone, and three trichothecenes by intact rumen fluid, rumen protozoa, and rumen bacteria. Applied and Environmental Microbiology. 47: 1070-1073.

King RR, McQueen RE, Levesque D, Greenhalgh R. 1984. Transformation of deoxynivalenol (vomitoxin) by rumen microorganisms. Journal of Agricultural and Food Chemistry. 32: 1181-1183.

Kolosova A, Stroka J. 2011. Substances for reduction of the contamination of feed by mycotoxins: a review. World Mycotoxin Journal. 4: 225-256.

Kuipergoodman T, Scott PM, Watanabe H. 1987. Risk assessment of the mycotoxin zearalenone. Regulatory Toxicology and Pharmacology. 7: 253-306.

Miller JD, Young JC, Sampson DR. 1985. Deoxynivalenol and Fusarium head blight resistance in spring cereals. Journal of Phytopathology. 113: 359-367.

Oldenburg E, Bramm A, Valenta H. 2007. Influence of nitrogen fertilization on deoxynivalenol contamination of winter wheat - experimental field trials and evaluation of analytical methods. Mycotoxin Research. 23: 7-12.

Oldenburg E, Valenta H, Sator C. 2000. Risikoabschätzung und Vermeidungsstrategien bei der Futtermittelerzeugung. In: Dänicke S, Oldenburg E, Editors. Risikofaktoren für die Fusariumtoxinbildung und Vermeidungsstrategien bei der Futtermittelerzeugung und Fütterung. Landbauforschung Völkenrode. SH 216: 5-34.

Oliver WT, Miles JR, Diaz DE, Dibner JJ, Rottinghaus GE, Harrell RJ. 2012. Zearalenone enhances reproductive tract development, but does not alter skeletal muscle signaling in prepubertal gilts. Animal Feed Science and Technology. 174: 79-85.

Olsen M, Malmlof K, Pettersson H, Sandholm K, Kiessling KH. 1985. Plasma and urinary levels of zearalenone and alpha-zearalenol in a prepubertal gilt fed zearalenone. Acta Pharmacologica Et Toxicologica. 56: 239-243.

Pei SC, Lee WJ, Kim SS. 2005. Reduction of deoxynivalenol in barley by chemical treatments and malting. Food Science and Biotechnology. 14: 823-827.

Pestka JJ. 2010. Deoxynivalenol: mechanisms of action, human exposure, and toxicological relevance. Archives of Toxicology. 84: 663-679.

Pestka JJ, Zhou HR, Moon Y, Chung YJ. 2004. Cellular and molecular mechanisms for immune modulation by deoxynivalenol and other trichothecenes: unraveling a paradox. Toxicology Letters. 153: 61-73.

Pinton P, Tsybulskyy D, Lucioli J, Laffitte J, Callu P, Lyazhri F, Grosjean F, Bracarense AP, Kolf-Clauw M, Oswald IP. 2012. Toxicity of Deoxynivalenol and Its Acetylated Derivatives on the Intestine: Differential Effects on Morphology, Barrier Function, Tight Junction Proteins, and Mitogen-Activated Protein Kinases. Toxicological Sciences. 130: 180-190.

Polak M, Gajecki M, Kulik T, Luczynski MK, Obremski K, Gora M, Gajecka M, Jakimiuk E, Zielonka L. 2009. The evaluation of the efficacy of sodium carbonate as zearalenone destructor in feeding stuffs. Polish Journal of Veterinary Sciences. 12: 103-111.

Prelusky DB, Trenholm HL. 1991. Tissue distribution of deoxynivalenol in swine dosed intravenously. Journal of Agricultural and Food Chemistry. 39: 748-751.

Ragab WSM, Drusch S, Schnieder F, Beyer M. 2007. Fate of deoxynivalenol in contaminated wheat grain during preparation of Egyptian 'balila'. International Journal of Food Sciences and Nutrition. 58: 169-177.

Ramos AJ, Hernandez E, PlaDelfina JM, Merino M. 1996. Intestinal absorption of zearalenone and *in vitro* study of non-nutritive sorbent materials. International Journal of Pharmaceutics. 128: 129-137.

Razzazi E, Böhm J, Kettner B, Hochsteiner W, Kahlbacher H. 2002. Metabolisierung von Deoxynivalenol beim Schwein: Bestimmung von DON und DOM-1 im Urin vom Schwein. Mycotoxin Research. 18: 84-88.

Rios G, Pinson-Gadais L, Abecassis J, Zakhia-Rozis N, Lullien-Pellerin V. 2009. Assessment of dehulling efficiency to reduce deoxynivalenol and Fusarium level in durum wheat grains. Journal of Cereal Science. 49: 387-392.

Rose AH. 1993. Sulphur dioxide and other preservatives. Journal of Wine Research. 4: 43-47.

Rotter BA, Prelusky DB, Pestka JJ. 1996. Toxicology of deoxynivalenol (vomitoxin). Journal of Toxicology and Environmental Health. 48: 1-34.

Rotter RG, Rotter BA, Thompson BK, Prelusky DB, Trenholm HL. 1995. Effectiveness of density segregation and sodium.carbonate treatment on the detoxification of Fusarium-

contaminated corn fed to growing pigs. Journal of the Science of Food and Agriculture. 68: 331-336.

Schaafsma AW, Fregeau-Reid J, Phibbs T. 2004. Distribution of deoxynivalenol in Gibberella-infected food-grade corn kernels. Canadian Journal of Plant Science. 84: 909-913.

Schwartz HE, Hametner C, Slavik V, Greitbauer O, Bichl G, Kunz-Vekiru E, Schatzmayr D, Berthiller F. 2013. Characterization of Three Deoxynivalenol Sulfonated Formed by Reaction of Deoxynivalenol with Sulfur Reagents. Journal of Agricultural and Food Chemistry. 61: 8941-8948.

Shier WT, Shier AC, Xie W, Mirocha CJ. 2001. Structure-activity relationships for human estrogenic activity in zearalenone mycotoxins. Toxicon. 39: 1435-1438.

Silverstone T, Goodall E. 1986. Serotoninergic mechanisms in human feeding: The pharmacological evidence. Appetite. 7, Supplement: 85-97.

Starke K, Hofmann F, Förstermann U, Kleinert H, Eichelbaum M, Schwab M, Schubert R, Dilger K. 2009. Allgemeine Pharmakologie und Toxikologie. In: Aktories K, Förstermann U, Hofmann F, Starke K, Editors. Allgemeine und spezielle Pharmakologie und Toxikologie, vol. 10. Auflage. Urban & Fisher, München, 1-104.

Steyn PS. 1998. The biosynthesis of mycotoxins. Revue De Medecine Veterinaire. 149: 469-478.

Stob M, Baldwin RS, Gillette KG, Andrews FN, Tuite J. 1962. Isolation of an anabolic, uterotrophic compound from corn infected with Gibberella zeae. Nature. 196: 1318-&.

Teixeira LC, Montiani-Ferreira F, Locatelli-Dittrich R, Santin E, Alberton GC. 2011. Effects of zearalenone in prepubertal gilts. Pesquisa Veterinaria Brasileira. 31: 656-662.

The Commission of the European Communities. 2006a. Commission Recommendation of 17 August 2006 on the presence of deoxynivalenol, zearalenone, ochratoxin A, T-2 and HT-2 and fumonisins in products intended for animal feeding. Official Journal of the European Union. L229: 7-9.

The Commission of the European Communities. 2006b. Verordnung (EG) Nr. 1881/2006 der Kommission vom 19. Dezember 2006 zur Festsetzung der Höchstgehalte für bestimmte Kontaminanten in Lebensmitteln. Amtsblatt der Europäischen Union. L 364/5.

Tiemann U, Brussow KP, Jonas L, Pohland R, Schneider F, Danicke S. 2006. Effects of diets with cereal grains contaminated by graded levels of two Fusarium toxins on selected

immunological and histological measurements in the spleen of gilts. Journal of Animal Science. 84: 236-245.

Tiemann U, Dänicke S. 2007. *In vivo* and *in vitro* effects of the mycotoxins zearalenone and deoxynivalenol on different non-reproductive and reproductive organs in female pigs: A review. Food Additives and Contaminants. 24: 306-314.

Trenholm HL, Charmley LL, Prelusky DB, Warner RM. 1991. 2 physical methods for the decontamination of 4 cereals contaminated with deoxynivalenol and zearalenone. Journal of Agricultural and Food Chemistry. 39: 356-360.

Ueno Y. 1984. Toxicological features of T-2 toxin and related trichothecenes. Fundamental and Applied Toxicology. 4: S124-S132.

Ueno Y, Sato N, Ishii K, Sakai K, Tsunoda H. 1973. Biological and chemical detection of trichothecene mycotoxins of Fusarium species. Applied microbiology. 25: 699-704.

Uhlig S, Jestoi M, Knutsen AK, Heier BT. 2006. Multiple regression analysis as a tool for the identification of relations between semi-quantitative LC-MS data and cytotoxicity of extracts of the fungus Fusarium avenaceum (syn. F-arthrosporioides). Toxicon. 48: 567-579.

Urry WH, Wehrmeister HL, Hodge EB, Hidy PH. 1966. The structure of zearalenone. Tetrahedron Letters. 7: 3109-3114.

Wang DF, Zhang NY, Peng YZ, Qi DS. 2012. Interaction of zearalenone and soybean isoflavone in diets on the growth performance, organ development and serum parameters in prepubertal gilts. Journal of Animal Physiology and Animal Nutrition. 96: 939-946.

Weidenbörner M. 2001. Encyclopedia of food mycotoxins. Springer, Berlin Heidelberg.

Young JC. 1986a. Formation of sodium bisulfite addition-products with trichothecenes and alkaline-hydrolysis of deoxynivalenol and its sulfonate. Journal of Agricultural and Food Chemistry. 34: 919-923.

Young JC. 1986b. Reduction in levels of deoxynivalenol in contaminated corn by chemical and physical treatment. Journal of Agricultural and Food Chemistry. 34: 465-467.

Young JC, Subryan LM, Potts D, McLaren ME, Gobran FH. 1986. Reduction in levels of deoxynivalenol in contaminated wheat by chemical and physical treatment. Journal of Agricultural and Food Chemistry. 34: 461-465.

Young JC, Trenholm HL, Friend DW, Prelusky DB. 1987. Detoxification of deoxynivalenol with sodium bisulfite and evaluation of the effects when pure mycotoxin or

contaminated corn was treated and given to pigs Journal of Agricultural and Food Chemistry. 35: 259-261.

Zöllner P, Jodlbauer J, Kleinova M, Kahlbacher H, Kuhn T, Hochsteiner W, Lindner W. 2002. Concentration levels of zearalenone and its metabolites in urine, muscle tissue, and liver samples of pigs fed with mycotoxin-contaminated oats. Journal of Agricultural and Food Chemistry. 50: 2494-2501.

Zwierzchowski W, Przybylowicz M, Obremski K, Zielonka L, Skorska-Wyszynska E, Gajecka M, Polak M, Jakimiuk E, Jana B, Rybarczyk L, Gajecki M. 2005. Level of zearalenone in blood serum and lesions in ovarian follicles of sexually immature gilts in the course of zearalenone micotoxicosis. Polish Journal of Veterinary Sciences. 8: 209-218.

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EIDESSTATTLICHE ERKLÄRUNG/ *DECLARATION UNDER OATH*

Ich erkläre an Eides statt, dass ich die Arbeit selbstständig und ohne fremde Hilfe verfasst, keine anderen als die von mir angegebenen Quellen und Hilfsmittel benutzt und die den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

I declare under penalty of perjury that this thesis is my own work entirely and has been written without any help from other people. I used only the sources mentioned and included all the citations correctly both in word or content.

Halle/Saale, 26.01.2014 Inga Rempe

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