Inactivation of deoxynivalenol (DON) in contaminated maize grain using sodium sulfite: implications on health and performance of pigs

Dissertation
zur Erlangung des
Doktorgrades der Naturwissenschaften (Dr. rer. nat.)

der
Naturwissenschaftlichen Fakultät III,
Institut für Agrar- und Ernährungswissenschaften,
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der
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Tag der Verteidigung: 12.06.2023

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I ABBREVIATIONS

Alb	Albumin
ANOVA	Analysis of variance
Abbr.	Abbreviations
AST	Aspartate amino-transferase
BW	Body weight
CON	Control maize
CD	Cluster of differentiation
Da	Dalton
DOM-1	De-epoxy-deoxynivalenol
DM	Dry matter
DON	Deoxynivalenol
DONS	DON-sulfonate
EC	European Commission
EFSA	European Food Safety Authority
FAO	Food and Agriculture Organization
FACS	Fluorescence activated cell sorting
FRAP	Ferric reducing ability of plasma
FUS	*Fusarium* toxin-contaminated maize
GfE	Gesellschaft für Ernährungsphysiologie
GLDH	Glutamate dehydrogenase
γ-GT	γ-Glutamyltransferase
HPLC	High-performance liquid chromatography
LC-MS/MS	Liquid chromatography-mass spectrometry
LOD	Limit of detection
LOQ	Limit of quantification
LPS	Lipopolysaccharide
LSmeans	Least square of means
mAb	Monoclonal antibodies
NaCl	Sodium chloride
NO	Nitric oxide

PBMC	Peripheral blood mononuclear cells
PMN	Polymorphonuclear cells
PSEM	Pooled standard error of means
ROS	Reactive oxygen species
SBS	Sodium metabisulfite
SoS	Sodium sulfite
TNF- α	Tumor necrosis factor-alpha
ZAN	Zearalanone
α-ZAL	Alpha-zearalanol
β-ZAL	Beta-zearalanol
α-ZEL	Alpha-zearalenol
β-ZEL	Beta-zearalenol
ZEN	Zearalenone

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1. INTRODUCTION

Mycotoxins are the secondary products of various fungi including field fungi (e.g. *Fusarium* and *Alternaria*) and storage fungi (e.g. *Aspergillus* and *Penicillium)* (Oldenburg et al., 2007). They are commonly found both in cereal grains, in animal feed and in human food products (Kabak et al., 2006). Depending on intake levels, toxin species as well as time of exposure, the ingestion of mycotoxin-contaminated food or feed may cause an array of teratogenic, cancerogenic, estrogenic, neurotoxic and immunologic effects in humans and animals (Kabak et al., 2006). About 25% of the world´s agricultural commodities are affected by mycotoxins resulting in significant economic losses (Rotter et al., 1996). Deoxynivalenol (DON) is the most common type B trichothecene mycotoxin produced by *Fusarium* species, mainly *Fusarium graminearum* (*Gibberella zea*) and *Fusarium culmorum* (JECFA, 2001). These pathogenic fungi induce *Fusarium* head blight in wheat and *Gibberella* ear rot in maize (Oldenburg et al., 2017). Temperature plays a crucial role for the geographical distribution of both toxins as *Fusarium graminearum* prefers to appear in warmer climates. The other critical factors for the toxin production are moisture at the time of flowering and the timing of rainfall (JECFA, 2001). Thus, such DON contaminations cannot be completely prevented. DON is found particularly in cereal crops such as wheat, maize and barley, which are normally used as the main source for human food and animal feed. Moreover, DON is quite stable and resistant to standard milling and processing procedures (Dänicke et al., 2000). Therefore, DON has been involved in incidents of mycotoxicoses in both human and farm animals (JECFA, 2001). In comparison to poultry and ruminants, pigs are the species responding most sensitively to DON with a striking reduction of feed intake (Prelusky, 1994; Rotter et al., 1996). With regard to animal health, DON is capable to inhibit the protein synthesis as well as to modulate the immune system depending on its dosage and duration of exposure (Tiemann and Dänicke 2007).

Based on the frequent occurrence of DON and the adverse effects on pigs, effective inactivation methods are needed in order to protect animal health and to ensure productivity.

2. BACKGROUND

Trichothecenes mycotoxins represent a large family of structurally related sesquiterpenoids which are produced by several species of *Fusarium*, *Cephalosporum*, *Myrothecium*, *Stachybotrys*, *Trichoderma*, *Trichothecium* and *Verticimonosporium* (Pestka, 2007; Döll and Dänicke 2011). Approximately 180 members of this family are identified. Generally, all trichothecenes are low molecular weight substances (≈ 200 – 500 Da) and commonly contain a 9, 10 double bond and 12, 13 epoxide ring but they also have various substituents as important components responsible for their potential toxicity (see Figure 1; Pestka, 2010). According to chemical properties, they can be divided into four types (A-D). *Fusarium* species are the main source for the formation of both type A and B trichothecenes. The main difference between these types is observed at the C-8 position: type A (e.g. T-2 toxin) is characterized by the presence of hydroxyl, while type B (e.g. DON) consists of a keto group (details see Figure 1 and Table1) (JECFA, 2001). As shown in the literature, type A and B trichothecenes are dominant and commonly distributed in cereals and feeds as natural pollutants, while type C and D trichothecenes rarely occur in food and feed (Krska et al., 2001).

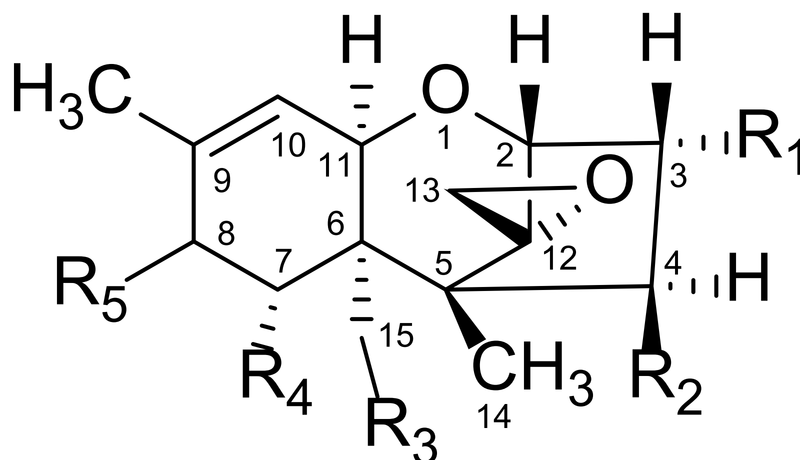

Figure 1. Basic structures of trichothecenes (according to Krska et al., 2001)

Table 1. Chemical structures of type A and B trichothecenes (adapted from He et al., 2010)

Mycotoxin	Abbr.	R1	R2	R3	R4	R5
Type A						
T-2 toxin	T-2	OH	OCOCH	OCOCH	H	- OCOCH2CH(CH3)$_2$
HT-2 toxin	HT-2	OH	OH	OCOCH	H	- OCOCH2CH(CH3)$_2$
Diacetoxyscirpenol	DAS	OH	OCOCH	OCOCH	H	H
Type B						

Mycotoxin	Abbr.	R1	R2	R3	R4	R5
Deoxynivalenol	DON	OH	H	OH	OH	= O
3-acetyldeoxynivalenol	3-ADON	OCOCH	H	OH	OH	= O
15-acetyldeoxynivalenol	15-ADON	OH	H	OCOCH	OH	= O
Nivalenol	NIV	OH	OH	OH	OH	= O
Fusarenon X	FX	OH	OCOCH	OH	OH	= O

2.1. Description of DON as hazard

DON belongs to the B-trichothecenes mycotoxins which are mainly produced from *Fusarium* species. Recently, a mycotoxin survey reported that approximately 67% of samples of European cereals and feed ingredients were contaminated with DON (Biomin, 2012). Due to its widespread diffusion in cereals especially in the temperate climate regions, DON is one of the most important mycotoxins and causes loss of performance and also animal diseases.

2.2. Chemical and physical characteristics

DON´s chemical structure is described as 12, 13-epoxy-3α, 7α, 15-trihydroxy-trichothec-9-ene-8-one ($C_{15}H_{20}O_6$; molecular weight: 296.32; Figure 2; EFSA, 2004). DON crystallizes as colorless needles and is stable at high temperatures (melting point at 151-153°C). Additionally, it is soluble in water and in some polar solvents such as aqueous methanol, acetonitrile and ethyl acetate (EFSA, 2004).

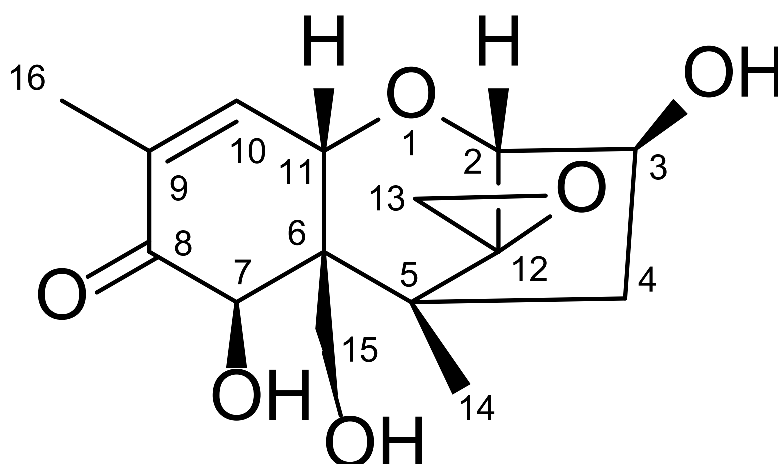

Figure 2. Chemical structure of deoxynivalenol (according to Rotter et al., 1996)

2.3. Mode of action

Inhibition of protein synthesis is well known to be the main effect of trichothecenes including DON at cellular level. Trichothecenes can bind to the 60S subunit of eukaryotic ribosomes and thus inhibit the peptidyl transferase function (Feinberg and McLaughlin, 1989). It is considered that the 9, 10-double bond and the 12, 13-epoxide ring are responsible for its toxicity (Rotter et al., 1996). Moreover, based on their substituents trichothecenes are able to prevent either the initiation or the elongation and termination step of the protein synthesis (Figure 3; Dänicke et al., 2000). Among them, DON is classified as elongation inhibitor, whereas most trichothecenes are predominantly initiation inhibitors (Ehrlich and Daigle, 1987).

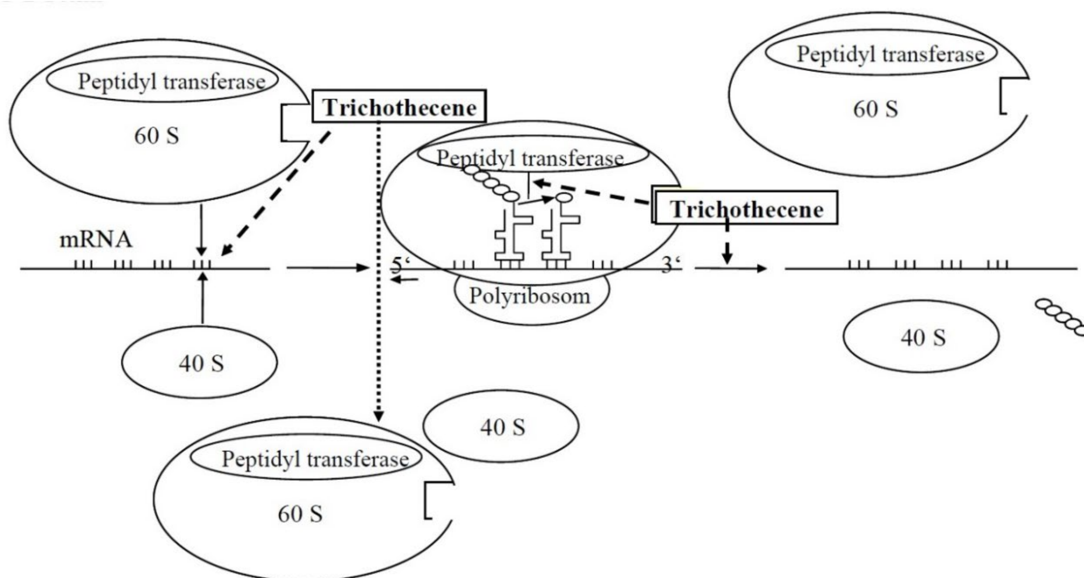

Figure 3. Mechanism of protein synthesis inhibition by trichothecenes (adapted from Dänicke et al., 2000). ---> = inhibitory effects

On the other hand, binding to the ribosome has been suggested to induce a ribotoxic stress response. DON binds to ribosomes which transduce a signal for RNA-activated protein kinase (PKR) and hematopoietic cell kinase (HCK). In turn, these kinases possibly trigger the mitogen-activated protein kinase (MAPK) activation. Subsequently, the activated MAPK interacts with the transcription factors (TFs) and thus leads to an upregulation of proinflammatory genes, activating apoptosis and resulting in immuno-suppression (Figure 4; Pestka, 2007). In particular, leukocytes as the functional repertoire of the immune system appear susceptible to

DON effects. Various studies with mice suggested that DON can induce either immune-stimulation or immune-suppression in a dose- and time-dependent manner (Pestka et al., 2004).

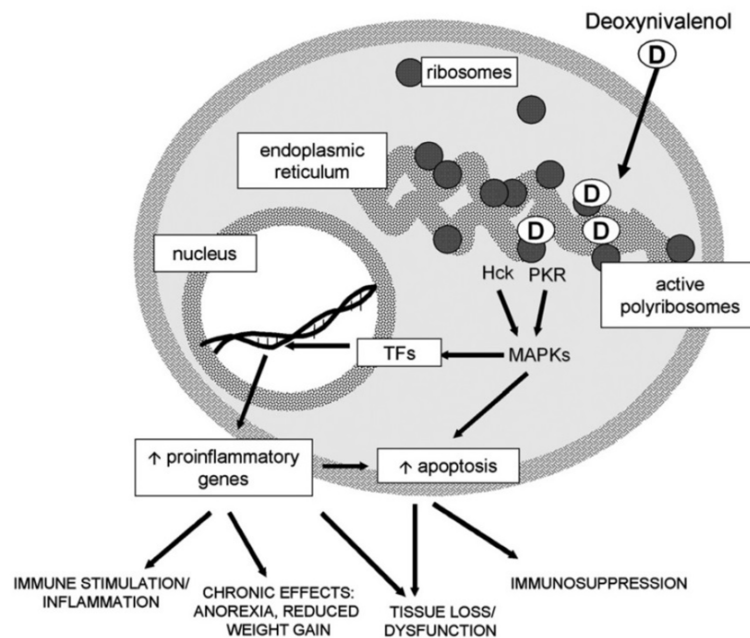

Figure 4. Molecular mechanism of deoxynivalenol (according to Pestka et al., 2007)

In pigs, results from different studies pointed out that the most striking effects of DON lead to reduced feed intake, decreased body weight gain and so far, resulting in a loss in performance. Accordingly, Dänicke et al. (2008) demonstrated that the voluntary feed intake of pigs fed DON contaminated feed decreased by 5.4% per 1 mg DON/kg feed compared to the respective control groups irrespective of other experimental factors (e.g. varying exposure time, age and breed of pigs). DON intoxication was first discovered in a maize batch infected with *Fusarium* in the 1970s, which caused emesis (vomitoxin) in pigs (Rotter et al., 1996). Regarding to emetic response, it is suggested that the serotoninergic system plays a critical role, because DON increases the serotonin concentration (5HT, 5-hydroxytryptamine) in the brain of rats as well as its metabolites, 5-hydroxyindoleacetic acid (5HIAA) in the cerebral spinal fluid of pigs (Rotter et al., 1996). However, literature reviews on these metabolites also reported contradictory results in dependence on DON exposure (Dänicke et al., 2012b; Etienne and Wache, 2008). Another possible hypothesis related to the interference of DON with the immune system. Recently, more evidence showed that DON refers to the upregulation of the pro-inflammatory cytokines like IL-1 and TNF-α. These cytokines also represent anorectic factors;

therefore, it could be suggested that DON impairs the feed intake via modulation of expression of pro-inflammatory cytokines (Dänicke et al., 2012b; EFSA, 2004).

2.4. Toxicokinetics and metabolism

DON toxicity was investigated in different experimental animals and the sensitivity towards DON varies from species to species and ranks as follow (from the most to the least sensitivity): swine > mice > rats > poultry ≈ ruminants (Prelusky, 1994). This variation in susceptibility may be explained by differences in absorption, distribution, metabolism and elimination of DON (Pestka and Smolinski, 2005).

In pigs, DON absorption from the gastrointestinal tract is very rapid and could be observed in plasma within 15-30 min after oral dosing (Prelusky et al., 1988). DON is absorbed up to 82% into the systemic circulation based on the dose urinary recovery (Rotter et al., 1996). However, the distribution of DON in pigs also differs from other species. Only a small amount of the dose is detectable in the blood, although this toxin is extensively absorbed. Despite the extensive distribution in various organs, the effect is very transient (Pestka and Smolinski, 2005; Rotter et al., 1996). The plasma elimination half-life of DON varies between 1.2 and 9.95 h depending on the experimental designs as reviewed by Dänicke and Brezina (2013). The main route of DON excretion occurs via urine with up to 43.2% of the ingested DON whereas it amounts only up to 3% with faeces (Dänicke and Brezina, 2013; Goyarts and Dänicke, 2005b). In principle, the ingested DON can either be de-epoxidated by anaerobic bacteria or conjugated to glucuronid acid by mammalian UDP glucuronosyltransferases (UGTs) (Dänicke and Brezina, 2013). De-epoxy DON (DOM-1) is the primary metabolite of DON and formed by ruminal or intestinal microbiota rather than by liver or other organs (Pestka and Smolinski, 2005). De-epoxy DON is known as the detoxified metabolite of DON (Figure 5; King et al., 1984). In pigs, the microbes in the digestive tract are also capable of forming de-epoxy-DON whereby capacity increases in the proximo-distal direction (Dänicke et al., 2004a; Eriksen et al., 2002). However, Dänicke et al. (2004a) suggested that the high hindgut capacity for the de-epoxy DON formation is obviously not relevant for the pig as the majority of unmetabolized DON is already absorbed from the proximal small intestine. Conjugation of DON with glucuronic acid is another metabolism product of DON and is less toxic than DON. This metabolite is formed by the liver and is detectable in blood and urine (Dänicke and Brezina 2013).

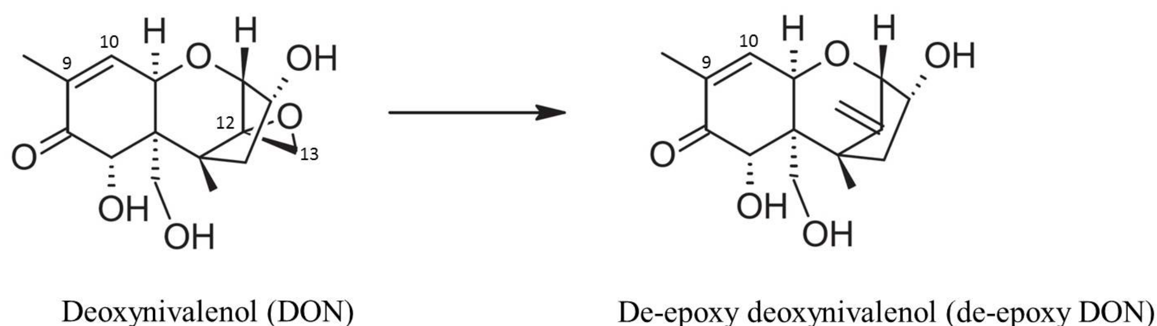

Figure 5. Chemical structure of DON and its metabolite de-epoxy DON (King et al., 1984)

2.5. Relevance of DON in animal nutrition

2.5.1. Animal species-related sensitivity to DON and guidance values for critical levels in feed

As already mentioned above the sensitivity towards DON is considered to generally decrease in the order pigs >> poultry ~ ruminants (Prelusky, 1994). 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 (DOM-1). The rumen thus enables the inactivation 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).

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 2.

Table 2. Guidance values for DON in animal feed

Mycotoxin	Animal feed	Guidance value (mg/kg)
DON	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

2.5.2. Occurrence of DON in feed in relation to critical levels

In general, DON is less toxic than other trichothecenes; however, extremely high DON doses can induce shock-like death of mice when orally administered with 46 mg DON/kg BW (Pestka, 2007). Moreover, DON is commonly the most relevant mycotoxin in both human and farm animal products, because of its frequent occurrence in toxicological relevant levels. Besides, DON is rarely degraded during the milling procedure (Rotter et al., 1996). Recently, there has been found more evidence for the effect of thermal food processing on DON degradation which however depends on different factors such as temperature, baking time, moisture content and compression rate during extrusion as well as pH conditions (Wu et al. 2017). Furthermore, this process is not considered as inactivation process.

Poorer quality grain is frequently used for animal feed instead for food products leading to higher DON exposure of livestock (EFSA, 2004). Besides, DON is found at higher concentrations in by-products (e.g. bran), which are often used as animal feed (EFSA, 2004). Among the livestock, the proportion of cereal grains in pig and poultry diets is normally higher than that used for ruminants. The dietary exposure to DON for both animal species is shown in Table 3. In pigs, the most sensitive species to DON exposure, the chronic DON exposure ranges from 10.2 to 15.5 µ/kg BW per day and the acute exposure from 32.8 to 66.3 µg/kg BW per day. For poultry, these DON exposure levels are much higher for both chronic and acute exposition and range between 15.5 and 43.5 µg/kg BW per day and 132.3 and 137.9 µg/kg BW per day, respectively (EFSA 2013). It is considered that ingestion of highly DON contaminated feed can induce vomiting. However, the major effects of dietary exposure of pig and poultry to

DON are feed intake reduction, lowered growth performance and altered nutritional efficiency (EFSA 2013).

Table 3. Dietary exposure to DON expressed in µg/kg BW per day (adapted from EFSA 2013)

Animals' species	Chronic exposure in µg/kg BW per day	Acute exposure in µg/kg BW per day
Pig	10.2 – 15.5	32.7 – 66.3
Poultry	15.5 – 43.5	132.3 – 137.9

2.6. Prevention strategies

In order to prevent the occurrence of mycotoxins including DON, a wide variety of treatments has been suggested. In the first line, the control of mold development on the plants or plant products is one of the main targets. Selection of good crop varieties which can resist mold attacks or choosing the right sowing date are effective strategies. Furthermore, pre-and post-harvests also represent a possible preventive approach (Jard et al. 2011).

2.6.1. Pre-harvest prevention

Generally, using the adequate cultivation techniques has been shown to be an effective strategy to prevent the fungal contamination and the inoculum in the field. For example, the agricultural waste of the previous crops should be removed. In addition, ploughing and crop rotation are effective to decrease mold development (Kabak et al. 2006). It has been reported that insects can act as an influential factor due to their feature of attacking the external husk of kernels and thus enhance the entrance and colonization of mycotoxin-producing toxins. Therefore, the application of insecticides can avoid these attacks (Jard et al. 2011). Currently, biocontrol techniques based on microorganisms are also developed. It has been reported that using non-aflatoxigenic strains in the soil treatment obviously decreased the pre-harvest aflatoxin contamination (Dorne and Cole 2002).

2.6.2. Post-harvest prevention

In order to prevent cereals from fungal contamination during storage, the storage conditions should be managed. Moisture has to be decreased to less than 15% in order to avoid the mold development. Moreover, a low oxygen concentration (< 1%) and a higher carbon dioxide concentration have positive effects. Furthermore, mixing grains and a long-time storage should be prevented (Jard et al. 2011). It is obvious that contaminated cereals have different colors

compared to safe cereals. Therefore, sorting of these grains is suggested. Other methods such as washing can reduce the mycotoxin levels, too. For example, washing the wheat in spaghetti production can remove 23% of DON (**Visconti et al**. 2004).

2.7. Inactivation strategies

2.7.1. General demand and legal background

Although various treatments are reported to decrease specific mycotoxin contamination in crops, the complete elimination of this mycotoxin contamination cannot be achieved. Therefore, inactivation processes are needed before the use of the final products.

There are some criteria which have to be fulfilled for an acceptable inactivation strategy (Bata and Lasztity, 1999; Döll and Dänicke, 2011):

- ✓ Destroy, inactivate or remove the mycotoxin due to transformation to non-toxic compounds
- ✓ Not produce or leave toxic, carcinogenic or mutagenic residues in final products or in food products
- ✓ Retain the nutritive value and acceptability of the product as well as not substantially alter important technological properties
- ✓ Destroy fungal spores and mycelia which could, under favorable conditions proliferate and form new toxins
- ✓ The process should be readily available, easily utilized and inexpensive

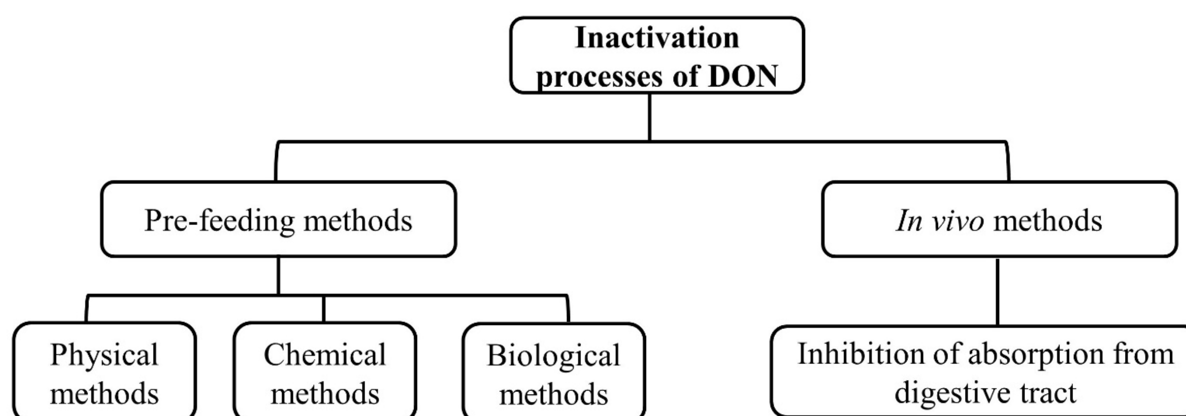

Figure 6. Inactivation strategies for DON in animal feed (according to Awad et al., 2010)

Moreover, inactivation processes can be further divided into two main strategies: pre-feeding methods and the *in vivo* approach (Figure 6). Both have widely been investigated and proposed

as potential inactivation strategies and can be distinguished from each other as described in the following.

2.7.2. Pre-feeding methods

Pre-feeding strategies consist of physical, chemical and biological methods, which are clearly defined and must fulfill the criteria of EU commission regulation for a proper strategy (The Commission of the European Communities, 2015).

2.7.2.1. Physical methods

Diverse physical methods have been applied for mycotoxin decontamination (Kabak et al., 2006). Like with other mycotoxins, the most widespread location of DON is in the outer husk layer (Aakre et al., 2005), thus only few physical approaches are working well to remove a part of DON from contaminated cereals. Trenholm (1991) provided the evidences that de-hulling decreased the DON content in contaminated-barley up to 88% whereas sieving reduced approximately 67% DON concentration in contaminated ground barley. In addition, density segregation also offers an efficient process with 79% and 96% DON reduction of contaminated-corn and wheat, respectively (Huff and Hagler, 1985). On the other hand, physical treatments remove valuable fractions of the grain leading to a loss of nutrients (Awad et al., 2010). For the mentioned reasons, these disadvantages prevent the success of physical methods.

2.7.2.2. Biological methods

Biological and chemical decontamination of mycotoxins may, however, overcome the named disadvantages because they are capable to transform the mycotoxin molecular structures into compounds with reduced toxicity. Biological attempts are known as biodegradation or biotransformation and have already been studied. Principally, some microorganisms are able to degrade mycotoxins into less or non-toxic compounds (Awad et al., 2010; He et al., 2010). Treatment with soil bacterium Agrobacterium-Rhizobium, strain E3-39, can transform DON into 3-keto-DON under aerobic conditions (Shima et al., 1997). Another effective microorganism, the Eubacterium sp., strain BBSH 797, which was isolated from bovine rumen fluid, has been currently developed into a commercial product for detoxifying trichothecenes in animal feed (He et al., 2010).

2.7.2.3. Chemical methods

The most effective chemical method is the use of sulfite salts, especially sodium bisulfite and sodium metabisulfite (SBS) (Dänicke et al., 2012b; Dänicke et al., 2005; Young, 1986). In earlier studies, Young (1986) treated aqueous sodium bisulfite in DON-contaminated wheat for 24 h and showed that the DON content obviously was decreased up to 94%. Hydrothermal treatment with SBS significantly reduced the DON concentration in contaminated ground wheat and ground maize (Dänicke et al., 2005; Rempe et al., 2013a). Likewise, wet preservation of DON-contaminated triticale and wheat with SBS showed a high reduction of DON up to 99% (Dänicke et al., 2008; Dänicke et al., 2010b). Moreover, when pigs were fed SBS treated DON-contaminated diets the level of their feed intake and live weight gain indicated an effective decontamination method in comparison with the control groups (Dänicke et al., 2008; Rempe et al., 2013b). However, results from feeding trials revealed several unspecific effects of SBS treatment independent of the initial DON contaminations. These effects include a stimulation of liver functions, increased plasma protein concentrations, reduced stimulation ability of peripheral blood mononuclear cells (PBMC) and a slight increase in proportion of sclerotic glomeruli in pigs fed SBS treated diets, irrespective of DON (Dänicke et al., 2012b). Recently, treatment with aqueous sodium sulfite (SoS) clearly reduced the DON content in the Fusarium-contaminated maize and may offer an alternative suitable tool for the DON reduction (Paulick et al., 2015a, b).

In addition to major application of SBS and SoS for DON reduction through DONS formation, these sulfiting agents are already used as food preservatives (Nair 2003). The termed sulfites also known as total sulfites including sulfur dioxide (SO_2), bisulfite (HSO_3^-) and sulfite (SO_3^{2-}) are easily released from sulfiting agents such as SBS and SoS when brought into solution. The formation of these sulfite derivates is strongly dependent on the pH value (Rose 1993; Dänicke et al. 2012). Under acidic conditions SO_2 is formed, while the HSO_3^- and SO_3^{2-} are more pronounced under neutral and alkaline pH values (Figure 7). The mode of action of these sulfiting agents as food preservatives are based on the permeation across the membrane of yeast and bacteria. Once inside the cell, they are dissociated to the anion forms due to the higher surrounding pH level leading to membrane disruption, inhibition of metabolic pathways and stress on the pH homeostasis (Brul and Coote 1999). It has been suggested that only SO_2 is able to cross yeast cell membranes and to be converted to the anion forms due to a higher pH value inside the cell (Rose, 1993). The other mechanism of HSO_3^- is due to the induction of an

energetically expensive stress to the cell (Maier et al. 1986, Rose, 1993). However, data from practical experiments showed no consistent ability to prevent yeasts and moulds growth (Dänicke et al. 2009; Dänicke et al. 2010). The reason for that difference could be the experimental conditions, whereby the pH value can be considered as key factor. SBS treatment without propionic acid only slightly decreased the pH value from approximately 6.6 to 6.1 (Dänicke et al. 2012). At this pH value, the total sulfites are almost only HSO_3^- and SO_3^{2-} (Figure 7). Even in the presence of propionic acid with lower pH value of 4.8, the SO_2 level is insufficient to perform its preserving effect. Therefore, the presence of propionic acid in the wet preserved cereal grains is required to avoid microbial spoilage.

2.7.2.3.1. Characterization of DON-sulfonate (DONS) as the derivation of DON and sulfite salts

It has been demonstrated that the formation of DON-sulfonates (DONS) is result of the reaction between DON and sulfite salts (Dänicke et al., 2010a; Young et al., 1987). Principally, the sulfonate group was bound to the DON molecule at C10 or at the keto group yielding the 10-DONS and 8-DONS, respectively (Figure 8, Young et al., 1987; Beyer et al., 2010). However, due to its chemical structure elucidation, the 10-DONS is commonly the major metabolite between DON and SBS, while the 8-DONS derivate is rarely formed (Young et al., 1986a, Young et al., 1987, Beyer et al., 2010).

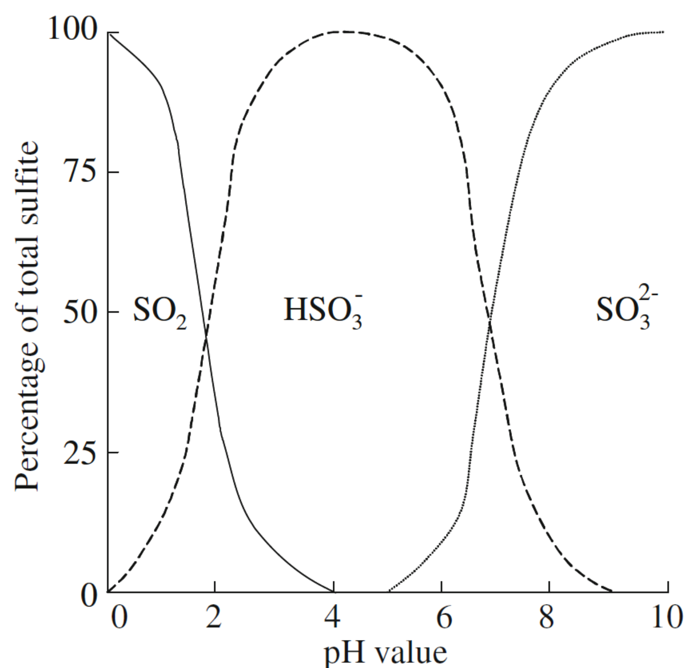

Figure 7. Percentage of three sulfite metabolite forms: SO_2, HSO_3^- and SO_3^{2-} in which total sulfites exist in solution, generated as a function of pH value (adapted from Rose 1993)

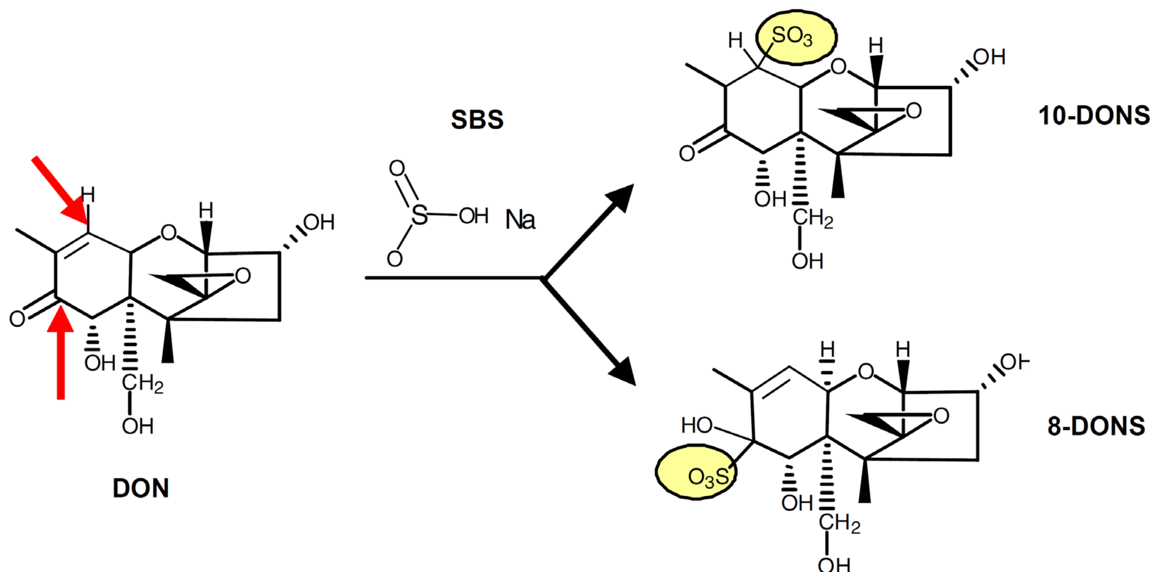

Figure 8. Structure of SBS-derived metabolites of DON: 10-DONS and 8-DONS. The red arrows display the C8 and C10 positions on the DON molecule and the yellow marked circles show the binding position between SBS and DON molecules (adapted from Beyer et al., 2010)

Recently, Schwartz et al. (2013) identified three different DONS including DONS-1, -2 and -3 after treatment with SoS (Figure 9, Schwartz et al., 2013). DONS-1 is characterized by loss of the epoxide group, while DONS-2 is identical by the formation of a hemiketal. DONS-3 is known as a mixture compound of two isomers, a ketone and a hemiketal (Schwartz et al., 2013). Moreover, the same authors also found that the pH value plays an important role for the formation and its stability of these derivatives (Schwartz-Zimmermann et al., 2014b). For instance, DONS-3 is preferentially formed under weak acid conditions with optimal pH value of six, whereas the alkaline pH value is advantageous for the DONS-1 and -2 formation (Schwartz-Zimmermann et al., 2014b). Moreover, these derivatives can be reversed depending on the pH value. Although DONS-3 is the predominant reaction product, DONS-3 can be converted to DONS-1 and -2 when pH value ≥ 6. Additionally, DONS-3 can be degraded to DON under physiological conditions (Schwartz-Zimmermann et al. 2014b). This degradation can be minimized when a longer preservation time of more than 8 weeks is applied. Regarding to the toxicity, it has been demonstrated that DONS-1 is non-toxic, while DONS-2 and -3 are much less toxic than DON (Schwartz-Zimmermann et al., 2014a). Therefore, the stability of DONS formation under acid conditions might be maintained in the presence of propionic acid as the additional effect besides its antimicrobial effect in the wet-preservation experiments.

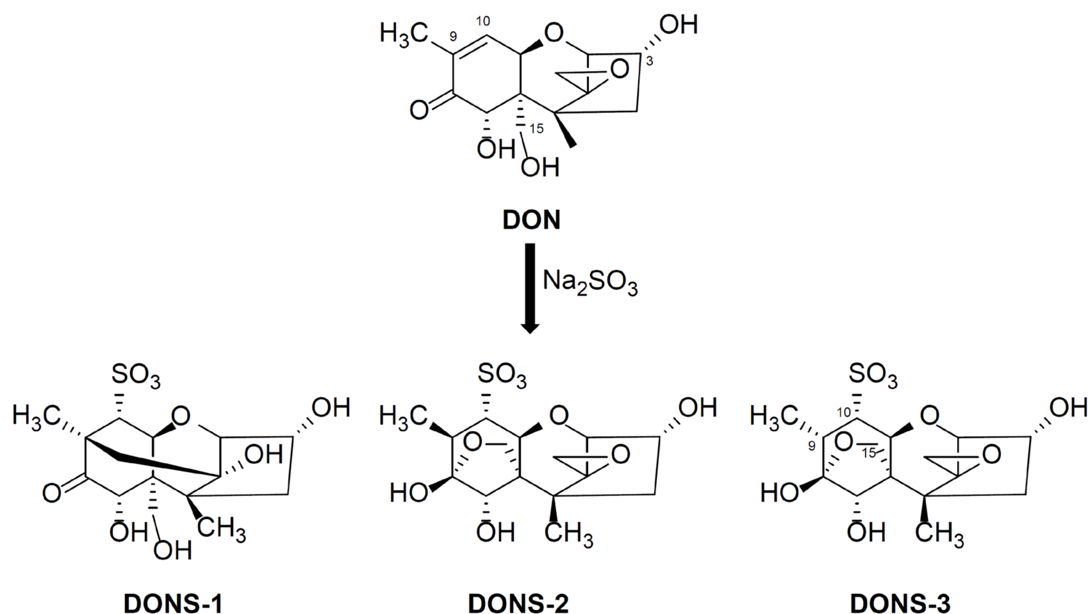

Figure 9. Structures of DON and DONS-1, -2 and -3 (Schwartz et al., 2013, modified)

2.7.3. In vivo methods

The *in vivo* approaches are defined as the addition of suitable substances to the contaminated feed which is then fed to the animals. During the passage through the digestive tract, these feed additives can inactivate the adverse effects of the mycotoxins due to adsorption (The commission of the European Communities, 2009). The common sorbent substances include organic (e.g. yeast or lactic acid bacteria) and inorganic (e.g. activated charcoal or silicate binders) additives to the feed. These substances can bind to the mycotoxins and consequently transport them through the digestive tracts. The other mechanism of these toxin-binders is due to degradation of the toxins into their non-toxic metabolites via enzymes or microbes as has been reviewed recently (Awad et al., 2010; Bata and Lasztity, 1999; Jard et al., 2011). The common adsorbents are activated carbon, hydrated sodium calcium aluminosilicate, zeolites and bentonite. The binding efficiency of absorbents is obviously dependent on the mycotoxin structure: high affinity for the polar mycotoxins such as aflatoxin, but less effective with the non-polar mycotoxins like ZEN and DON (Kabak et al. 2006). Therefore, the efficiency of such adsorbents is still uncertain (Dänicke et al., 2012a; Dänicke and Döll, 2010; Dänicke et al., 2004b; Döll et al., 2005).

3. SCOPE OF THE THESIS

Considering the literature, there is an ongoing search for an effective inactivation procedure for DON contaminated feedstuffs. Among the various strategies, wet-preservation of DON contaminated feed materials with sulfiting agents such as sodium sulfite is most promising and could be performed at the farm level.

Proving the efficacy and safety of the procedure requires to demonstrate the reduction of DON in feedstuffs prior to feeding, the stability of the formed DON sulfonates when passing through the digestive tract, and to evaluate the effects of the procedure itself on health and performance of pigs.

In order to address these issues a six weeks lasting feeding trial with rearing piglets was conducted. Results were evaluated and structured according to the main questions and published in three papers:

Paper I reports the impact of inactivation of DON-contaminated maize with SoS on piglet health. Hereby, different health parameters including blood profile, clinical biochemistry and global oxidative status via NO production as well as pH in digesta along the intestinal tract were investigated. Moreover, the efficacy of this SoS inactivated-DON treatment was also examined through analysis of DON, DOM1, ZEN, ZEN-metabolites residues in physiological matrices such as bile, urine and liquor.

There is evidence that DON modulates the immune system particularly under inflammatory conditions. Therefore, the next aim of this thesis was to elucidate whether the six-week feeding period with diets containing DONS and/or SoS lead to similar modulations on the immune system in pigs. For this purpose, an intraperitoneal LPS-challenge was carried out and monitored for 2h in order to examine the potential modulation. As a result, clinical symptoms, leukogram, clinical chemistry, TNF-alpha as well as the influence of SoS-treatment were published in **Paper II**.

In addition to these traits, the reaction of the innate immune system, especially the alteration of granulocytes and monocytes was another further investigated aspect of this thesis. Thus, the function of neutrophils and monocytes including ROS-production and phagocytosis were examined via flowcytometric methods. Besides, the phenotyping of T-cell subsets, B-cells were also evaluated. These outcome data were summarized and published in **Paper III**.

4. PAPER I

Detoxification of *Fusarium* contaminated maize with sodium sulfite – *in vivo* efficacy with special emphasis on mycotoxin residues and piglet health

Anh Tuan Tran[1], Jeannette Kluess[1], Andreas Berk[1], Marleen Paulick[1], Jana Frahm[1], Dian Schatzmayr[2], Janine Winkler[1], Susanne Kersten[1], Sven Dänicke[1]

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The full article can be found in the followed link:
https://www.tandfonline.com/doi/abs/10.1080/1745039X.2017.1418047?journalCode=gaan20

Abstract: A feeding experiment with piglets was performed to examine the efficacy of a wet preservation of *Fusarium* (FUS)-contaminated maize with sodium sulphite (SoS) based on deoxynivalenol (DON) and zearalenone (ZEN) residue levels in urine, bile and liquor and health traits of piglets. For this purpose, 80 castrated male piglets (7.57 ± 0.92 kg BW) were assigned to four treatment groups: CON− (control diet, with 0.09 mg DON and <0.01 mg ZEN/kg diet), CON+ (diet CON−, wet-preserved with 5 g SoS/kg maize; containing 0.05 mg DON and <0.01 mg ZEN/kg diet), FUS− (diet with mycotoxin-contaminated maize; containing 5.36 mg DON and 0.29 mg ZEN/kg diet), and FUS+ (diet FUS−, wet-preserved with 5 g SoS/kg maize; resulting in 0.83 mg DON and 0.27 mg ZEN/kg diet). After 42 d, 40 piglets (n = 10 per group) were sampled. A clear reduction of DON levels by approximately 75% was detected in all specimens of pigs fed diet FUS+. ZEN was detected in all urine, bile and liquor samples, while their metabolites were only detectable in urine and bile. Additionally, their concentrations were not influenced by SoS treatment. Among the health-related traits, feeding of FUS diets increased the total counts of leukocytes and segmented neutrophil granulocytes irrespective of SoS treatment. SoS treatment increased the total blood protein content slightly with a similar numerical trend in albumin concentration. These effects occurred at an obviously lower level in FUS-fed groups. Moreover, SoS treatment recovered the reduction of NO production induced by feeding diet FUS− indicating an effect on the redox level. As this effect only occurred in group FUS+, it is obviously related to the adverse effects of the *Fusarium* toxins. In conclusion, treatment of FUS-contaminated maize with SoS

decreased the inner exposure with DON as indicated by the lower DON levels in various piglet specimens. However, health-related traits did not consistently reflect this decreased exposure.

This paper is distinguished from the printed version dissertation and has been taken in the pages from 17 to 35 of the printed version dissertation.

5. PAPER II

Effects of a *Fusarium* toxin-contaminated maize treated with sodium sulfite on male piglets in the presence of an LPS-induced acute inflammation

Anh Tuan Tran[1], Jeannette Kluess[1], Andreas Berk[1], Marleen Paulick[1], Jana Frahm[1], Dian Schatzmayr[2], Susanne Kersten[1], Sven Dänicke[1]

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Toxins (Basel)
2018
Volume 10 (10)
419
DOI: 10.3390/toxins10100419

Article

Effects of a *Fusarium* Toxin-Contaminated Maize Treated with Sodium Sulfite on Male Piglets in the Presence of an LPS-Induced Acute Inflammation

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Received: 15 August 2018; Accepted: 12 October 2018; Published: 18 October 2018

Abstract: We investigated the effects of feeding sodium sulfite (SoS) treated uncontaminated and *Fusarium* contaminated maize in a porcine lipopolysaccharide (LPS) challenge model. Eighty piglets (7.59 ± 0.92 kg body weight [BW]) were equally assigned to one of four experimental diets containing 10% maize, either uncontaminated and untreated (CON−, 0.09 mg deoxynivalenol [DON]/kg diet) or uncontaminated and SoS-treated (CON+, wet-preserved with 5 g SoS/kg maize; 0.05 mg DON/kg diet), or prepared with 10% of a *Fusarium* contaminated maize containing mainly deoxynivalenol (DON), either contaminated and untreated (FUS−, 5.36 mg DON/kg diet), or contaminated and SoS-treated (FUS+, wet-preserved with 5 g SoS/kg maize; 0.83 mg DON/kg diet). At day 42 of experiment, ten pigs of each group were injected intraperitoneally with either 7.5 µg LPS/kg BW or placebo (0.9% NaCl). At 120 min after injection, blood samples were collected to analyse TNF-α, hematological profile, clinical biochemistry as well as the redox status. A significant increase in body temperature and cytokine TNF-α concentration was observed in the LPS-injected piglets. Results for hematology, clinical chemistry and redox status indicate no effects of SoS treatment, with exception of neutrophil counts being significantly more pronounced after feeding the SoS treated FUS maize. In conclusion, SoS treatment of maize did not modulate the LPS-induced acute inflammation.

Keywords: deoxynivalenol; sodium sulfite; detoxification; piglets; lipopolysaccharide

Key Contribution: Feeding piglet diets containing sodium sulfite-treated maize did not alter the response of the porcine innate immune system upon an endotoxin-challenge.

1. Introduction

The mycotoxin deoxynivalenol (DON) is mainly produced by *Fusarium* species and commonly found in agricultural commodities and finished feed [1]. Pigs are the most sensitive species with respect to DON, with prominent reduction of feed intake resulting in reduced weight gain [1]. As *Fusarium* infection of cereal strongly depends on the climate conditions, especially on temperature and humidity, such mycotoxin contamination cannot be completely avoided. Moreover, DON is rather stable under milling, processing and heating [2]. Therefore, decontamination strategies are needed in order to detoxify DON contaminated cereal grains before mixing into the diet [3]. Although many methods of decontamination are applied, an effective method at farm level is still lacking. Previous studies

Toxins **2018**, *10*, 419; doi:10.3390/toxins10100419 www.mdpi.com/journal/toxins

suggested that wet-preservation of *Fusarium* toxin-contaminated maize with sodium sulfite (Na_2SO_3, SoS) clearly reduced its DON content due to the transformation of DON into DON-sulfonates (DONS), which are considerably less toxic than DON [4–6]. Moreover, when piglets consumed SoS-treated maize, a decreased DON- and an increased DONS level was observed in plasma [7]. This was accompanied by an improved feed intake and an increased weight gain of animals [8]. Additionally, the reduced-DON concentrations in plasma were paralleled by lower DON levels in various specimens (urine, bile and liquor) supporting an efficient detoxification with SoS [9]. However, the health-related parameters did not consistently reflect the positive effects of SoS treatment [9].

Similar to SoS, sodium bisulfite (SBS) has been already investigated in various studies and was shown as an effective substance to detoxify DON contaminated cereal grains [6,10–12]. On the other hand, SBS also increased the plasma protein concentration, stimulated the functional liver capacity as determined by the ^{13}C-methacetin breath test and stimulation ability of peripheral blood mononuclear cells in pigs fed an SBS treated diet indicating side effects of SBS treatment [12,13].

Based on these non-specific effects in general, and the critical role of the liver as secondary immunological organ and its involvement in the initiation and mediation of the acute phase response as an innate immune mechanism in particular, the consequences for inflammatory conditions remain to be elucidated. Experimentally, a strong inflammatory response can be induced by systemic application of lipopolysaccharides (LPS). LPS, acting as an endotoxin, is a major component of the outer membrane of Gram-negative bacteria [14]. LPS is known as a pathogen-associated molecular pattern (PAMP) capable of inducing an acute phase response [15]. Additionally, it has been suggested that LPS possibly interacted with DON and resulted in an upregulation of pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α) in mice [16,17] as well as in porcine hepatic Kupffer cell cultures [18]. Furthermore, co-exposure to LPS and DON amplified the toxicity of DON in mice through an increase in apoptosis in lymphoid organs (thymus, spleen and Peyer's patches) when mice were intraperitoneally injected with LPS (0.5 mg LPS/kg body weight [BW]) and DON (25 mg DON/kg BW) [19]. In swine, higher DON content was observed in blood of pigs infused with DON (0.1 mg DON/kg BW) and LPS (7.5 μg LPS/kg BW) [20] as well as an augmentation of lactic acidosis was reported in pigs fed a DON diet (4.59 mg DON/kg feed) infused with LPS (7.5 μg LPS/kg BW) [21].

Based on the reported non-specific effects of SBS on metabolism such effects might also occur as a consequence of a SoS treatment. Moreover, the course of a systemic inflammation might be modified by SoS treatment and further modulated by the discussed interactions between DON and LPS. Therefore, the aim of present study was to investigate the effects of feeding SoS treated uncontaminated and contaminated maize under inflammatory stress conditions.

2. Results

2.1. Clinical Signs

Typical clinical signs of an inflammatory response were recorded in a time dependent manner and scored for their presence in all experimental groups. The sum of all symptoms was calculated over time and deemed as cumulative clinical score (CCS), whereby the highest achievable score of 88 represents the most severe clinical presentation. Data on CCS are presented in Figure 1. The highest CCS was only 12.2 for the group FUS−/LPS. In contrast, the lowest CCS with 2.6 was found in group CON+/NaCl, whereas the other groups (CON−/NaCl, FUS−/NaCl, FUS+/NaCl, CON−/LPS, CON+/LPS and FUS+/LPS) showed similar values of CCS, which resulted in a significant interaction between maize batch, SoS treatment and LPS ($p_{maize \times treatment \times injection}$ = 0.017). In general, the respiratory rate ranged above the physiological value 30 breaths/min [22] and statistically only a significant time effect (p_{time} < 0.001) was apparent. Starting from an initial value of 54.4 ± 2.8 breaths/min at −30 min (LSMeans), the respiratory rate was significantly increased at 15 min (66.0 ± 2.7 breaths/min) and 45 min (64.3 ± 2.7 breaths/min), but returned to its initial rate at 75 min (56.3 ± 2.7 breaths/min) and remained there (105 min: 58.2 breaths/min).

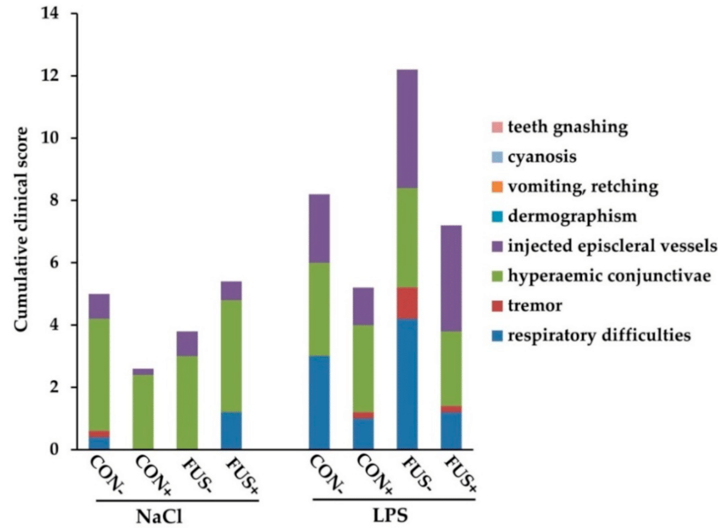

Figure 1. Cumulative clinical score of male castrated piglets fed diets containing untreated control (CON−) or *Fusarium* toxin-contaminated maize (FUS−), or sodium sulfite (SoS) wet-preserved treated control (CON+) and FUS maize (FUS+). Pigs were injected intraperitoneally with either 7.5 µg LPS/kg BW or 0.9% NaCl (LSMeans, $n = 5$). The entire clinical symptoms are presented as the cumulative clinical score (CCS). Each CCS bar consists of maximum 8 symptoms scored at four time points over the entire observation period of 120 min for each group. ANOVA, p-values: $p_{\text{maize}} = \mathbf{0.001}$; $p_{\text{treatment}} = \mathbf{0.034}$; $p_{\text{injection}} < \mathbf{0.001}$; $p_{\text{time}} = \mathbf{0.011}$; $p_{\text{maize}\times\text{treatment}} = 0.940$; $p_{\text{maize}\times\text{injection}} = 0.066$; $p_{\text{treatment}\times\text{injection}} = 0.090$; $p_{\text{maize}\times\text{treatment}\times\text{injection}} = \mathbf{0.017}$; $p_{\text{maize}\times\text{treatment}\times\text{injection}\times\text{time}} = 0.245$.

As shown in Figure 2, the body temperature was increased in a time-dependent manner due to LPS administration, irrespective of maize batch and SoS treatment. At 105 min after LPS administration, all LPS-injected pigs showed an increase in rectal temperature ($p_{\text{maize}\times\text{treatment}\times\text{injection}\times\text{time}} < 0.001$) compared to saline-injected groups.

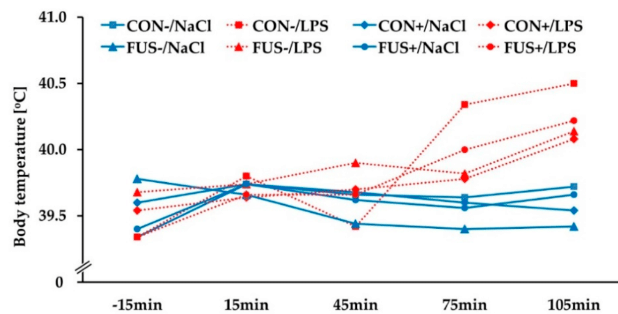

Figure 2. Rectal temperature of male castrated piglets fed diets containing untreated control (CON−, ■) or *Fusarium* toxin-contaminated maize (FUS−, ▲), or sodium sulfite (SoS) wet-preserved treated control (CON+, ⧫) and FUS maize (FUS+, ●). Pigs were i.p. injected with either 7.5 µg LPS/kg BW (red dotted lines) or 0.9% NaCl (blue solid lines) (LSMeans, $n = 5$). ANOVA, p-values: $p_{\text{maize}} = 0.764$; $p_{\text{treatment}} = 0.699$; $p_{\text{injection}} = \mathbf{0.025}$; $p_{\text{time}} < \mathbf{0.001}$; $p_{\text{maize}\times\text{treatment}} = 0.797$; $p_{\text{maize}\times\text{injection}} = 0.764$; $p_{\text{treatment}\times\text{injection}} = 0.467$; $p_{\text{maize}\times\text{treatment}\times\text{injection}} = 0.965$; $p_{\text{maize}\times\text{treatment}\times\text{injection}\times\text{time}} < \mathbf{0.001}$.

2.2. TNF-α

Generally, LPS-injected piglets showed significantly higher TNF-α concentrations compared to the saline-injected animals with exception of group CON+/LPS (Figure 3).

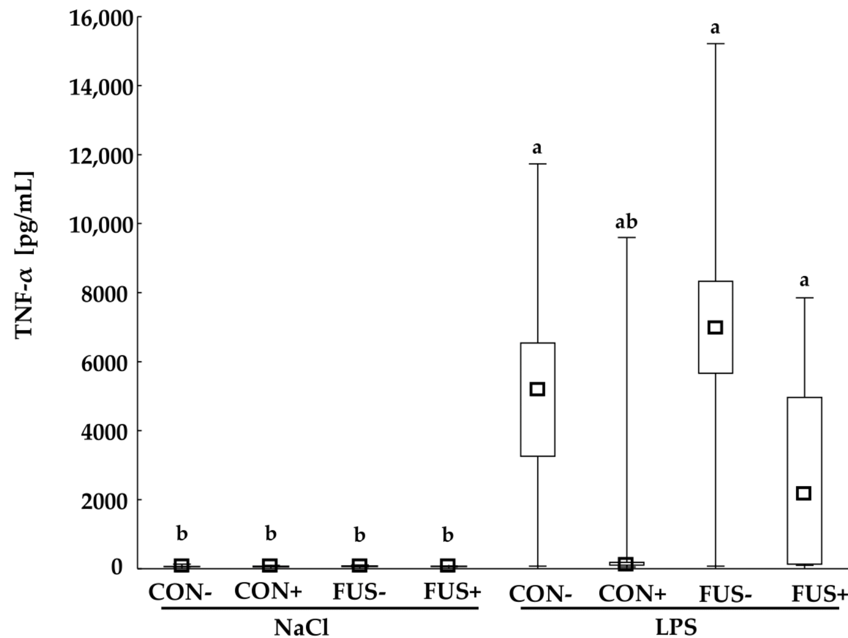

Figure 3. Pro-inflammatory cytokine TNF-α concentration (pg/mL) in serum of male castrated piglets fed diets containing untreated control (CON−) or *Fusarium* toxin-contaminated maize (FUS−), or sodium sulfite (SoS) wet-preserved treated control (CON+) and FUS maize (FUS+). Pigs were i.p. injected with either 7.5 μg LPS/kg BW or 0.9% NaCl. Squares, boxes and whiskers represent medians, 25–75th percentile, and minimum and maximum values, respectively. [a,b] Values with no common superscripts are significantly different (Mann-Whitney *U*-test, $p < 0.05$).

2.3. Organ Weights

The relative organ weights relate the absolute organ weights to the live weight of animals (Table 1). Live weight of the pigs fed diets containing untreated *Fusarium* contaminated maize (FUS−) was reduced, compared to the other three groups (CON−, CON+ and FUS+) which exhibited comparable body weights (LSMeans, CON−: 30.1 ± 1 kg; CON+: 30.3 ± 1 kg; FUS−: 26.1 ± 1 kg and FUS+: 30.9 ± 1 kg), resulting in a significant interaction between maize batch and SoS treatment. LPS-injected pigs showed a significantly higher relative spleen weight compared to the saline-injected pigs (pooled LSMeans of LPS-injected pigs: 1.8 ± 0.06 g/kg BW and saline-injected pigs: 1.6 ± 0.06 g/kg BW). Similarly, a significant increase in relative emptied stomach weight was observed in the LPS-injected groups (pooled LSMeans of LPS-injected pigs: 8.6 ± 0.2 g/kg BW and saline-injected pigs: 8.0 ± 0.2 g/kg BW). In addition, the relative emptied stomach weight of feeding group FUS− was increased compared to group FUS+ which appeared similar to the respective weight of control groups (LSMeans, CON−: 7.6 ± 0.3 g/kg BW; CON+: 7.8 ± 0.3 g/kg BW; FUS−: 9.8 ± 0.3 g/kg BW and FUS+: 7.9 ± 0.3 g/kg BW). These relationships resulted in a significant interaction between maize batch and SoS treatment. Furthermore, a significant interaction between maize batch and SoS treatment was also observed for the relative lung weight ($p_{\text{maize}\times\text{treatment}}$ =

0.009), whereby FUS+ showed a decrease compared to FUS−, which in turn was comparable to CON+ (LSmeans in g/kg BW, CON−: 12.1 ± 0.8; CON+: 13.8 ± 0.8; FUS−: 14.2 ± 0.8, FUS+: 11.7 ± 0.8).

Table 1. Live weight and relative organ weights (g/kg BW) of male castrated piglets fed diets containing control (CON−) or *Fusarium* toxin-contaminated maize (FUS−), or sodium sulfite (SoS) wet-preserved treated control (CON+) and FUS maize (FUS+). Pigs were i.p. injected with either 7.5 µg LPS/kg BW or 0.9% NaCl (LSMeans, n = 5).

Maize	Treatment	Injection	BW [kg]	Heart	Liver	Spleen	Kidney	Lung	Stomach
CON	−	NaCl	30.6	4.8	25.5	1.5	4.8	13.0	7.2
CON	−	LPS	29.6	4.7	28.9	2.0	4.6	11.1	8.0
CON	+	NaCl	29.8	4.7	26.3	1.6	4.6	13.8	7.4
CON	+	LPS	30.8	4.7	25.4	1.6	4.3	13.8	8.3
FUS	−	NaCl	25.8	4.8	25.9	1.6	4.8	14.6	9.4
FUS	−	LPS	26.3	4.4	27.4	1.8	4.8	13.8	10.1
FUS	+	NaCl	31.4	4.7	26.1	1.6	4.5	13.1	7.9
FUS	+	LPS	30.4	4.7	26.4	1.7	4.6	10.3	7.9
ANOVA (p-value)									
Maize			0.087	0.712	0.897	0.888	0.482	0.995	**<0.001**
Treatment			**0.015**	0.846	0.261	0.141	0.126	0.645	**0.010**
Injection			0.495	0.503	0.181	**0.015**	0.642	0.082	**0.048**
Maize × treatment			**0.023**	0.697	0.524	0.881	0.831	**0.009**	**0.001**
Maize × injection			0.495	0.606	0.834	0.590	0.382	0.580	0.392
Treatment × injection			0.495	0.388	0.081	0.082	0.890	0.973	0.636
Maize × treatment × injection			0.742	0.668	0.319	0.242	0.793	0.211	0.545
PSEM #			1.4	0.2	1.1	0.1	0.2	1.1	0.4

Notes: # PSEM, Pooled standard error of means, LSMeans, least square means.

2.4. Haematology

Overall, the red haemogram remained unaffected by either treatment with exception of haemoglobin concentration (Supplementary Table S1). Although LPS administration significantly increased the haemoglobin concentration as compared to its placebo counterparts from 12.1 g/dL to 12.9 g/dL, haemoglobin was still in the physiological range (10.8–14.8 g/dL).

Results for the white differential blood count are presented in Table 2. The total leukocyte counts of LPS-challenged pigs were strongly decreased in some groups (CON−/LPS, FUS−/LPS) even below the physiological reference range (10–22 × 10^9/L). However, total leukocytes of CON+/LPS were seemingly not affected, which resulted in a significant interaction between maize batch, SoS treatment and LPS injection. Similar significant relationships were also observed for lymphocytes ($p_{\text{maize}\times\text{treatment}\times\text{injection}}$ = 0.012) and monocytes ($p_{\text{maize}\times\text{treatment}\times\text{injection}}$ = 0.011). A further interaction between maize batch and SoS treatment was found for the segmented neutrophils due to an increase in the feeding group FUS+ when compared to other feeding groups (LSMeans, CON−: 3.5 ± 0.5 × 10^9/L; CON+: 3.1 ± 0.5 × 10^9/L; FUS−: 3.1 ± 0.5 × 10^9/L and FUS+: 5.2 ± 0.5 × 10^9/L). Similarly, the feeding group FUS+ showed a significantly higher concentration of banded neutrophils with 0.8 ± 0.1 × 10^9/L, while the other feeding groups (CON−, CON+ and FUS−) remained unaltered with 0.5 ± 0.1 × 10^9/L, 0.5 ± 0.1 × 10^9/L and 0.4 ± 0.1 × 10^9/L, respectively. Moreover, both neutrophils (segmented and banded neutrophils) were decreased in all LPS-injected piglets, while the eosinophils remained unaffected by either treatment.

Table 2. Total and differential white blood cell counts [10^9/L] of male castrated piglets fed diets containing untreated control (CON−) or *Fusarium* toxin-contaminated maize (FUS−), or sodium sulfite (SoS) wet-preserved treated control (CON+) and FUS maize (FUS+). Pigs were i.p. injected with either 7.5 μg LPS/kg BW or 0.9% NaCl (LSMeans, *n* = 5).

Maize	Treatment	Injection	Leukocytes (10–22) $^{\Phi}$	Lymphocytes (6–16) $^{\Phi}$	Segmented Neutrophils (1–8.2) $^{\Phi}$	Banded Neutrophils (0–1.5) $^{\Phi}$	Monocytes (0–1) $^{\Phi}$	Eosinophils (0–1.3) $^{\Phi}$
CON	−	NaCl	18.7	11.9	4.7	0.5	0.7	0.3
CON	−	LPS	9.8	6.6	2.3	0.4	0.2	0.2
CON	+	NaCl	13.4	8.7	3.7	0.4	0.2	0.3
CON	+	LPS	14.0	10.2	2.6	0.5	0.4	0.3
FUS	−	NaCl	14.4	8.4	4.8	0.6	0.4	0.2
FUS	−	LPS	7.8	6.0	1.3	0.2	0.2	0.1
FUS	+	NaCl	20.8	12.2	6.8	0.9	0.7	0.3
FUS	+	LPS	13.0	8.0	3.7	0.7	0.3	0.2
ANOVA (*p*-value)								
Maize			0.974	0.388	0.091	0.126	0.836	0.218
Treatment			**0.034**	0.062	0.067	**0.028**	0.960	0.258
Injection			**<0.001**	**0.003**	**<0.001**	**0.049**	**0.002**	0.380
Maize × treatment			**0.012**	0.112	**0.011**	**0.019**	**0.022**	0.691
Maize × injection			0.206	0.425	0.095	0.112	0.491	0.843
Treatment × injection			0.092	0.132	0.366	0.299	0.064	0.948
Maize × treatment × injection			**0.033**	**0.012**	0.664	0.809	**0.011**	0.858
PSEM #			1.7	1.1	0.7	0.1	0.1	0.1

Notes: $^{\Phi}$ Reference values according to Kraft and Dürr (2014) [23]; LSMeans, least square means; # PSEM, Pooled standard error of means.

2.5. Clinical Chemistry

Total protein and albumin remained unaffected by any treatment (Table 3). Activities of glutamate dehydrogenase (GLDH) were below the LOD (1.0 U/L) in all groups (data not shown). The activity of aspartate aminotransferase (AST) was recorded to be above the reference range of 35 U/L [21] in all groups. The AST activity increased in LPS-injected pigs fed CON diets (CON/LPS) compared to the respective saline-injected pigs (CON/NaCl), whereas the opposite was detected in the FUS fed pigs (LSMeans, CON/NaCl: 38.3 ± 2.9 U/L; CON/LPS: 48.0 ± 2.9 U/L; FUS/NaCl: 49.0 ± 2.9 U/L and FUS/LPS: 45.5 ± 2.9 U/L) as indicated by the significant interaction between maize batch and LPS. In addition, in LPS-injected pigs, significantly increased γ-glutamyltransferase (γ-GT) activity (pooled LSMeans of NaCl injection: 25 U/L and LPS injection: 27.8 U/L) and total bilirubin concentrations (pooled LSMeans of NaCl injection: 2.8 μmol/L and LPS injection: 3.5 μmol/L) were determined.

Table 3. Serum biochemical parameters of male castrated piglets fed diets containing untreated control (CON−) or *Fusarium* toxin-contaminated maize (FUS−), or sodium sulfite (SoS) wet-preserved treated control (CON+) and FUS maize (FUS+). Pigs were i.p. injected with either 7.5 μg LPS/kg BW or 0.9% NaCl (LSMeans, *n* = 5).

Maize	Treatment	Injection	Protein [g/L] < 86 $^{\Phi}$	Albumin [g/L] (19–39) $^{\Phi}$	AST † [U/L] < 35 $^{\Phi}$	γ-GT ‡ [U/L] < 45 $^{\Phi}$	Total Bilirubin [μmol/L] < 4.3 $^{\Phi}$
CON	−	NaCl	48.0	32.7	36.8	24.0	3.1
CON	−	LPS	45.5	32.4	46.3	29.1	3.5
CON	+	NaCl	45.0	32.4	39.7	25.4	2.4
CON	+	LPS	45.2	33.0	49.4	24.4	3.0
FUS	−	NaCl	46.0	31.2	49.6	24.0	3.0
FUS	−	LPS	41.1	29.7	50.0	28.1	4.2
FUS	+	NaCl	49.2	34.0	49.0	26.8	2.6
FUS	+	LPS	43.7	32.8	40.8	29.5	3.5
ANOVA (*p*-value)							
Maize			0.583	0.438	0.153	0.259	0.305
Treatment			0.700	0.073	0.744	0.845	0.087
Injection			0.060	0.466	0.334	**0.031**	**0.024**
Maize × treatment			0.181	0.105	0.184	0.130	0.949
Maize × injection			0.220	0.363	**0.028**	0.594	0.345
Treatment × injection			0.755	0.713	0.481	0.130	0.949
Maize × treatment × injection			0.616	0.876	0.464	0.332	0.784
PSEM #			2.3	1.2	4.2	1.7	0.5

Notes: $^{\Phi}$ Reference values according to Kraft and Dürr (2014) [23]; † AST, Aspartate aminotransferase, ‡ γ-GT, γ-glutamyltransferase; LSMeans, least square means; # PSEM, Pooled standard error of means.

2.6. Redox Status

Nitric oxide (NO) production, determined as the release of nitrite, is illustrated in Figure 4A. The NO production remained unaffected by all treatments and ranged between 14.3 ± 3.7 µM to 20.0 ± 3.7 µM.

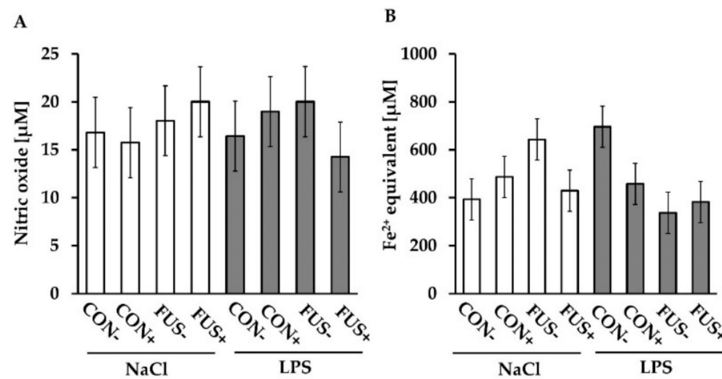

Figure 4. Nitric oxide (NO) production (**A**) and ferric reducing ability (FRA) of serum (**B**) of male castrated piglets fed diets containing untreated control (CON−) or *Fusarium* toxin-contaminated maize (FUS−), or sodium sulfite (SoS) wet-preserved treated control (CON+) and FUS maize (FUS+). Pigs were i.p. injected with either 7.5 µg LPS/kg BW or 0.9% NaCl (LSmeans ± SEM, *n* = 5). ANOVA of NO production (A): p_{maize} = 0.673; $p_{\text{treatment}}$ = 0.825; $p_{\text{injection}}$ = 0.931; $p_{\text{maize}\times\text{treatment}}$ = 0.614; $p_{\text{maize}\times\text{injection}}$ = 0.526; $p_{\text{treatment}\times\text{injection}}$ = 0.690; $p_{\text{maize}\times\text{treatment}\times\text{injection}}$ = 0.279 and ANOVA of FRA levels (B): p_{maize} = 0.329; $p_{\text{treatment}}$ = 0.207; $p_{\text{injection}}$ = 0.748; $p_{\text{maize}\times\text{treatment}}$ = 0.921; $p_{\text{maize}\times\text{injection}}$ = **0.015**; $p_{\text{treatment}\times\text{injection}}$ = 0.765; $p_{\text{maize}\times\text{treatment}\times\text{injection}}$ = **0.021**.

Ferric reducing ability (FRA) is considered as an indicator of the total non-enzymatic antioxidant capacity of serum and is measured as Fe^{2+} equivalents (µM) per L serum. Results of FRA in serum are displayed in Figure 4B. Saline-injected pigs fed diets containing untreated FUS maize showed a higher FRA (FUS−/NaCl: 643.4 ± 86.0 µM) compared to its CON-fed counterparts (CON−/NaCl: 393.0 ± 86.0 µM). In contrast, pigs fed diets containing SoS wet-preserved treated maize exhibited a similar FRA level independent of *Fusarium* toxin and LPS injection, and amounted to 487.1 ± 86.0 µM, 429.4 ± 86.0 µM, 457.6 ± 86.0 µM, and 382.6 ± 86.0 µM in groups CON+/NaCl, FUS+/NaCl, CON+/LPS and FUS+/LPS, respectively. Additionally, the FRA level was increased in untreated CON-fed pigs challenged with LPS (CON−/LPS) with 696.3 ± 86.0 µM and therefore resulted in a three factorial interaction between maize batch, SoS treatment and LPS.

3. Discussion

Recently, wet preservation of *Fusarium* contaminated maize with SoS was clearly shown to reduce the DON content of contaminated maize in vitro [4], while the in vivo efficiency of this procedure was tested in a feeding experiment with rearing piglets [12]. While the untreated FUS-diet clearly reduced feed intake and weight gain of piglets, SoS treatment of the FUS-maize could overcome these negative effects resulting in growth performance levels comparable to the control groups [8]. Furthermore, as a specific indicator for the successful detoxification, SoS treatment of *Fusarium* toxin-contaminated maize significantly reduced the exposure to DON as indicated by the reduced toxin residue levels in feed and in various physiological samples of pigs [8,9].

Based on the reported unspecific effects of SBS treatment on the liver and the proliferative ex vivo response of porcine PBMC, we hypothesized that SoS treatment of maize would affect biochemical indicators of liver function and integrity, the white blood count, as well the redox status.

Therefore, as part of the cited feeding experiment [12] we investigated the interactions between *Fusarium* contaminated maize and SoS treatment in the absence and presence of an LPS- induced acute inflammation in the present study to understand the treatment effects under inflammatory stress conditions.

TNF-α plays an essential role in the acute phase response and represents one of the first pro-inflammatory cytokines which is released in response to LPS, and is therefore suited to verify the intended initiation of a systemic inflammation. Although our data show significantly higher TNF-α concentration in most pigs challenged with LPS, the TNF-α concentration in LPS-challenged pigs fed a CON+ diet (CON+/LPS) did not completely reflect this observation. Looking closer, only one out of five pigs in this group showed a higher TNF-α concentration of 9596 pg/mL, the other four pigs exhibited lower values of TNF-α of approximately 111 pg/mL. This high variation might be due to the timing of blood collection for TNF-α analysis as well as the route of application. The peak TNF-α concentration appears often approximately 1 h after intravenous LPS-infusion [24] in pigs. In the present study blood samples were collected later, 2 h after intraperitoneal LPS-injection. The rationale behind this timing was to consider the time required for LPS-absorption from the abdominal cavity into the blood stream when injecting intraperitoneally rather than intravenously. Taking into account an individual vulnerability as well as LPS-absorption kinetics, it might well be that peak concentrations of TNF-α were not covered in each of the challenged pigs. However, the mean increase in body temperature in group CON+/LPS was comparable to that of the other LPS-challenged groups supporting the view that the systemic inflammation was initiated [25]. Moreover, the cumulative clinical score would rather suggest a less pronounced systemic inflammation in LPS-challenged groups fed SoS treated uncontaminated and contaminated maize.

Organ weights do not support this interpretation. Viewing data of the emptied stomach weights, it appeared that SoS treatment only of the contaminated maize obviously prevented the LPS-induced weight increase observed in all other groups including group CON+/LPS. While the LPS-associated increase in stomach weight might be due to oedema [26], the mechanism why feeding of SoS treated contaminated maize prevented stomach oedema remains to be clarified but seems to be related to the presence of DONS, both in stomach chyme and in systemic circulation. The LPS-induced increase in spleen weight might be due to a splenic congestion [27] which was not affected by SoS treatment of maize. The lower lung weights detected in groups fed the diets containing the SoS treated contaminated maize probably reflect an effect of chronic feeding while an acute LPS effect was not visible. In addition, neither clinical-chemical nor haematological traits support the view that treatment of maize with SoS influenced the outcome of the LPS-induced systemic inflammation.

It has been suggested that the alteration of clinical chemistry represents secondary effects of an LPS-induced acute phase reaction [7,24]. In agreement with these reports, in the present study the total bilirubin and γ-GT concentrations were significantly increased in LPS-injected animals indicating cholestasis relative to the saline-injected CON groups. Other indicators of hepatocyte integrity (GLDH, AST) and liver function (albumin concentration) remained unaltered, irrespective of LPS challenge and SoS treatment of maize, leading to the conclusion that the observed LPS-induced effects on the liver were neither modified nor directly influenced by SoS treatment.

The present results demonstrated significantly three factorial interactions for some parameters of white blood counts (the total leukocyte counts, lymphocytes and monocytes). It needs to be stressed however that these observations were characterized by a high individual variation. Feeding of SoS treated FUS maize—which contained mostly DON-sulfonates (DONSs)—caused significant effects and interactions for both segmented and banded neutrophils and would suggest a DONS effect. Previous studies have demonstrated that DONSs are considered less toxic derivatives of DON [4,6–8,13,28]. The DONS effect on increased neutrophil counts is not consistent with data from the other part of our study [9], whereby feeding the diets containing SoS treated FUS maize did not show any impact on the neutrophils counts. Such results were also reported for the impact of FUS maize treated with SBS, mono-methylamine and calcium hydroxide in diets for female piglets [29]. In the present experiment

LPS injection induced neutropenia in almost LPS-injected piglets with exception in the group FUS+. Looking closer, an increase in neutrophils was found in the group FUS+. Thus, another explanation for the enhancement of neutrophil counts might be due to the increased influx from the bone marrow and/or a decreased apoptosis of neutrophils compared to the control groups. As the DON content in the control groups was very low, this observation is seemingly related to the DONS effect.

Besides the white blood count, the ferric reducing ability (FRA) of serum was influenced by treatment factors in an interactive manner whereby SoS treatment effects appeared to be less consistent. Therefore, both sulfite residues and DONS might have influenced FRA in dependence on SoS treatment and maize batch. It was considered that the antioxidative effect of sulfite is due to the formation of sulfite (SO_3^{2-}) [30]. In animals, sulfite is rapidly reduced by the enzyme sulfite oxidase to sulfate (SO_4^{2-}) [31], which might contribute to this antioxidative capacity of sulfite. On the other hand, sulfite also caused oxidative stress due to sulfite oxidation into a sulfite radical ($SO_3^{-\blacksquare}$) [13,30]. Therefore, the effect of sulfite on the redox status in pigs was of interest and examined in the present study. Although our data showed the interaction between FUS maize, SoS treatment and LPS injection for the FRA level, it needs to be stressed that this finding was characterized by high individual variation. Moreover, the NO production, which represents another indicator for oxidative stress, remained unaffected irrespective of SoS treatment and LPS injection leading to the suggestion that SoS treatment of maize did not modulate the redox status, neither in LPS-stimulated nor in unstimulated animals.

Taken together, we could demonstrate that SoS treatment of maize did not have an impact on our investigated parameters of liver function and integrity, redox status and blood cell counts, with the exception of neutrophil counts that were generally increased in FUS+ fed piglets irrespective of the LPS-induced systemic inflammation. Thus, this effect is most likely due to the presence of DONS in FUS+ diets, but the underlying mechanism for this relative neutrophilia requires further elucidation.

4. Materials and Methods

The piglet experiment was performed at the Institute of of Animal Nutrition, Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Braunschweig, Germany according to the European Community regulations concerning the protection of experimental animals and was approved by the Lower Saxony State Office for Consumer Protection and Food Safety (file number: 33.92-42502-04-13/1153, date of approval: 11.07.2013).

4.1. Experimental Design and Procedures

The present manuscript is part of a comprehensive study, investigating the efficacy of detoxification of DON-contaminated maize with sodium sulfite. Experimental design, SoS treatment in *Fusarium* toxin contaminated maize as well as procedures have been already described in detail in Paulick et al. [8] and Tran et al. [9]. Briefly, a total of eighty male castrated weaned crossbred piglets ((German Landrace × German Large White) × Piétrain) from the Bundeshybridzuchtprogramm (BHZP) with an average initial BW of 7.59 ± 0.92 kg were group-housed (4 pigs/pen) and equally allotted to one of four experimental diets, fed over a period of 42 days: CON− (diet with 10% control maize; 0.09 mg DON/kg and <0.01 mg ZEN/kg feed), CON+ (diet with 10% control maize, wet-preserved with 5 g SoS/kg maize; 0.05 mg DON/kg and <0.01 mg ZEN/kg feed), FUS− (diet with 10% mycotoxin-contaminated maize; 5.36 mg DON/kg; 0.09 mg DONS-2/kg; 0.06 mg DONS-3/kg and 0.29 mg ZEN/kg feed) and FUS+ (diet with 10% contaminated maize, wet-preserved with 5 g SoS/kg maize; 0.83 mg DON/kg; 2.62 mg DONS-2/kg; 1.98 mg DONS-3/kg and 0.27 mg ZEN/kg feed). Diets consisted of 35% barley, 27.3% wheat, 10% maize, 23% soybean meal, 1.5% soya bean oil, 1% mineral and vitamin premix, 2.2% amino acid and phytase. More details about the diets are described in Tran et al. [9]. Feed and water were offered ad libitum.

At the last day of the feeding experiment, on day 42, ten pigs of each feeding group were used for the LPS challenge. An overview about the LPS challenge is illustrated in Figure 5.

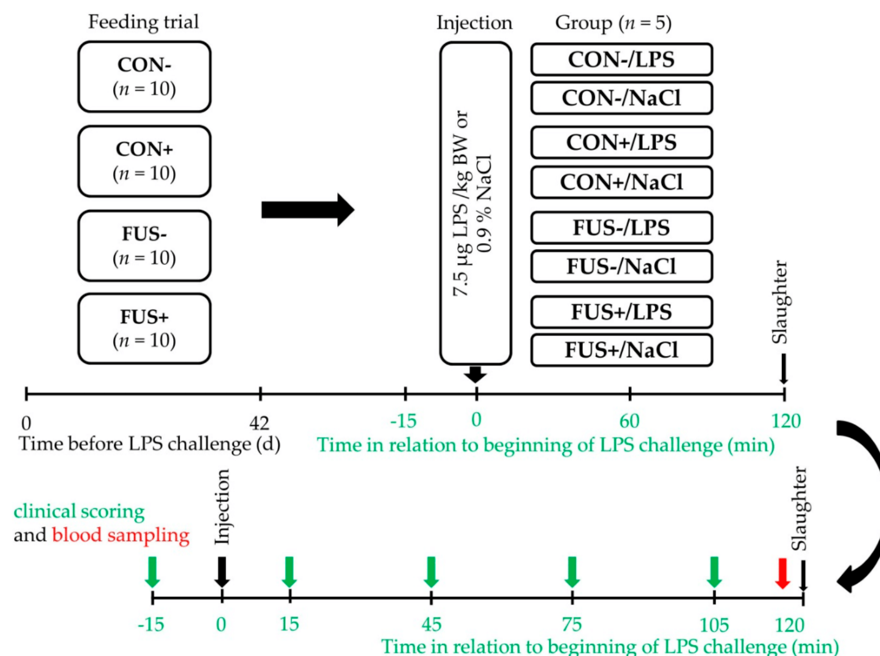

Figure 5. The feeding trial included diets containing 10% control maize (CON−) or *Fusarium* toxin-contaminated maize (FUS−), or sodium sulfite (SoS) wet-preserved treated control (CON+) and FUS maize (FUS+). The feeding trial lasted for 42 days before LPS challenge (upper panel). On day 42, ten pigs of each group were injected intraperitoneally either with 7.5 µg LPS/kg BW (*Escherichia coli* serotype O111:B4, Sigma-Aldrich, Steinheim, Germany, L 2630) or with 0.9% NaCl (volume ~6.5 mL/animal). The lower panel displays the LPS challenge, over a period of 2 h, starting 15 min before LPS challenge until 120 min after injection clinical symptoms were recorded and blood samples were taken once direct before slaughter.

The dose of LPS was chosen for induction of a reliable systemic inflammation as described in previous studies [24,25]. Over a period from −15 min before to 120 min after injection, different clinical signs were scored (Table 4), respiratory rate was taken, and rectal temperature measured using a digital thermometer (Geratherm Rapid GT-195, Geratherm Medical AG, Geschwenda, Germany) at −15, 15, 45, 75 and 105 min. At 120 min, all animals were electrically stunned and blood samples were taken immediately via venipuncture of *Vena cava cranialis*. Then, pigs were sacrificed by exsanguination and organs dissected from the thoracic and abdominal cavity. Weights of emptied stomach, liver (without gallbladder), spleen, kidneys, heart and lungs were recorded.

Table 4. Clinical score: Cumulative score calculated from all scores of each symptom over the whole observation period (8 symptoms × 4 times, maximum score 88) or of each symptom for every point in time (8 symptoms per time, maximum score 22).

Clinical Symptom	Grade	Score
Respiratory difficulties	None	0
	Low labored breathing	1
	Medium labored breathing	2
	Severe labored breathing	3
	Open-mouth breathing	4
Tremor	None	0
	Low shivering	1
	Medium shivering	2
	Severe shivering	3
	Spasms	4
Conjunctivae	Physiological (pale-rose)	0
	(Rose) red	1
	Red	2
Episcleral vessels	Physiological (not injected)	0
	Slightly injected	1
	Medium injected	2
	Highly injected	3
Cyanosis	None	0
	Low cyanosis	1
	Medium cyanosis	2
	Severe cyanosis	3
Vomiting, retching	None	0
	Smacking, foam-forming, retching	1
	Vomiting of slime	2
	Vomiting of feed/digesta	3
	Vomiting of slime and feed/digesta	4
Dermographism	None	0
	Skin colouring pattern present	1
Teeth gnashing	None	0
	Teeth gnashing present	1
Maximum clinical score (each animal each time point)		22
Maximum clinical score (each animal for all time points)		88

4.2. Analyses

4.2.1. Haematology and Biochemichal Analysis

Blood samples were collected into EDTA-tubes (Sarstedt AG&Co, Nümbrecht, Germany). Red blood cell counts and total leukocytes counts were measured on a Celltac-α (MEK 6450, Nihon Kohden, Qinlab Diagnostik, Weichs, Germany). In order to differentiate white blood cells, blood smears were prepared and stained according to PAPPENHEIM: Slides were fixed and stained in MAY-GRÜNWALD solution for 3 min and subsequently rinsed in distilled water (pH 7.2) to remove staining solution. Then, slides were counterstained in a GIEMSA solution for 15 min and again rinsed in distilled water (pH 7.2). Dried specimens were microscopically analyzed (Nikon Eclipse E200, Nikon Europe b.v., Badhoevedorp, The Netherlands) and at least 200 cells counted per slide (100× magnifications) based on their morphological characteristics. The differential blood counts were firstly calculated as percentage of total leukocytes. Data of differential blood counts are presented in absolute values and were calculated as the total leukocytes multiplied with the proportion of corresponding cell subpopulation.

Further blood samples were collected into serum tubes (Serum Z, Sarstedt AG&Co, Nümbrecht, Germany), left clotting for 60 min at room temperature and 30 min at 30 °C, subsequently centrifuged at 2123× *g* for 15 min (15 °C) and stored at −80 °C until analysis. Serum was analyzed for total protein, albumin, total bilirubin, aspartate-aminotransferase (AST), glutamate-dehydrogenase (GLDH) and γ-glutamyl transferase (γ-GT) using photometric methods (Eurolyser, CCA180, Eurolab, Austria). The cytokine tumor necrosis factor α (TNF-α) was determined by commercially available ELISA (Quantikine® ELISA, R&D systems) using the specific immunoassay kit for pigs.

4.2.2. Oxidative Status

Griess Assay

The release of nitric oxide was measured with the Griess assay employing to enzymatic reduction of nitrate to nitrite and reaction of nitrite with Griess reagent. Briefly, 80 µL serums (duplicates) was pipetted in a 96-well MTP, 10 µL nitrate reductase cofactor and 10 µL nitrate reductase enzymes (both Cayman Chemical, Ann Arbor, MI, USA) were added to each well. After three hours incubation in the dark, 100 µL of Griess solution (1% sulfanilamide and 0.1% *N*-(1-naphthyl) ethylenediamine dihydrochloride) were added to each well and incubated for 10 min at room temperature and absorbance was measured at 540 nm (Tecan Infinite® 200, Tecan Group Ltd., Männedorf, Switzerland).

Ferric Reducing Ability

The ferric reducing ability (FRA) of serum was measured according to the specifications of Benzie and Strain [32] with in-house modifications. Briefly, 10 µL serums (triplicate) were placed into wells of a 96-well MTP and mixed with 30 µL distilled water (pH 7.2). After incubating for 5 min at 37 °C, 300 µL FRA reagent including ferric (III) chloride hexahydrate, acetate buffer and 2,4,6-tripyridyl-s-triazine (TPTZ) (pre-warmed for 30 min at 37 °C) were added to each well and the absorbance of this mixture was measured at 593 nm for 15 min (Tecan Infinite® 200, Tecan Group Ltd., Männedorf, Switzerland).

4.3. Statistics

Generally, data were statistically analyzed with a 2 × 2 × 2-factorial design using PROC MIXED in SAS software (SAS Institute 2013, Cary, NC, USA), presented as least square means (LSMeans) and pooled standard error of means (PSEM). Maize batch (control maize or *Fusarium* toxin-contaminated maize), SoS treatment (with or without SoS) and injection (injection of NaCl or LPS) as well as their interactions were defined as fixed factors. The student's *t*-test was used for post-hoc testing of differences between LSMeans and considered significant at $p < 0.05$.

Data of cytokine TNF-α were not normally distributed and thus evaluated using a Mann-Whitney U-test (Statistica 13, Dell Inc., Tulsa, OK, USA). Corresponding data were presented as median and respective ranges (minimum-maximum values).

Supplementary Materials: The following are available online at http://www.mdpi.com/2072-6651/10/10/419/s1, Table S1: Red haemogram of male castrated piglets fed diets containing untreated control (CON−) or Fusarium toxin contaminated maize (FUS−), or sodium sulfite (SoS) wet-preserved treated control (CON+) and FUS maize (FUS+). Pigs were i.p. injected with either 7.5 µg LPS/kg BW or 0.9% NaCl (LSMeans, $n = 5$).

Author Contributions: The project was conceived and designed by J.K., J.F., S.K., D.S. and S.D., M.P., A.B., J.K., J.F. organized and performed the experiments. A.-T.T., J.K., J.F., S.K. and S.D. analyzed and interpreted the data. The manuscript was written by A.-T.T. All authors read and approved the final manuscript.

Funding: This research was funded by Biomin Holding GmbH, Tulln, Austria.

Acknowledgments: The assistance of the co-workers of the Institute of Animal Nutrition and the experimental station of the Friedrich-Loeffler-Institute in Braunschweig in performing the experiment and analysis is gratefully acknowledged.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. EFSA. Opinion of the Scientific Panel on Contaminants in the Food Chain on a request from the Commission related to Deoxynivalenol (DON) as undesirable substance in animal feed. *EFSA J.* **2004**, *73*, 1–41.
2. Rotter, B.A.; Prelusky, D.B.; Pestka, J.J. Toxicology of deoxynivalenol (vomitoxin). *J. Toxicol. Environ. Health* **1996**, *48*, 1–34. [CrossRef] [PubMed]
3. Awad, W.A.; Ghareeb, K.; Bohm, J.; Zentek, J. Decontamination and detoxification strategies for the Fusarium mycotoxin deoxynivalenol in animal feed and the effectiveness of microbial biodegradation. *Food Addit. Contam. Part A Chem.* **2010**, *27*, 510–520. [CrossRef] [PubMed]
4. Paulick, M.; Rempe, I.; Kersten, S.; Schatzmayr, D.; Schwartz-Zimmermann, H.E.; Dänicke, S. Effects of increasing concentrations of sodium sulfite on deoxynivalenol and deoxynivalenol sulfonate concentrations of maize kernels and maize meal preserved at various moisture content. *Toxins* **2015**, *7*, 791–811. [CrossRef] [PubMed]
5. Paulick, M.; Winkler, J.; Kersten, S.; Schatzmayr, D.; Schwartz-Zimmermann, H.E.; Dänicke, S. Studies on the bioavailability of deoxynivalenol (DON) and DON sulfonate (DONS) 1, 2, and 3 in pigs fed with sodium sulfite-treated DON-contaminated maize. *Toxins* **2015**, *7*, 4622–4644. [CrossRef] [PubMed]
6. Young, J.C.; Trenholm, H.L.; Friend, D.W.; Prelusky, D.B. 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.* **1987**, *35*, 259–261. [CrossRef]
7. Dänicke, S.; Beyer, M.; Breves, G.; Valenta, H.; Humpf, H.U. Effects of oral exposure of pigs to deoxynivalenol (DON) sulfonate (DONS) as the non-toxic derivative of DON on tissue residues of DON and de-epoxy-DON and on DONS blood levels. *Food Addit. Contam. Part A Chem.* **2010**, *27*, 1558–1565. [CrossRef] [PubMed]
8. Paulick, M.; Winkler, J.; Kersten, S.; Schatzmayr, D.; Frahm, J.; Kluess, J.; Schwartz-Zimmermann, H.E.; Dänicke, S. Effects of oral exposure to sodium sulphite-treated deoxynivalenol (DON)-contaminated maize on performance and plasma concentrations of toxins and metabolites in piglets. *Arch. Anim. Nutr.* **2018**, *72*, 42–57. [CrossRef] [PubMed]
9. Tran, A.T.; Kluess, J.; Berk, A.; Paulick, M.; Frahm, J.; Schatzmayr, D.; Winkler, J.; Kersten, S.; Dänicke, S. Detoxification of Fusarium-contaminated maize with sodium sulphite–in vivo efficacy with special emphasis on mycotoxin residues and piglet health. *Arch. Anim. Nutr.* **2018**, *72*, 58–75. [CrossRef] [PubMed]
10. Dänicke, S.; Brezina, U. Kinetics and metabolism of the Fusarium toxin deoxynivalenol in farm animals: Consequences for diagnosis of exposure and intoxication and carry over. *Food Chem. Toxicol.* **2013**, *60*, 58–75. [CrossRef] [PubMed]
11. Dänicke, S.; Brüssow, K.P.; Valenta, H.; Ueberschar, K.H.; Tiemann, U.; Schollenberger, M. 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. *Mol. Nutr. Food Res.* **2005**, *49*, 932–943. [CrossRef] [PubMed]
12. Dänicke, S.; Beineke, A.; Goyarts, T.; Valenta, H.; Beyer, M.; Humpf, H.U. Effects of a Fusarium toxin-contaminated triticale, either untreated or treated with sodium metabisulphite ($Na_2S_2O_5$, SBS), on weaned piglets with a special focus on liver function as determined by the (13)C-methacetin breath test. *Arch. Anim. Nutr.* **2008**, *62*, 263–286. [CrossRef] [PubMed]
13. Dänicke, S.; Kersten, S.; Valenta, H.; Breves, G. Inactivation of deoxynivalenol-contaminated cereal grains with sodium metabisulfite: A review of procedures and toxicological aspects. *Mycotoxin Res.* **2012**, *28*, 199–218. [CrossRef] [PubMed]
14. Palsson-McDermott, E.M.; O'Neill, L.A. Signal transduction by the lipopolysaccharide receptor, Toll-like receptor-4. *Immunology* **2004**, *113*, 153–162. [CrossRef] [PubMed]
15. Kuzmich, N.N.; Sivak, K.V.; Chubarev, V.N.; Porozov, Y.B.; Savateeva-Lyubimova, T.N.; Peri, F. TLR4 Signaling Pathway Modulators as Potential Therapeutics in Inflammation and Sepsis. *Vaccines* **2017**, *5*, 34. [CrossRef] [PubMed]
16. Islam, Z.; Pestka, J.J. LPS priming potentiates and prolongs proinflammatory cytokine response to the trichothecene deoxynivalenol in the mouse. *Toxicol. Appl. Pharmacol.* **2006**, *211*, 53–63. [CrossRef] [PubMed]
17. Zhou, H.R.; Harkema, J.R.; Yan, D.; Pestka, J.J. Amplified proinflammatory cytokine expression and toxicity in mice coexposed to lipopolysaccharide and the trichothecene vomitoxin (deoxynivalenol). *J. Toxicol. Environ. Health A* **1999**, *57*, 115–136. [PubMed]

18. Döll, S.; Schrickx, J.A.; Dänicke, S.; Fink-Gremmels, J. Deoxynivalenol-induced cytotoxicity, cytokines and related genes in unstimulated or lipopolysaccharide stimulated primary porcine macrophages. *Toxicol. Lett.* **2009**, *184*, 97–106. [CrossRef] [PubMed]
19. Zhou, H.R.; Harkema, J.R.; Hotchkiss, J.A.; Yan, D.; Roth, R.A.; Pestka, J.J. Lipopolysaccharide and the Trichothecene Vomitoxin (Deoxynivalenol)Synergistically Induce Apoptosis in Murine Lymphoid Organs. *Toxicol. Sci. Off. J. Soc. Toxicol.* **2000**, *53*, 253–263. [CrossRef]
20. Dänicke, S.; Valenta, H.; Ganter, M.; Brosig, B.; Kersten, S.; Diesing, A.K.; Kahlert, S.; Panther, P.; Kluess, J.; Rothkötter, H.J. Lipopolysaccharides (LPS) modulate the metabolism of deoxynivalenol (DON) in the pig. *Mycotoxin Res.* **2014**, *30*, 161–170. [CrossRef] [PubMed]
21. Bannert, E.; Tesch, T.; Kluess, J.; Frahm, J.; Kersten, S.; Kahlert, S.; Renner, L.; Rothkötter, H.J.; Dänicke, S. Metabolic and hematological consequences of dietary deoxynivalenol interacting with systemic Escherichia coli lipopolysaccharide. *Toxins* **2015**, *7*, 4773–4796. [CrossRef] [PubMed]
22. Baumgartner, W. *Klinische Propädeutik der Haus-und Heimtiere [Clinical Propaedeutic of Domestic and Pet Animals]*, 7th ed.; MVS Medizinverlage Stuttgart GmbH & Co., KG: Stuttgart, Germany, 2009.
23. Kraft, W.; Dürr, U.M. *Klinische Labordiagnostik in der Tiermedizin. [Clinical Laboratory Diagnostician in Veterinary Medicine]*, 7th ed.; Schattauer GmbH: Stuttgart, Germany, 2014.
24. Stanek, C.; Reinhardt, N.; Diesing, A.K.; Nossol, C.; Kahlert, S.; Panther, P.; Kluess, J.; Rothkotter, H.J.; Kuester, D.; Brosig, B.; et al. A chronic oral exposure of pigs with deoxynivalenol partially prevents the acute effects of lipopolysaccharides on hepatic histopathology and blood clinical chemistry. *Toxicol. Lett.* **2012**, *215*, 193–200. [CrossRef] [PubMed]
25. Stelter, K.; Frahm, J.; Paulsen, J.; Berk, A.; Kleinwachter, M.; Selmar, D.; Dänicke, S. Effects of oregano on performance and immunmodulating factors in weaned piglets. *Arch. Anim. Nutr.* **2013**, *67*, 461–476. [CrossRef] [PubMed]
26. Saetre, T.; Hovig, T.; Roger, M.; Gundersen, Y.; Aasen, A.O. Hepatocellular damage in porcine endotoxemia: Beneficial effects of selective versus non-selective nitric oxide synthase inhibition? *Scand. J. Clin. Lab. Investig.* **2001**, *61*, 503–512. [CrossRef]
27. Hinshaw, L.B.; Emerson, T.E., Jr.; Chang, A.C.; Duerr, M.; Peer, G.; Fournel, M. Study of septic shock in the non-human primate: Relationship of pathophysiological response to therapy with anti-TNF antibody. *Circ. Shock.* **1994**, *44*, 221–229. [PubMed]
28. Dänicke, S.; Hegewald, A.-K.; Kahlert, S.; Kluess, J.; Rothkotter, H.J.; Breves, G.; Döll, S. 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 Chem. Toxicol.* **2010**, *48*, 2154–2162. [PubMed]
29. Rempe, I.; Kersten, S.; Valenta, H.; Dänicke, S. 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.* **2013**, *29*, 169–175. [CrossRef] [PubMed]
30. Elmas, O.; Aslan, M.; Caglar, S.; Derin, N.; Agar, A.; Aliciguzel, Y.; Yargicoglu, P. The prooxidant effect of sodium metabisulfite in rat liver and kidney. *Regul. Toxicol. Pharmacol.* **2005**, *42*, 77–82. [CrossRef] [PubMed]
31. Tejnorova, I. Sulfite oxidase activity in liver and kidney tissue in five laboratory animal species. *Toxicol. Appl. Pharmacol.* **1978**, *44*, 251–256. [CrossRef]
32. Benzie, I.F.F.; Strain, J.J. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": The FRAP assay. *Anal. Biochem.* **1996**, *239*, 70–76. [CrossRef] [PubMed]

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6. PAPER III

Sodium sulfite (SoS) as decontamination strategy for *Fusarium*-toxin contaminated maize and its impact on immunological traits in pigs challenged with lipopolysaccharide (LPS)

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Mycotoxin Research
2020
Volume 36 (4)
429- 442
DOI: 10.1007/s12550-020-00403-x

Mycotoxin Research
https://doi.org/10.1007/s12550-020-00403-x

ORIGINAL ARTICLE

Sodium sulfite (SoS) as decontamination strategy for *Fusarium*-toxin contaminated maize and its impact on immunological traits in pigs challenged with lipopolysaccharide (LPS)

Anh-Tuan Tran[1] · Jeannette Kluess[1] · Susanne Kersten[1] · Andreas Berk[1] · Marleen Paulick[1] · Dian Schatzmayr[2] · Sven Dänicke[1] · Jana Frahm[1]

Received: 3 December 2019 / Revised: 16 July 2020 / Accepted: 22 July 2020
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Abstract
The main objective of this study was to evaluate the effects of sodium sulfite (SoS) treatment of maize and its impact on the porcine immune system in the presence of an LPS-induced systemic inflammation. Control maize (CON) and *Fusarium*-toxin contaminated maize (FUS) were wet-preserved (20% moisture) for 79 days with (+) or without (−) SoS and then included at 10% in a diet, resulting in four experimental groups: CON−, CON+, FUS−, and FUS+ with deoxynivalenol (DON) concentrations of 0.09, 0.05, 5.36, and 0.83 mg DON/kg feed, respectively. After 42-day feeding trial (weaned barrows, $n = 20$/group), ten pigs per group were challenged intraperitoneally with either 7.5 µg LPS/kg BW or placebo (0.9% NaCl), observed for 2 h, and then sacrificed. Blood, mesenteric lymph nodes, and spleen were collected for phenotyping of different T cell subsets, B cells, and monocytes. Phagocytic activity and intracellular formation of reactive oxygen species (ROS) were analyzed in both polymorphonuclear cells (PMN) and peripheral blood mononuclear cells (PBMC) using flow cytometry. Our results revealed that the impact of DON was more notable on $CD3^+CD4^+CD8^+$ T cells in lymphoid tissues rather than in blood T cells. In contrast, SoS treatment of maize altered leukocyte subpopulations in blood, e.g., reduced the percentage and fluorescence signal of $CD8^{high}$ T cells. Interestingly, SoS treatment reduced the amount of free radicals in basal ROS-producing PMNs only in LPS-challenged animals, suggesting a decrease in basal cellular ROS production ($p_{SoS*LPS} = 0.022$).

Keywords Sodium sulfite · Deoxynivalenol · Immune cells · Lipopolysaccharide · Piglet

Abbreviations

CON	Control maize
DON	Deoxynivalenol
FUS	*Fusarium*–toxin contaminated maize
LOD	Level of detection
LPS	Lipopolysaccharide
MFI	Mean fluorescence intensity
PBMC	Peripheral blood mononuclear cells
PMN	Polymorphonuclear cells
ROS	Reactive oxygen species
SoS	Sodium sulfite

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s12550-020-00403-x) contains supplementary material, which is available to authorized users.

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Introduction

Deoxynivalenol (DON) belongs to the B-trichothecene mycotoxins produced from *Fusarium* species. Among the farm animals, pigs are known as the most susceptible species to DON exposure, responding with a reduction in feed intake and lower weight gain, resulting in economic losses for the farmer (EFSA 2004). In the temperate climate regions, DON often occurs in cereal grains, especially wheat and maize (EFSA 2013). Moreover, DON contamination in the field cannot

Published online: 09 September 2020

completely be avoided due to weather conditions. Additionally, DON is only negligibly degraded by feed processing and therefore decontamination methods are required to inactivate DON before cereal grains are used for the production of complete feeds (Awad et al. 2010). Although various decontamination strategies were investigated, an effective procedure is still needed (Kabak et al. 2006; He et al. 2010). Moreover, easy-to-use inactivation procedures are required at farm level where cereals are directly used for feeding. The simple wet preservation of *Fusarium*-toxin contaminated cereal grains with sodium sulfite (Na_2SO_3, SoS) or sodium metabisulfite ($Na_2S_2O_5$, SBS), using a defined moisture content and acidification with propionic acid, has shown to decrease DON concentration via the formation of sulfonated derivatives of DON, the so-called DON sulfonates (DONS) (Young et al. 1987; Schwartz et al. 2013; Schwartz-Zimmermann et al. 2014;). The SoS- and SBS-induced decrease of DON concentration in cereal grains was reflected by a concomitant detection of low DON concentrations in blood and other physiological specimen (Dänicke et al. 2005; Dänicke et al. 2008; Paulick et al. 2018; Tran et al. 2018a) as well as an improved performance comparable with that observed in control groups fed non-contaminated diets (Paulick et al. 2018).

Despite positive effects on the piglet performance, several non-specific effects of SBS treatment were recorded when pigs were fed SBS-treated diets irrespective of DON contamination (Dänicke et al. 2008; Dänicke et al. 2012), such as a plasma protein increase, stimulated liver function as determined by the ^{13}C-methacetin breath test, and an increased stimulation capacity of peripheral blood mononuclear cells (PBMC). The liver, besides its function as a main metabolic organ, also acts as a secondary immunological organ, which initiates and mediates the acute phase response as an innate immune mechanism (Crispe 2009); therefore, a consequential interference of SBS treatment with the immune system remains to be elucidated.

Lipopolysaccharides (LPS) are a major component of the cell wall of Gram-negative bacteria (Palsson-McDermott and O'Neill 2004) and are known as pathogen-associated molecular patterns (PAMPs), capable of inducing an acute phase response. The latter consists of immediate effects such as releasing soluble mediators like tumor necrosis factor-alpha (TNF-α) and interferon-β (IFN-β) from immune cells into the circulation as well as secondary inflammatory effects, for example, an increased aspartate aminotransferase (AST) activity and bilirubin concentration leading to tissue and cellular injuries (Dänicke et al. 2013; Kuzmich et al. 2017). Due to these characteristics, LPS is experimentally often used as a defined immune challenge in animal models investigating the responsiveness of the immune system (Wyns et al. 2015).

Applying this LPS model, we could show recently that both, SoS treatment of maize and LPS-induced systemic inflammation, altered the differential white blood cell counts (total leukocytes, lymphocytes, granulocytes, monocytes) of piglets in an interactive manner (Tran et al. 2018b). However, based on this general evaluation of the white blood count, no information about possible mechanisms of these global changes could be gathered. Thus, lymphocytes were further phenotyped investigating the effects of SoS wet-preservation on lymphocyte subpopulations, such as T helper and cytotoxic T cells. As granulocytes and monocytes directly or indirectly influence the number and function of lymphocytes and other immune cells, we further examined their main functions such as phagocytic activity and capability to mount an oxidative burst.

Material and methods

Animal experiment

The experiment was performed at the experimental station of the Institute of Animal Nutrition, Friedrich-Loeffler-Institute (FLI), Brunswick, Germany, in compliance with the European Community regulations concerning the protection of experimental animals and was approved by the Lower Saxony State Office for Consumer Protection and Food Safety (LAVES), Germany (file number: 33.92-42502-04-13/1153).

All samples were derived from a weaned piglet trial, set up in a 2 × 2 × 2 factorial design (maize batch × sodium sulfite × LPS challenge) comprising an acute LPS challenge at the end of a 42-day feeding period (Tran et al. 2018b). Briefly, two maize batches were produced, a control batch (CON) with a background contamination of the two major *Fusarium* mycotoxins deoxynivalenol (DON) and zearalenone (ZEN) and a batch experimentally inoculated with *Fusarium* spores (FUS), containing high levels of DON and ZEN (Paulick et al. 2015). Each batch of maize kernels was subdivided and wet-preserved (20% moisture, 15 g propionic acid/kg maize), either with or without (+/−) the addition of 5 g SoS/kg maize (Na_2SO_3: CAS-no. 7757-83-7, ≥ 98%, p.a., ACS, water free; Carl Roth GmbH & Co KG, Karlsruhe/Germany) for 79 days. After preservation, the resulting four maize batches were ground and blended into experimental diets containing 35% barley, 27.3% wheat, 10% maize, 23% soybean meal, 1.5% soya bean oil, 1% vitamin and mineral premix, 2.2% synthetic amino acids (HCl-lysine, DL-methionine, L-threonine, L-tryptophan), and a commercial phytase. This resulted in four experimental diets: CON− (control maize, without SoS; 0.09 mg DON/kg feed, ZEN < LOD), CON+ (control maize, with SoS; 0.05 mg DON/kg feed, ZEN < LOD), FUS− (contaminated maize, without SoS; 5.36 mg DON/kg feed, 0.29 mg ZEN/kg feed), and FUS+ (contaminated maize, with SoS; 0.83 mg DON/kg feed, 0.27 mg ZEN/kg feed).

Eighty male castrated crossbred piglets (7.59 ± 0.92 kg) were allotted equally—based on body weight—to the four experimental groups, group-housed (4 piglets/pen; 20 pigs/group = 5 pens/group), and offered feed and water for *ad libitum* consumption. After 42 days, ten pigs of each dietary group were allocated to the challenge trial, while the remaining pigs (n = 10/diet) were slaughtered for further aspects (Tran et al. 2018a). Detailed procedures of the LPS challenge were described in Tran et al. (2018b). Briefly, these piglets were injected intraperitoneally either with 7.5 μg LPS/kg BW (*Escherichia coli* serotype O111:B4, Sigma-Aldrich, Steinheim, Germany, L 2630; CON−/LPS, CON+/LPS, FUS−/LPS, FUS+/LPS; n = 5 per treatment group) or with 0.9% NaCl as placebo (volume ~ 6.5 mL/animal; CON−/NaCl, CON+/NaCl, FUS−/NaCl, FUS+/NaCl; n = 5 per treatment group). Two hours after the challenge, animals were electrically stunned, blood samples taken immediately from neck vessels for further analyses, and pigs then sacrificed by exsanguination. Pigs were immediately dissected, tissue samples of mesenteric lymph nodes and spleen collected and directly placed into a cardioplegia solution (Custodiol, Dr. Franz Köhler Chemie GmbH, Bensheim, Germany) until cell preparation procedures.

Analytical methods

Cell preparations

Cells from mesenteric lymph nodes were isolated by a modified method according to Solano-Aguilar et al. (2000). Briefly, serosa and surrounding tissues from mesenteric lymph nodes were removed. Then, mesenteric lymph nodes were transferred into a culture dish with cold PBS (phosphate-buffered saline), cut medial and longitudinal with a scalpel to obtain a cell suspension. The suspension was filtered with a sieve kept on ice and centrifuged at 300x*g* at 4 °C for 10 min. Next, the supernatant was removed and the pellet was resuspended in HEPES-buffered saline (HBS). After one filtration, this cell suspension was used for staining with antibodies for flow cytometry.

Isolation of splenic cells was performed according to Renner et al. (2012). In brief, spleen tissue was minced with a scalpel, placed into sterile HEPES-buffered solution (HBS), and smoothed through a cell strainer into Petri dishes for erythrocytes' lysis. After centrifugation (250×*g*, 5 min) and washing in HBS, cells were resuspended in RPMI 1640 medium, supplemented with fetal bovine serum, penicillin-streptomycin, mercapto-ethanol, and L-glutamine (Biochrom AG, Berlin, Germany). Subsequently, these cells were stained with antibodies for flow cytometric analysis.

Phenotyping of leukocyte subsets

For phenotyping of leukocyte subsets, single cell suspensions (1×10^6 cells/mL, in duplicate/sample) of cells from either mesenteric lymph node or splenocytes or 50 μl EDTA whole blood were incubated with monoclonal antibodies (Table 1) as double stain (monocytes, B cells) or triple stain (T cells) for 30 min on ice in the dark. After washing, the cell suspensions from tissue samples were resuspended in HBS and analyzed with a FACSCanto™ II flow cytometer (BD Biosciences, San Jose, CA, USA). The whole blood samples were further processed by incubating for 10 min with lysis buffer (BD Pharm Lyse, BD Biosciences, San Jose, CA, USA), centrifugation (5 min, 250×*g*, 4 °C), and resuspension in HBS.

For measurement, an acquisition gate was set for blood peripheral mononuclear cells (PBMC = lymphocytes, monocytes) according to its side scatter and forward scatter characteristics for each tissue (blood, spleen, mesenteric lymph node). A minimum of 10,000 cells was evaluated with FACSDiva™ software 6.1.3 (BD Biosciences, San Jose, CA, USA). The gating strategies for T cells (S1) and B cells (S2) are detailed in supplementary material.

B cells ($CD21^+$) and monocytes ($CD14^+$) were characterized by the expression of their respective epitope. Four T cell subsets were defined as follows: $CD3^+CD4^+CD8^-$ (T helper cells), $CD3^+CD4^-CD8^+$ (cytotoxic T cells), $CD3^+CD4^-CD8^-$ (undifferentiated T cells), and $CD3^+CD4^+CD8^+$ (double-positive T cells). The expression density of each CD epitope per cell was characterized by their mean fluorescence intensity (MFI). The total $CD3^+CD4^-CD8^+$ cells were further separated in two subsets according to their fluorescence intensity classifying cells of low ($CD8^{low}$) or high expression ($CD8^{high}$) of the CD8 marker. Furthermore, the ratio between $CD3^+CD4^+CD8^-$ and $CD3^+CD4^-CD8^+$ cells ($CD4^+/CD8^+$) was calculated.

Intracellular production of reactive oxygen species

Dihydrorhodamine 123 (DHR, Molecular Probes, Eugene, Oregon, USA) is a non-fluorescent molecule capable of passing the cell membrane and can be oxidized to its fluorescent product rhodamine 123 ($R123^+$) by ROS. Thus, DHR is routinely used to measure intracellular ROS generation by flow cytometry. The capacity of ROS production in polymorphonuclear cells (PMN = granulocytes) and PBMC was determined using a FACSCanto™ II flow cytometer (BD Biosciences, San Jose, CA, USA). Fifty microliters of whole blood samples (in duplicates) were incubated for 15 min at 37 °C with 40 mM DHR alone (basal production) or with 20 nM tetradecanoyl-12,13-phorbol acetate (TPA, Sigma Aldrich, Taufkirchen, Germany) in order to stimulate an oxidative burst. After 10 min lysis of erythrocytes (BD Pharm Lyse,

Table 1 List of monoclonal antibodies (mAb) as applied in flow cytometric analysis of leukocytes (FACS™ II Canto flow cytometer, BD Biosciences, San Jose, CA, USA)

Specificity	mAb	Fluorescent dye	Manufacturer
CD3	Mouse Anti-Pig CD3ε	Alexa Fluor®647	BD Bioscience, San Jose, USA
CD4	Mouse Anti-Pig CD4	FITC	AbD, seroTEC, Oxford, UK
CD8	Mouse Anti-Pig wCD8	RPE	AbD, seroTEC, Oxford, UK
CD21	Mouse Anti-Human CD21	PE	BD Biosciences, San Jose, USA
CD14	Mouse Anti-Pig CD14	FITC	AbD, seroTEC, Oxford, UK
Isotype control	mouse IgG1	Alexa Fluor®647	AbD, seroTEC, Oxford, UK
Isotype control	mouse IgG2a	RPE	AbD, seroTEC, Oxford, UK
Isotype control	mouse IgG2b	FITC	AbD, seroTEC, Oxford, UK

BD Biosciences, San Jose, CA, USA), cells were washed with HBS and the formation of $R123^+$ was measured. At least 10,000 cells of each population were analyzed selecting the appropriate gates. Percentage of ROS-producing PMN and PMBC and the intracellular level of ROS production (MFI) were determined.

Phagocytosis assay

The phagocytic activity of PMN and PBMC was assessed by a commercial kit, Phagotest™ (Glycotope Biotechnology, Heidelberg, Germany) according to the manufacturer's instructions. Briefly, 100 µl heparinized whole blood (in duplicates) was incubated with FITC-labelled *E. coli* bacteria for either 10 min at 37 °C or 10 min in an ice bath for negative control. After quenching and lysis of erythrocytes, cells were washed and propidium iodide (PI) was added to stain the DNA. Fluorescence intensity was measured using FACSCanto™ II (BD Biosciences, San Jose, CA, USA). PMN and PBMC cells were gated according to their size and granularity based on measurements of forward and size scatter (S1). At least 10,000 cells of each population were analyzed with FACSDiva™ software 6.1.3 (BD Biosciences, San Jose, CA, USA). Percentage of phagocyting PMN and PMBC as well as the phagocytic capacity per cell was characterized by their MFI.

Statistics

Data were statistically analyzed as a 2 × 2 × 2 factorial design, using PROC MIXED (SAS Institute 2004), with maize batch (control maize vs. *Fusarium*-toxin contaminated maize [FUS]), SoS treatment (with vs. without Na_2SO_3 [SoS]), and LPS challenge (NaCl vs. LPS [LPS]) as well as all their interactions (FUS*SoS, FUS*LPS, SoS*LPS, FUS*SoS*LPS) as fixed factors. Differences were deemed significant at $p < 0.05$ and Student's *t* test was applied as *post hoc* procedure. Results are presented as least square means (LSmeans) and pooled standard error of the mean (PSEM).

Results

T cell subsets in peripheral blood and lymphatic organs

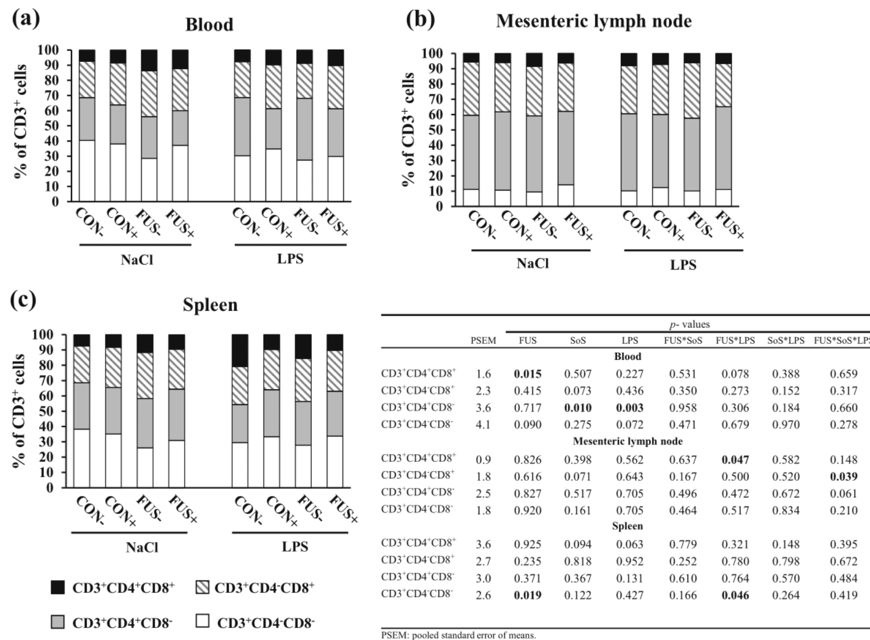
T cells in peripheral blood and lymphatic tissues were characterized by their expression of the CD3 protein complex and further differentiated in four subsets according to the co-expression of CD4 and CD8 epitopes. The distribution of T cell phenotypes as percentage of all $CD3^+$ cells in blood, mesenteric lymph node, and spleen, including their respective *p*-values, are presented in Fig. 1.

In blood (Fig. 1a), we observed a significant impact of the factor FUS ($p_{FUS} = 0.015$), with an increase in double-positive cells ($CD3^+CD4^+CD8^+$) in pigs receiving FUS-contaminated diets (8.4 vs. 11.2%). Furthermore, $CD4^+$ cells ($CD3^+CD4^+CD8^-$) were decreased by SoS treatment ($p_{SoS} = 0.010$, 33.7 vs. 26.6%) but increased in LPS-challenged pigs ($p_{LPS} = 0.003$, 26.0 vs. 34.2%). No interactions were evident in blood T cell subsets.

In mesenteric lymph node (Fig. 1b), a significant interaction between FUS and LPS challenge ($p_{FUS*LPS} = 0.047$) was observed for the double-positive cells ($CD3^+CD4^+CD8^+$) due to a decline in the pooled group FUS/NaCl while the other three groups remained unaltered. Moreover, $CD8^+$ cells ($CD3^+CD4^-CD8^+$) significantly displayed an interaction between FUS, SoS treatment, and LPS challenge ($p_{FUS*SoS*LPS} = 0.039$) due to a clear increase in group FUS−/LPS and a strong reduction in group FUS+/LPS while the other groups remained unchanged.

In spleen (Fig. 1c), the percentage of double-negative cells ($CD3^+CD4^-CD8^-$) was lower in pooled groups FUS/NaCl, CON/LPS, and FUS/LPS compared with group CON/NaCl, which resulted in a significant interaction between the main factors FUS and LPS challenge ($p_{FUS*LPS} = 0.046$).

The intensity of the fluorescence signal as an indicator of cellular activity remained unaffected in total $CD8^+$ T cells (S3a) in lymph node ($4.5 \pm 0.3 \times 10^3$) and spleen ($3.1 \pm 0.8 \times 10^3$). However, in blood, statistical analysis revealed a significant increase of MFI in $CD8^+$ cells due to FUS feeding ($p_{FUS} = 0.008$). Factor SoS showed a contrasting effect ($p_{SoS} =$



	PSEM	*p*- values						
		FUS	SoS	LPS	FUS*SoS	FUS*LPS	SoS*LPS	FUS*SoS*LPS
Blood								
$CD3^+CD4^+CD8^+$	1.6	**0.015**	0.507	0.227	0.531	0.078	0.388	0.659
$CD3^+CD4^-CD8^+$	2.3	0.415	0.073	0.436	0.350	0.273	0.152	0.317
$CD3^+CD4^+CD8^-$	3.6	0.717	**0.010**	**0.003**	0.958	0.306	0.184	0.660
$CD3^+CD4^-CD8^-$	4.1	0.090	0.275	0.072	0.471	0.679	0.970	0.278
Mesenteric lymph node								
$CD3^+CD4^+CD8^+$	0.9	0.826	0.398	0.562	0.637	**0.047**	0.582	0.148
$CD3^+CD4^-CD8^+$	1.8	0.616	0.071	0.643	0.167	0.500	0.520	**0.039**
$CD3^+CD4^+CD8^-$	2.5	0.827	0.517	0.705	0.496	0.472	0.672	0.061
$CD3^+CD4^-CD8^-$	1.8	0.920	0.161	0.705	0.464	0.517	0.834	0.210
Spleen								
$CD3^+CD4^+CD8^+$	3.6	0.925	0.094	0.063	0.779	0.321	0.148	0.395
$CD3^+CD4^-CD8^+$	2.7	0.235	0.818	0.952	0.252	0.780	0.798	0.672
$CD3^+CD4^+CD8^-$	3.0	0.371	0.367	0.131	0.610	0.764	0.570	0.484
$CD3^+CD4^-CD8^-$	2.6	**0.019**	0.122	0.427	0.166	**0.046**	0.264	0.419

PSEM: pooled standard error of means.

Fig. 1 Distribution pattern of four T cell subsets (% of $CD3^+$ cells) in blood (**a**), mesenteric lymph node (**b**), and spleen (**c**) in piglets receiving experimental diets for 5 weeks and subjected to a subsequent acute LPS challenge. Diets contained control (CON) or *Fusarium*-toxin contaminated maize (FUS), wet-conserved with or without 5 g SoS/kg maize sodium sulfite (+/−) and piglets were injected with 7.5 µg LPS/kg BW or 0.9% NaCl. Data represent LSmeans (n = 5) for each T cell subset

0.001) with a decrease in MFI levels due to SoS treatment. In $CD4^+$ cells, the MFI (S3b) was unaltered by any treatment with $2.0 \pm 0.1 \times 10^3$ in blood, $2.4 \pm 0.1 \times 10^3$ in lymph node, and $2.6 \pm 0.1 \times 10^3$ in spleen.

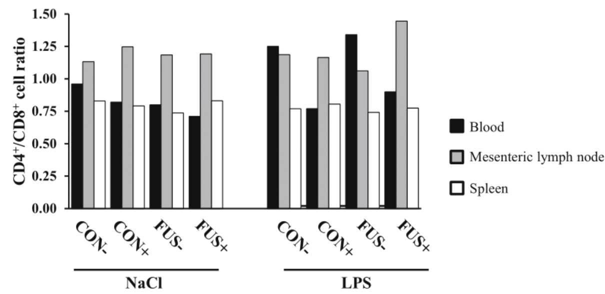
Although some alterations of T cell subpopulations were found in the specimen analyzed, the $CD4^+/CD8^+$ ratio (Fig. 2) was only significantly affected by treatments in blood samples rather than spleen and mesenteric lymph node: SoS treatment (p_{SoS} = 0.003) decreased the $CD4^+/CD8^+$ ratio due to a decrease in the $CD4^+$ cells, whereas LPS (p_{LPS} = 0.011) increased the ratio, likely due to the increase in $CD4^+$ and constancy of $CD8^+$ cells. There was no interaction between the main factors. Furthermore, $CD4^+/CD8^+$ ratio differed significantly due to site of cell residence: the highest $CD4^+/CD8^+$ ratio was observed in the mesenteric lymph node, whereas the $CD4^+/CD8^+$ ratio in blood and spleen appeared comparable ($p_{Localization} < 0.001$).

In pigs, $CD8^+$ T cells are unique as they show two subpopulations with high and with low expression ($CD8^{low}$ and $CD8^{high}$) of the CD8 subsets (Lunney and Pescovitz 1987; Gerner et al. 2009), denoting differences in cellular function. These subpopulations and their respective MFI in peripheral blood are displayed in Fig. 3. The main factor SoS treatment showed a significant effect on the percentage of both $CD8^{low}$ and $CD8^{high}$ cells: $CD8^{low}$ cells were increased whereas $CD8^{high}$ cells were conversely decreased (Fig. 3a). A significant interaction between FUS, SoS treatment, and LPS challenge was found for the MFI (Fig. 3b) of $CD8^{low}$ cells due to an increase in group FUS−/NaCl and a drop in group FUS −/LPS, whereas the other groups remained unaltered. Changes in MFI of $CD8^{high}$ cells were also observed: MFI was increased in pigs receiving FUS-contaminated diets (13.6×10^3 vs. 15.6×10^3), whereas a depressing effect of SoS treatment was shown (15.7×10^3 vs. 13.5×10^3). In mesenteric lymph node, there were no alterations in percentage and MFI of $CD8^{low}$ (67.4 ± 5.8%, $2.7 \pm 0.2 \times 10^3$) and $CD8^{high}$ cells (32.5 ± 5.8%, $8.1 \pm 0.4 \times 10^3$). Similarly, the percentage and MFI of both $CD8^{low}$ and $CD8^{high}$ cells in spleen remained unaltered ($CD8^{low}$: 86.3 ± 3.6%, $2.0 \pm 0.1 \times 10^3$; $CD8^{high}$: 13.5 ± 3.5%, $9.3 \pm 1.0 \times 10^3$).

B cell and monocytes in blood and lymphatic organs

B cells were characterized by the expression of $CD21^+$ epitope. The percentage of $CD21^+$ cells was altered in blood only (Fig. 4a), with a significant interaction between

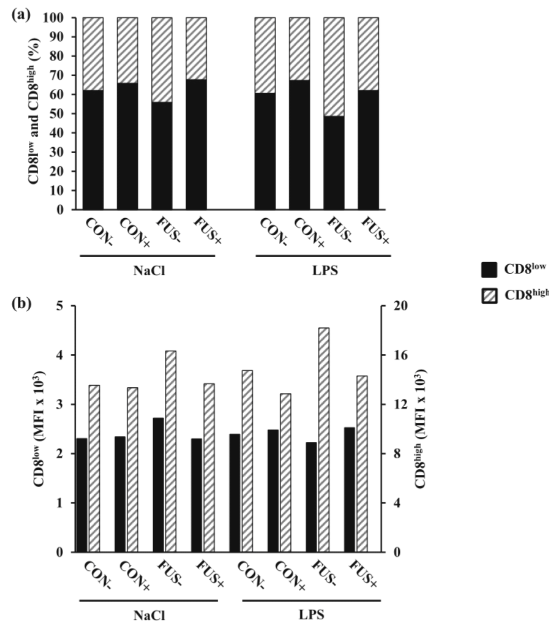
Fig. 2 Relationship between T helper and cytotoxic T cells ($CD4^+$ to $CD8^+$ ratio) in blood, mesenteric lymph node, and spleen (LSMeans, $n = 5$) in piglets receiving experimental diets for 5 weeks and subjected to a subsequent acute LPS challenge. Diets contained control (CON) or *Fusarium*-toxin contaminated maize (FUS), wet-conserved with or without 5 g SoS/kg maize sodium sulfite (+/−) and piglets were injected with 7.5 µg LPS/kg BW or 0.9% NaCl



	PSEM	*p*- values FUS	SoS	LPS	FUS*SoS	FUS*LPS	SoS*LPS	FUS*SoS*LPS
Blood	0.10	0.918	**0.003**	**0.011**	0.808	0.187	0.061	0.982
Mesenteric lymph node	0.10	0.593	0.096	0.722	0.294	0.581	0.400	0.079
Spleen	0.10	0.624	0.580	0.659	0.572	0.980	0.954	0.551

PSEM: pooled standard error of means.

Fig. 3 Proportion of cytotoxic (**a**) $CD8^{low}$ and $CD8^{high}$ T cells in blood (LSMeans, $n = 5$) and their respective mean fluorescence intensity (MFI; **b**) in piglets receiving experimental diets for 5 weeks and subjected to a subsequent acute LPS challenge. Diets contained control (CON) or *Fusarium*-toxin contaminated maize (FUS), wet-conserved with or without 5 g SoS/kg maize sodium sulfite (+/−) and piglets were injected with 7.5 µg LPS/kg BW or 0.9% NaCl



	PSEM	*p*- values FUS	SoS	LPS	FUS*SoS	FUS*LPS	SoS*LPS	FUS*SoS*LPS
Percentage								
$CD8^{low}$ cells	4.0	0.071	**0.004**	0.277	0.211	0.273	0.694	0.911
$CD8^{high}$ cells	4.0	0.071	**0.004**	0.277	0.211	0.273	0.694	0.911
MFI								
$CD8^{low}$ cells	84.6	0.301	0.986	0.857	0.327	**0.050**	**0.003**	**0.010**
$CD8^{high}$ cells	849.9	**0.003**	**0.001**	0.197	0.076	0.470	0.237	0.857

PSEM: pooled standard error of means.

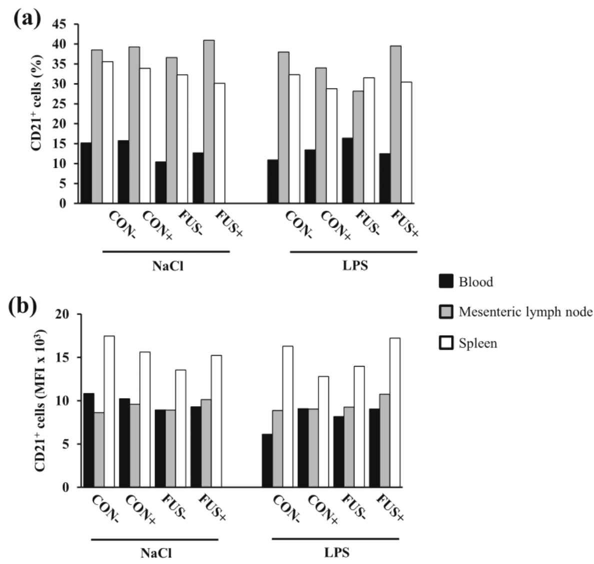


Fig. 4 Distribution of CD21$^+$ B cells (**a**) in blood, mesenteric lymph node, and spleen (LSMeans, n = 5) and their respective mean fluorescence intensity (MFI; **b**) in piglets receiving experimental diets for 5 weeks and subjected to a subsequent acute LPS challenge. Diets contained control (CON) or *Fusarium*-toxin contaminated maize (FUS), wet-conserved with or without 5 g SoS/kg maize sodium sulfite (+/−) and piglets were injected with 7.5 μg LPS/kg BW or 0.9% NaCl

		p- values						
	PSEM	FUS	SoS	LPS	FUS*SoS	FUS*LPS	SoS*LPS	FUS*SoS*LPS
Percentage								
Blood	1.6	0.466	0.748	0.851	0.296	**0.009**	0.351	0.077
Mesenteric lymph node	3.5	0.644	0.215	0.121	0.064	0.685	0.819	0.241
Spleen	2.7	0.423	0.278	0.256	0.790	0.304	0.920	0.699
MFI								
Blood	854.0	0.731	0.149	**0.008**	0.652	0.054	0.102	0.215
Mesenteric lymph node	748.3	0.176	0.080	0.761	0.469	0.550	0.800	0.618
Spleen	1656.4	0.644	0.930	0.738	**0.036**	0.181	0.985	0.497

PSEM: pooled standard error of means.

FUS and LPS: pooled groups FUS/NaCl and CON/LPS were lower compared with their respective counterparts CON/NaCl and FUS/LPS. The MFI of CD21$^+$ cells in blood was significantly reduced in LPS-challenged pigs compared with their placebo counterparts. In spleen, an interaction between FUS and SoS treatment was evident: in CON-fed groups, SoS treatment (CON+) decreased MFI, whereas in their FUS counterparts, the situation was vice versa. There were no changes in CD21$^+$ cells in mesenteric lymph node, neither in percentage nor in MFI.

Monocytes, identified as CD14$^+$ cells by flow cytometry, showed a marked difference between blood, mesenteric lymph node, and spleen (Fig. 5): < 5% in blood, ~ 20% in mesenteric lymph node, and ~ 30% in spleen. In blood, percentage of CD14$^+$ cells was significantly decreased in LPS-challenged pigs (NaCl vs. LPS: 3.9 vs. 2.0%). In both lymphatic tissues, feeding FUS diets significantly increased the CD14$^+$ proportion as compared with CON diets: in mesenteric lymph node from 17.5 to 21.5% and in spleen from 32.2 to 36.5%. There were no significant changes due to other factors or a significant interaction between factors. The MFI of CD14$^+$ cells in blood, mesenteric lymph node, and spleen was not altered by any treatment and its values averaged at $1.5 \pm 0.2 \times 10^3$, $2.5 \pm 0.3 \times 10^3$, and $2.6 \pm 0.2 \times 10^3$, respectively.

Intracellular production of reactive oxygen species

The basal ROS production (=R123$^+$ cells) was measured in PMN and PBMC and the percentage of positive cells as well as the MFI as functional marker is depicted in Fig. 6. Both the proportion of ROS-producing PMN (Fig. 6a) and PBMC (Fig. 6b) were significantly increased in LPS-challenged animals compared with their placebo counterparts. Moreover, the MFI in PMN (Fig. 6c) showed a significant interaction between SoS treatment and LPS challenge: whereas in NaCl

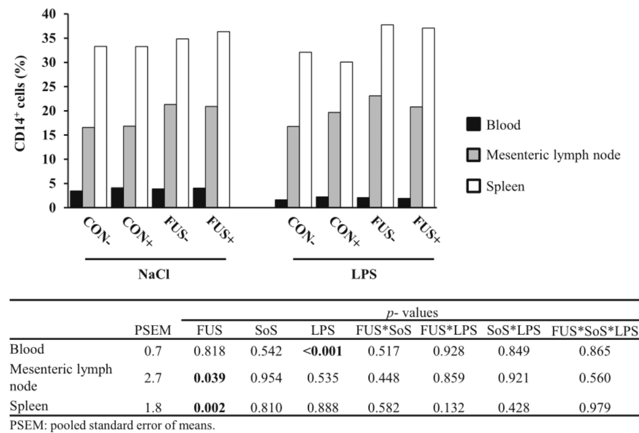


Fig. 5 Proportion of CD14$^+$ monocytes in blood, mesenteric lymph node, and spleen (LSMeans, $n = 5$) in piglets receiving experimental diets for 5 weeks and subjected to a subsequent acute LPS challenge. Diets contained control (CON) or *Fusarium*- toxin contaminated maize (FUS), wet-conserved with or without 5 g SoS/kg maize sodium sulfite (+/−) and piglets were injected with 7.5 μg LPS/kg BW or 0.9% NaCl

		p- values						
	PSEM	FUS	SoS	LPS	FUS*SoS	FUS*LPS	SoS*LPS	FUS*SoS*LPS
Blood	0.7	0.818	0.542	**<0.001**	0.517	0.928	0.849	0.865
Mesenteric lymph node	2.7	**0.039**	0.954	0.535	0.448	0.859	0.921	0.560
Spleen	1.8	**0.002**	0.810	0.888	0.582	0.132	0.428	0.979

PSEM: pooled standard error of means.

groups, there was no marked impact of SoS-treatment, SoS-treatment resulted in a significant decrease in LPS-challenged groups. In PBMC, there was no impact on MFI (Fig. 6d). Both PMN and PBCM were stimulated with TPA in order to induce an oxidative burst and both cell populations showed a clear increase in percentage (PMN 97.8 ± 0.7%; PBMC 24.8 ±

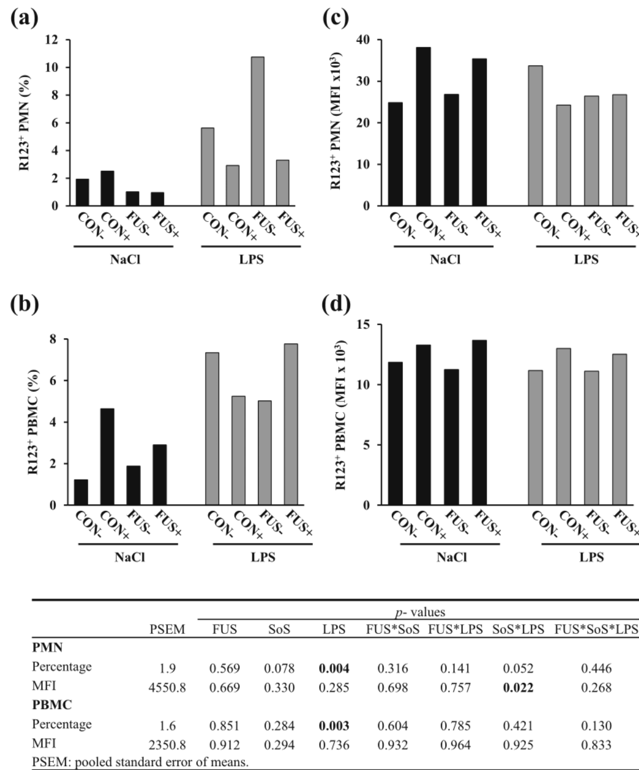


Fig. 6 Intracellular basal (= non-stimulated) ROS production was evaluated in PMN (= granulocytes) and PBMC (incl. monocytes) with the fluochrome rhodamine R123 (R123$^+$). The percentage of R123$^+$ PMN (**a**) and PBMC (**b**) and their respective MFI values (**c**, **d**) are presented as LSMeans ($n = 5$). Piglets received experimental diets for 5 weeks and were subjected to a subsequent acute LPS challenge. Diets contained control (CON) or *Fusarium*-toxin contaminated maize (FUS), wet-conserved with or without 5 g SoS/kg maize sodium sulfite (+/−) and piglets were injected with 7.5 μg LPS/kg BW or 0.9% NaCl

		p- values						
	PSEM	FUS	SoS	LPS	FUS*SoS	FUS*LPS	SoS*LPS	FUS*SoS*LPS
PMN								
Percentage	1.9	0.569	0.078	**0.004**	0.316	0.141	0.052	0.446
MFI	4550.8	0.669	0.330	0.285	0.698	0.757	**0.022**	0.268
PBMC								
Percentage	1.6	0.851	0.284	**0.003**	0.604	0.785	0.421	0.130
MFI	2350.8	0.912	0.294	0.736	0.932	0.964	0.925	0.833

PSEM: pooled standard error of means.

5.4%) and MFI (PMN 80.3 ± 6.8 × 10^3; PBMC 99.0 ± 21.3 × 10^3) after stimulation. This indicates that cells were capable of oxidative burst and responded well to the stimulation. However, stimulating properties and capacity of radical production in TPA-stimulated cells remained unaffected by any treatment in PMN and PBMC.

Phagocytosis of PMN and PBMC

Data on phagocytosis in PMN and PBMC population are represented in Fig. 7. The percentage of phagocytic PMN (Fig. 7a) was not altered by any treatment and remained at its mean value 69.5 ± 5.3%. However, we observed a significant increase in MFI of LPS-challenged pigs (Fig. 7c), indicating a higher phagocytic activity per cell in PMN. The percentage of phagocytic PBMC (Fig. 7b) was significantly altered as indicated by an interaction between factors FUS, SoS treatment, and LPS challenge: in CON-fed groups, LPS challenge decreased percentage of phagocytic cells, independent of SoS presence. However, in FUS-fed groups the LPS effect was dependent on SoS presence: in group FUS −, the percentage of phagocytic cells slightly increased after LPS challenge, whereas in FUS+, the percentage markedly dropped. PBMC of LPS-challenged pigs showed a decrease in MFI (Fig. 7d), indicating a lower phagocytic activity per cell.

Discussion

Based on the cytotoxic effects of DON and sulfiting agents in general, and the reported interactive effects between SoS treatment of maize and LPS-induced systemic inflammation on the leukocyte count subsets in particular (Tran et al. 2018b), we hypothesized that DON and/or SoS treatment of maize would affect the immune traits with regard to the proportion of lymphocyte subsets as well as cellular function of granulocytes and monocytes.

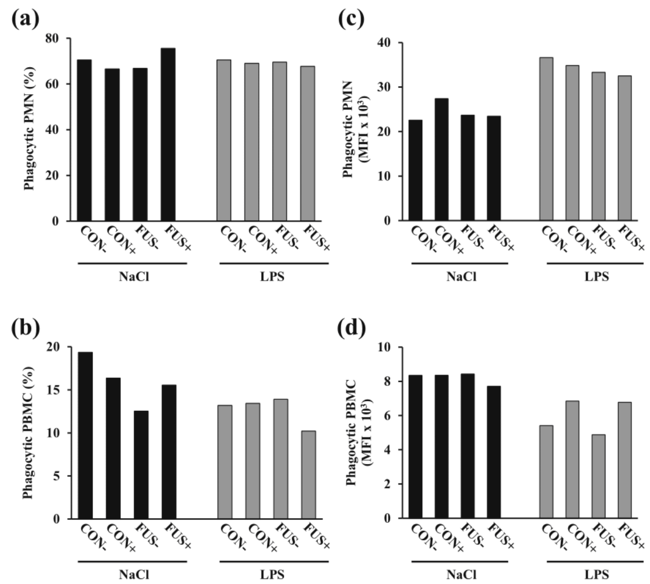


Fig. 7 Phagocytic activity was evaluated in PMN (= granulocytes) and PBMC (= monocytes) with the use of FITC-labelled *E. coli* bacteria. The percentage of phagocyting PMN (**a**) and PBMC (**b**) and their respective MFI values (**c**, **d**) are presented as LSMeans (n = 5). Piglets received experimental diets for 5 weeks and were subjected to a subsequent acute LPS challenge. Diets contained control (CON) or *Fusarium*-toxin contaminated maize (FUS), wet-conserved with or without 5 g SoS/kg maize sodium sulfite (+/−) and piglets were injected with 7.5 µg LPS/kg BW or 0.9% NaCl

	PSEM	*p*- values FUS	SoS	LPS	FUS*SoS	FUS*LPS	SoS*LPS	FUS*SoS*LPS
PMN								
Percentage	5.3	0.846	0.921	0.869	0.436	0.633	0.604	0.408
MFI	5047.2	0.575	0.894	**0.012**	0.786	0.852	0.633	0.690
PBMC								
Percentage	1.5	**0.034**	0.459	**0.008**	0.647	0.270	0.450	**0.038**
MFI	694.5	0.573	0.212	**<0.001**	0.899	0.986	0.059	0.568

PSEM: pooled standard error of means.

LPS-induced systemic inflammation is based on a cascade of release of inflammatory mediators, vascular and physiological changes, and a recruitment of immune cells (van Amersfoort et al. 2003) resulting in a redistribution of immune cells between blood, lymph nodes, spleen, other organs and the site of the stimulus. Initial LPS response includes an activation of monocytes and macrophages due to binding simultaneously to the sCD14 (soluble form) in the blood and to the mCD14 (membrane-bound form) (Metkar et al. 2012). LPS-activated macrophages produce intracellular oxygen-free radicals and microbicidal agents and release various inflammatory mediators, which in turn initiate an acute phase reaction resulting in an array of effects on surrounding tissues (van Amersfoort et al. 2003). In agreement with literature, our results revealed a lower percentage of $CD14^+$ cells in blood in LPS-challenged pigs. This observation is further substantiated by the LPS-reduced absolute monocyte counts in blood. Furthermore, our previous evaluation on the differential blood counts revealed an interactive effect between FUS maize, SoS treatment, and LPS injection for the monocyte counts with a general decrease due to LPS, a marked lower number in pooled groups FUS− and CON+ compared with control group CON−, and an increase in group FUS+ (Tran et al. 2018b). In the present study, a higher percentage of $CD14^+$ cells in both mesenteric lymph node and spleen in pigs fed FUS maize suggests a regulatory effect of DON on the migration of blood monocytes into lymphatic tissues.

As the first response of host defense, the innate immune cells including monocytes and neutrophils activate the phagocytosis and promote the release of oxygen free radicals, also called reactive oxygen species (ROS) in order to kill the invading microbial pathogen (Goodbourn et al. 2000; Fialkow et al. 2007). Although our results showed a significant three-factorial interaction between FUS maize, SoS treatment, and LPS injection for the percentage of phagocytosing PBMC, this effect was seemingly related to the LPS and DON impact with a general alteration in pooled LPS-challenged groups and in pooled FUS groups. Additionally, the significant lower MFI of phagocytosing PBMC in LPS-challenged pigs could reflect the impairment of LPS on the phagocytic activity per cell. In addition to phagocytosis, the release of ROS is responsible for killing the invading microorganism; however, it has been further suggested that an excessive ROS production leads to oxidative stress (Mishra et al. 2014). Besides, LPS-induced oxidative stress has been reported in various studies (Hsu and Wen 2002; Hsu and Liu 2004; Gasparrini et al. 2017). In accordance with the literature, our present results showed an increased proportion in basal ROS-generated PBMC in LPS-challenged pigs. Mitochondria are considered the center of metabolic pathway, a bioenergetic organelle with multiple functions such as energy supply, biosynthesis, and signaling platform for various innate immune signaling pathways. Recently, it has been reported that the mitochondrial respiratory chain in macrophages is adapted for contributing to antibacterial host defense due to ROS signaling to mitochondrial electron transport chain (Garaude et al. 2016). Thus, an increase for ROS generation in PBMC with LPS could reflect the increased metabolic activity and also increased energy demand in monocytes suggesting an impact of LPS on the mitochondrial respiratory chain.

Similar to monocytes/macrophages, the neutrophil granulocytes not only promote phagocytosis but also induce the ROS production in response to LPS (van Amersfoort et al. 2003). The significant effect of LPS on the MFI of phagocytosing granulocytes might be a reflection of a higher phagocytic activity per cell. Our results also revealed that LPS significantly increased the percentage of basal ROS production in granulocytes. Moreover, the significant interaction between SoS treatment and LPS on the MFI of basal ROS formation of PMN would suggest a SoS-modulated LPS effect on this cell type. These observations might be further supported by our previous data on the differential white blood counts, whereby an interactive effect between FUS maize and SoS treatment was observed for the amount of granulocytes with the higher number of granulocytes in the pooled group FUS+ compared with the group FUS− despite a general LPS reduction (Tran et al. 2018b). In a study with human PBMC, Winkler et al. (2006) concluded that SoS suppressed the release of cytokine INF-γ, which is the most important mediator responsible for ROS formation (Karin and Greten 2005; Winkler et al. 2006). Thus, SoS may act as an antioxidant preventing ROS formation due to a reduced INF-γ release, which however was not measured in our study.

The present data revealed that the alterations of T lymphocyte subsets mostly occurred in the peripheral blood samples. In blood, higher proportions of $CD4^+$ T cells, the so-called T helper cells, were observed in LPS-treated piglets, confirming an activated state of the immune system (Gerner et al. 2009; Gerner et al. 2015) after the LPS challenge. This interpretation is further supported by the $CD4^+/CD8^+$ cell ratio, which is regarded as critical in maintaining a stable immunological function (Dou et al. 2013). Looking closer, LPS injection induced an increase of $CD4^+/CD8^+$ cell ratio in LPS-challenged piglets, which agreed well with the findings of another study using a similar LPS challenge protocol in rearing piglets (Stelter et al. 2013). With regard to SoS treatment, lower proportions of $CD4^+$ T cells in blood were found in pigs receiving SoS treated maize, which resulted in lower $CD4^+/CD8^+$ ratio. Winkler et al. (2006) demonstrated in an in vitro study on human PBMC that the food preservative SoS had a suppressive impact on activated T helper cells of the type Th1. Therefore, the SoS-related reduction of $CD4^+$ T cells and a change of $CD4^+/CD8^+$ ratio in our study might be partially attributed to the impact on Th1 cell functionality. Moreover, the release of cytokines is considered the crucial factors for activation of T helper cell types such as IFN-γ, IL-

2, IL-12 (Th1) and IL-4, IL-5, IL-9 (Th2) which however were not measured in the present study (Romagnani 2004; Murr et al. 2005; Winkler et al. 2006).

In the literature, the higher percentage of porcine double-positive T cells ($CD3^+CD4^+CD8^+$) in blood and lymphoid organs as compared with humans has been reported (Zuckermann and Husmann 1996; Waters et al. 1999; Zuckermann 1999). Our results revealed an increase in double-positive T cells in blood when pigs were fed FUS-toxin contaminated maize. Moreover, the significant interaction between FUS maize and LPS challenge for double-positive cells in mesenteric lymph node might reflect a DON-related effect since the percentage of double-positive T cells was only decreased in the pooled group FUS/NaCl. Contrarily, a tendency of an interactive effect between FUS maize and LPS challenge in blood ($p_{FUS*LPS} = 0.078$, Fig. 1) was observed due to an increase in the pooled group FUS/NaCl. Therefore, the higher percentage of double-positive T cells in blood might be related to migrating cells from secondary lymphoid organs such as mesenterial lymph nodes into peripheral blood. The double-positive T cells are considered memory cells and play a role in protective immunity and immune regulation (Zuckermann 1999).

For the double-negative T cells ($CD3^+CD4^-CD8^-$), similar observations have been made: a decrease of this cell type in spleen, whereas a marked increase in blood in pigs fed FUS maize. It has been suggested that in thymus, the double-negative $CD4^-CD8^-$ precursor cells differentiate into $CD4^+CD8^+$ thymocytes, which upon further differentiation lose either CD4 or CD8, giving rise to the mature $CD4^+$ and $CD8^+$ single-positive cells (Gerner et al. 2015). In mise, the extrathymic double-negative T cells seemingly relate to defective differentiation. However, analyses of porcine T cells in blood and secondary lymphatic organs revealed several peculiarities compared with humans and rodent species, whereby the presence of double-negative T cells are predominant in spleen (Saalmüller et al. 1987). Moreover, it has been proposed that the double-negative cells are not homogeneous and might also comprise other subpopulations including B cells and monocytes (Arriëns et al. 1998). Thus, the immune-modulating effects of DON on the double-negative cells might be associated with the alteration of other immune cell types. Our data indicate that the significant impact of FUS maize was only evident in double-positive or double-negative T cell subsets. In the frame of our experimental setup, we are unable to address whether double-negative cells lose their epitopes and transform into double-negative cells or differentiate in other subsets (i.e., $CD8^+$).

Although the alteration of T cell subpopulation only occasionally occurred in the lymphatic tissues, the present results demonstrated significant three-factorial interaction for $CD8^+$ T cells in mesenteric lymph node. It seemed that SoS treatment only of the FUS-contaminated maize group (FUS+/LPS) prevented the LPS-induced increase of $CD8^+$ T cell proportion observed in group FUS−/LPS. This observation might be related to the presence of DON sulfonates, both in systemic and local immune system.

It has been suggested that swine showed a higher percentage of $CD8^+$ T cells in blood compared with humans (Lunney and Pescovitz 1987; Saalmüller et al. 1989; Lorenzen et al. 2015). The $CD8^+$ T cells are known as cytotoxic cells and can attack directly pathogen-infected cells (Gerner et al. 2009). Therefore, it was of interest to examine the $CD8^+$ T cell subsets, which can be subdivided into $CD8^{low}$ and $CD8^{high}$ subsets. $CD8^{high}$ subset is considered the cytotoxic T cells due to a strong cytolytic activity (Gerner et al. 2009), whereas $CD8^{low}$ cells might act as T helper–like cells due to the expression of IFN-γ and IL-4, the signature cytokines of type 1 (Th1) and 2 (Th2) T cells (Lunney and Pescovitz 1987). The effect of DON on both subsets was rarely examined and showed no effect on the $CD8^{high}$ subset in pigs fed diet containing 0.5 mg DON (Ferrari et al. 2009). Contrarily, present results suggested that the MFI (giving an indication of the density of CD epitopes) of $CD8^{high}$ cells in pigs fed FUS maize was increased which was paralleled by a slightly higher percentage of $CD8^{high}$ cells, suggesting that dietary exposure to DON might change the cytotoxic T cell function due to increased expression of CD8 receptor on the T cell surface. The DON effect on the immune response is considered to depend on dose, exposure frequency, and timing of functional immune assay (Pestka et al. 2004). Thus, the reason for the different results between studies might be associated with higher dosage of DON, which was about ten-fold higher in our study.

With regard to SoS treatment, our data revealed significant SoS effects on both $CD8^{low}$ and $CD8^{high}$ cells. Studies on the SoS toxicity showed that SoS has a very low level of mammalian toxicity and is free from carcinogenic activity (Nair and Elmore 2003). Once ingested, SoS and other sulfite salts are metabolized to sulfate by the enzyme sulfite oxidase which is excreted and less toxic than sulfite (Dänicke et al. 2012). However, this conversion is determined by the species-specific enzyme activity (Tejnorova 1978) and appears to be incomplete. Moreover, a very low level of sulfite oxidase activity was found in macrophages and neutrophils compared with other cell types such as hepatocytes. Consequently, non-metabolized sulfite might affect these cell types (Beckspeier et al. 1985). It was shown that sulfite did not suppress the cell viability (Winkler et al. 2006). Additionally, an amount of sulfite still existed in blood when pigs were fed SoS-treated maize, although a higher whole sulfuric concentration was not observed in groups compared with control groups (Tran et al. 2018a). Therefore, these present sulfite concentrations might affect immune cells due to the alteration of CD8 epitope expression.

CD21 is only expressed by mature B cells and the proportion of this cell type is regarded as an indicator for B cell development (Axcrona et al. 1996). Present results demonstrated that the $CD21^+$ B cell proportion in blood was altered due to an interactive effect between FUS and LPS. This observation is further supported by a similar alteration of lymphocyte counts in blood as published earlier (Tran et al. 2018b): a decrease of lymphocytes in group FUS−/NaCl and CON−/LPS, but an increase of this cell type in group FUS−LPS. In addition, a tendency ($p_{FUSxLPS}$ = 0.054) for opposing effects of FUS maize and LPS injection on MFI of $CD21^+$cells in blood was also observed with a similar reduction in pooled groups FUS/NaCl and CON/LPS, suggesting a suppressive effect of DON and LPS on the circulating B cells and might reflect the migration activity of this cell type. However, this putative migration was not paralleled by an altered B cell percentage in lymphatic organs. Interestingly, the significant interaction between FUS maize and SoS treatment for the MFI of splenic $CD21^+$ B cells could hint at a DON-related effect since the MFI level was higher in group FUS+, which reflects some alterations in the expression of CD21 molecules on the B cell surface in lymphatic organs. The CD21 epitope as an indicator for B cell development interacts with CD2 and forms the CD2/CD21 complex, leading to further differentiation of mature B cells. The latter CD2 expression, however, was not measured in our study.

DON might affect immune traits, especially the immunoglobulin A (IgA) production as a specific effect of DON (Döll and Dänicke 2011; Pestka 2003), whereas DON effects on B cells itself were rarely investigated. In horse, Khol-Parisini et al. (2012) observed no effect of DON on the $CD21^+$ B cells when horses were fed highly DON-contaminated oat with 20.2 mg DON/kg oat. Other studies with mice reported that DON has no direct impact on the primary B cells from Peyer's patches or spleen or in cloned B cells, but rather indirectly due to the influence on the T cell subpopulations and macrophages (Pestka 2003).

In conclusion, the results of this study indicate that alterations in the subpopulations of lymphocytes and monocytes mostly occurred in the peripheral blood and only occasionally in the investigated mesenteric lymph node and spleen tissues. In particular, SoS treatment of maize altered the T-lymphocyte subpopulations in blood with a more pronounced effect on $CD8^{low}$ and $CD8^{high}$ subsets, whereas impact of FUS was more notable on the double-positive T cells in the lymphatic tissues. Furthermore, SoS treatment of maize partially suppressed the magnitude of an LPS effect on cellular function, irrespective of diet. Further studies are needed to elucidate the observed effects, in particular with regard to cell function in the various subsets and tissues.

Acknowledgments The authors thank the co-workers of the Institute of Animal Nutrition and the experimental station of the Friedrich-Loeffler-Institut in Braunschweig for their assistance in performing the experiment and analyses.

Funding Open Access funding provided by Projekt DEAL. The authors would like to thank Biomin Holding GmbH (Tulln, Austria) for financial support.

Compliance with ethical standards

The experiment was performed at the experimental station of the Institute of Animal Nutrition, Friedrich-Loeffler-Institute (FLI), Brunswick, Germany, in compliance with the European Community regulations concerning the protection of experimental animals and was approved by the Lower Saxony State Office for Consumer Protection and Food Safety (LAVES), Germany (file number: 33.92-42502-04-13/1153).

Conflict of interest The authors declare that they have no conflict of interest.

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References

Awad WA, Ghareeb K, Böhm J, Zentek J (2010) Decontamination and detoxification strategies for the Fusarium mycotoxin deoxynivalenol in animal feed and the effectiveness of microbial biodegradation. Food Addit Contam 27:510–520

Arriëns MA, Summerfield A, McCullough KC (1998) Differential adhesion molecule expression on porcine mononuclear cell populations. Scand J Immunol 47:487–495

Axcrona K, Gray D, Leanderson T (1996) Regulation of B cell growth and differentiation via CD21 and CD40. Eur J Immunol 26:2203–2207

Beckspeier I, Hinze H, Holzer H (1985) Effect of sulphite on the energy-metabolism of mamalian-tissues in correlation to sulphite oxidase activity. Biochim Biophys Acta 841:81–89

Crispe IN (2009) The liver as a lymphoid organ. Annu Rev Immunol 27:147–163

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 (Na_2S2O_5, SBS), on weaned piglets with a special focus on liver function as determined by the ^{13}C-methacetin breath test. Arch Anim Nutr 62:263–286

Dänicke S, Brüssow KP, Valenta H, Ueberschar KH, Tiemann U, Schollenberger M (2005) 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. Mol Nutr Food Res 49:932–943

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

Dänicke S, Brosig B, Kersten S, Kluess J, Kahlert S, Panther P, Diesing A-K, Rothkötter H-J (2013) The *Fusarium* toxin deoxynivalenol (DON) modulates the LPS induced acute phase reaction in pigs. Toxicol Lett. 220:172–180

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

Dou X, Wang RB, Yan HJ, Jiang SM, Meng XJ, Zhu KL, Xu XQ, Mu DB (2013) Circulating lymphocytes as predictors of sensitivity to preoperative chemoradiotherapy in rectal cancer cases. Asian Pac J Cancer P 14:3881–3885

EFSA (2004) Opinion of the Scientific Panel on contaminants in the food chain on a request from the commission related to Deoxynivalenol (DON) as undesirable substance in animal feed. EFSA J 73:1–41. https://doi.org/10.2903/j.efsa.2004.73

EFSA (2013) Deoxynivalenol in food and feed: occurrence and exposure. EFSA J 11. https://doi.org/10.2903/j.efsa.2013.3379

Ferrari L, Cantoni AM, Borghetti P, De Angelis E, Corradi A (2009) Cellular immune response and immunotoxicity induced by DON (deoxynivalenol) in piglets. Vet Res Commun 33(Suppl 1):133–135

Fialkow L, Wang Y, Downey GP (2007) Reactive oxygen and nitrogen species as signaling molecules regulating neutrophil function. Free Radic Biol Med 42:153–164

Gasparrini M, Forbes-Hernandez TY, Giampieri F, Afrin S, Alvarez-Suarez JM, Mazzoni L, Mezzetti B, Quiles JL, Battino M (2017) Anti-inflammatory effect of strawberry extract against LPS-induced stress in RAW 264.7 macrophages. Food Chem Toxicol 102:1–10

Garaude J, Acín-Pérez R, Martínez-Cano S, Enamorado M, Ugolini M, Nistal-Villán E, Hervás-Stubbs S, Pelegrín P, Sander LE, Enríquez JA, Sancho D (2016) Mitochondrial respiratory-chain adaptations in macrophages contribute to antibacterial host defense. Nat Immunol 17:1037–1045

Gerner W, Kaser T, Saalmüller A (2009) Porcine T lymphocytes and NK cells–an update. Dev Comp Immunol 33:310–320

Gerner W, Talker SC, Koinig HC, Sedlak C, Mair KH, Saalmüller A (2015) Phenotypic and functional differentiation of porcine alphabeta T cells: current knowledge and available tools. Mol Immunol 66:3–13

Goodbourn S, Didcock L, Randall RE (2000) Interferons: cell signalling, immune modulation, antiviral response and virus countermeasures. J Gen Virol 10:2341–2364

He JW, Zhou T, Young JC, Boland GJ, Scott PA (2010) Chemical and biological transformations for detoxification of trichothecene mycotoxins in human and animal food chains: a review. Trends Food Sci Tech 21:67–76

Hsu DZ, Liu MY (2004) Sesame oil protects against lipopolysaccharide-stimulated oxidative stress in rats. Crit Care Med 32:227–231

Hsu HY, Wen MH (2002) Lipopolysaccharide-mediated reactive oxygen species and signal transduction in the regulation of interleukin-1 gene expression. J Biol Chem 277:22131–22139

Ji AJ, Savon SR, Jacobsen DW (1995) Determination of total serum sufite by HPLC with fluorescence detection. Clin Chem 41:897–903

Kabak B, Dobson AD, Var I (2006) Strategies to prevent mycotoxin contamination of food and animal feed: a review. Crit RevFood Sci 46:593–619

Karin M, Greten FR (2005) NF-kappaB: linking inflammation and immunity to cancer development and progression. Nat Rev Immunol 5:749–759

Khol-Parisini A, Hellweg P, Razzazi-Fazeli E, Saalmuller A, Strasser A, Tichy A, Zentek J (2012) Highly deoxynivalenol contaminated oats and immune function in horses. Arch Anim Nutr 66:149–161

Kuzmich NN, Sivak KV, Chubarev VN, Porozov YB, Savateeva-Lyubimova TN, Peri F (2017) TLR4 signaling pathway modulators as potential therapeutics in inflammation and sepsis. Vaccines 5:34–59

Lorenzen E, Follmann F, Jungersen G, Agerholm JS (2015) A review of the human vs. porcine female genital tract and associated immune system in the perspective of using minipigs as a model of human genital Chlamydia infection. Vet Res 46:116–129

Lunney JK, Pescovitz MD (1987) Phenotypic and functional characterization of pig lymphocyte populations. Vet Immunol Immunopathol 17:135–144

Mishra S, Dwivedi PD, Pandey HP, Das M (2014) Role of oxidative stress in Deoxynivalenol induced toxicity. Food Chem Toxicol 72: 20–29

Murr C, Schroecksnadel K, Winkler C, Ledochowski M, Fuchs D (2005) Antioxidants may increase the probability of developing allergic diseases and asthma. Med Hypotheses 64:973–977

Nair B, Elmore AR (2003) Final report on the safety assessment of sodium sulphite, potassium sulphite, ammonium sulphite, sodium bisulphite, ammonium bisulphite, sodium metabisulphite and potassium metabisulphite. Int J Toxicol 22:63–88

Palsson-McDermott EM, O'Neill LA (2004) Signal transduction by the lipopolysaccharide receptor, Toll-like receptor-4. Immunology 113: 153–162

Paulick M, Rempe I, Kersten S, Schatzmayr D, Schwartz-Zimmermann HE, Dänicke S (2015) Effects of increasing concentrations of sodium sulfite on deoxynivalenol and deoxynivalenol sulfonate concentrations of maize kernels and maize meal preserved at various moisture content. Toxins 7:791–811

Paulick M, Winkler J, Kersten S, Schatzmayr D, Frahm J, Kluess J, Schwartz-Zimmermann HE, Dänicke S (2018) Effects of oral exposure to sodium sulphite-treated deoxynivalenol (DON)-contaminated maize on performance and plasma concentrations of toxins and metabolites in piglets. Arch Anim Nutr 72:42–57

Pestka JJ (2003) Deoxynivalenol-induced IgA production and IgA nephropathy-aberrant mucosal immune response with systemic repercussions. Toxicol Lett 140-141:287–295

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

Renner L, von Soosten D, Sipka A, Döll S, Beineke A, Schuberth H-J, Dänicke S (2012) Effect of conjugated linoleic acid on proliferation and cytokine expression of bovine peripheral blood mononuclear cells and splenocytes *ex vivo*. Arch Anim Nutr 66:73–85

Romagnani S (2004) Immunologic influences on allergy and the TH1/TH2 balance. J. Allergy Clin. Immunol 113:395–400

Saalmüller A, Reddehase MJ, Bühring HJ, Jonjić S, Koszinowski UH (1987) Simultaneous expression of CD4 and CD8 antigens by a substantial proportion of resting porcine T lymphocytes. Eur J Immunol 17:1297–1301

Saalmüller A, Hirt W, Reddehase MJ (1989) Phenotypic discrimination between thymic and extrathymic CD4-CD8- and CD4+CD8+ porcine T lymphocytes. Eur J Immunol 19:2011–2016

Schwartz-Zimmermann HE, Paulick M, Dänicke S, Schatzmayr D, Berthiller F (2014) Determination of deoxynivalenol sulphonates in cereal samples: method development, validation and application. World Mycotoxin J 7:233–245

Schwartz HE, Hametner C, Slavik V, Greitbauer O, Bichl G, Kunz-Vekiru E, Schatzmayr D, Berthiller F (2013) Characterization of three deoxynivalenol sulfonates formed by reaction of deoxynivalenol with sulfur reagents. J Agric Food Chem 61:8941–8948

Solano-Aguilar GI, Vengroski KG, Beshah E, Lunney JK (2000) Isolation and purification of lymphocyte subsets from gut-associated lymphoid tissue in neonatal swine. J Immunol Methods 241:185–199

Stelter K, Frahm J, Paulsen J, Berk A, Kleinwachter M, Selmar D, Dänicke S (2013) Effects of oregano on performance and immunmodulating factors in weaned piglets. Arch Anim Nutr 67: 461–476

Tejnorova I (1978) Sulphite oxidase activity in liver and kidney tissue in five laboratory animal species. Toxicol Appl Pharmacol 44:251–256

Tran AT, Kluess J, Berk A, Paulick M, Frahm J, Schatzmayr D, Winkler J, Kersten S, Dänicke S (2018a) Detoxification of *Fusarium*-contaminated maize with sodium sulphite - *in vivo* efficacy with special emphasis on mycotoxin residues and piglet health. Arch Anim Nutr 72:58–75

Tran AT, Kluess J, Berk A, Paulick M, Frahm J, Schatzmayr D, Kersten S, Dänicke S (2018b) Effects of a *Fusarium*-toxin contaminated maize treated with sodium sulphite on male piglets in the presence of an LPS-induced acute inflammation. Toxins (Basel) 10:419. https://doi.org/10.3390/toxins10100419

van Amersfoort ES, van Berkel TJ, Kuiper J (2003) Receptors, mediators, and mechanisms involved in bacterial sepsis and septic shock. Clin Microbiol Rev 16:379–414

Waters WR, Pesch BA, Hontecillas R, Sacco RE, Zuckermann FA, Wannemuehler MJ (1999) Cellular immune responses of pigs induced by vaccination with either a whole cell sonicate or pepsin-digested *Brachyspira (Serpulina) hyodysenteriae* bacterin. Vaccine 18:711–719

Winkler C, Frick B, Schroecksnadel K, Schennach H, Fuchs D (2006) Food preservatives sodium sulphite and sorbic acid suppress mitogen-stimulated peripheral blood mononuclear cells. Food Chem Toxicol 44:2003–2007

Wyns H, Plessers E, de Backer P, Meyer E, Croubels S (2015) *In vivo* porcine lipopolysaccharide inflammation models to study immunomodulation of drugs. Vet Immunol Immunopathol 166: 58–69

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

Zuckermann FA (1999) Extrathymic CD4/CD8 double positive T cells. Vet Immunol Immunopathol 72:55–66

Zuckermann FA, Husmann RJ (1996) Functional and phenotypic analysis of porcine peripheral blood CD4/CD8 double-positive T cells. Immunology 87:500–512

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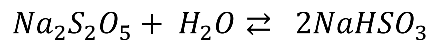
7. GENERAL DISCUSSION

7.1. Reduction of DON in contaminated cereal grains through sulfiting agents

7.1.1. Sodium metabisulfite (SBS)

Several treatments with sulfiting agents in mycotoxin-contaminated feed were found to be effective in reducing the toxin concentrations (Young et al. 1986; Rotter et al. 1995; Ragab et al. 2007). Already over 30 years ago, a study conducted by Young et al. (1986) showed a clear reduction of DON concentration by 98% when aqueous sodium bisulfite was supplemented to DON contaminated wheat on a laboratory scale. The same authors also examined the positive effects observed in another short feeding study where feed intake was restored to the control level when pigs were fed sodium bisulfite treated DON contaminated maize (Young et al., 1987). Sodium bisulfite is a sulfite salt but, in fact, it only exists and is stable in aqueous form. Due to its special chemical characteristics and stability, sodium bisulfite can be formed when other sulfite salts like sodium metabisulfite (SBS) is dissolved in water leading to the formation of two sodium bisulfite atoms as follows:



$$Na_2S_2O_5 + H_2O \rightleftarrows 2NaHSO_3$$

Based on the first success in DON reduction after sodium bisulfite treatment, Dänicke et al. (2005) examined the efficacy of DON reduction when DON-contaminated wheat (7.6 mg DON/kg) was treated with 10 g SBS/kg wheat under special conditions with higher temperature of 100ºC and with 22% humidity for 15 minutes. As a result, the DON concentration strongly decreased to the level of 0.28 mg DON/kg. Furthermore, a similar positive effect of thermal SBS treatment for DON reduction was also revealed in other investigations with DON-contaminated maize (Rempe et al. 2013b).

In addition to thermal treatment, due to the simple application, SBS wet preservation seemingly appears as an efficient procedure for DON reduction at farm level, especially in Germany, where almost 50% of used cereal grains are directly applied on farms (Deutscher Verband Tiernahrung E.V. [DVT] 2009). The first SBS wet preservation study with triticale kernels contaminated with 6.63 mg DON/kg revealed the highest DON reduction by 96% as compared to the initial DON concentration in the presence of 5 g SBS/kg and in combination with 10 g propionic acid/kg at a moisture content of 15% for 63 days (Dänicke et al. 2009). This result is supported by another study with similar wet-preserved conditions (Dänicke et al., 2010c). In this study, approximately 0.04 mg DON/kg of the initial 2.09 mg DON/kg was found as final result corresponding to approximately 2 mg DONS/kg, suggesting a DON reduction due to a

parallel increase in DONS concentration. This observation is further accompanied by another preservation experiment with wheat kernels (Beyer et al. 2010).

Interestingly, the preservation conditions including storage time, moisture and the presence of organic acid might additionally affect the DON reduction due to SBS treatment (Dänicke et al. 2012; Schwartz-Zimmermann et al. 2014b). For a DON reduction by approximately 80% it has been suggested that the storage time should last between 21 and 28 days while the needed moisture content should vary between 13 and 20% (Dänicke et al. 2009; Dänicke et al. 2010c; Beyer et al. 2010; Dänicke et al. 2012).

7.1.2. Sodium sulfite (SoS)

Sodium sulfite (E 221) belongs to the sulfiting agents. It is commonly used as reducing agent in cosmetic formulations (Nair and Elmore, 2003). Besides, it also has anti-oxidative functions and thus is largely used as an antioxidant in many food products, especially those that are fermented (Nair and Elmore, 2003).

As another option besides SBS treatment efficacy regarding DON reduction, also SoS treatment has been investigated in several studies (Schwartz et al., 2013; Schwartz-Zimmermann et al., 2014a, b; Paulick et al. 2015a, b). At laboratory conditions, the efficacy of SoS regarding DON reduction is comparable to that with SBS treatment. For example, in a small-scale experiment over 13 weeks kernel maize containing 44 mg DON/kg was mixed either with 0.5 % (w/w) SoS or SBS in the presence of 15 g/kg propionic acid at 30% moisture content and proved a similar effect of SoS and SBS treatment: after storage for 1 week, DON reduction was approximately 80% and achieved up to 90% after 6 weeks (Schwartz-Zimmermann et al., 2014b). Results from a large scale wet-preservation experiment with SoS also showed a similar DON reduction in the variants treated with ≥ 5 g SoS/kg (Paulick et al. 2015a). Similarly, our recent preservation experiment also revealed the efficacy of SoS treatment on DON reduction when the same preservation conditions were applied (Paper I).

Like SBS treatment, the mode of action of SoS treatment is based on the DONS formation immediately after adding SoS whereby the DON content is reduced (Schwartz et al., 2013; Schwartz-Zimmermann et al. 2014b; Paulick et al. 2015a, b). Schwartz-Zimmermann (2014b) conducted a laboratory scale experiment and revealed that the formation of DONS-3 was pronounced and rapidly reached approximately 50% after 1 day. The concentration of formed DONS-2 increased steadily and exceeded that of DONS-3 after 6 weeks of storage, while DONS-1 was rarely formed. A comparable result was revealed in another preservation study where the variant was stored at 30% moisture (Paulick et al., 2015a). Interestingly, the formed

DONS-3 level decreased continuously during the preservation period, while the DONS-2 level increased at the same time. Schwartz et al. (2013) described that DONS-3 was seemingly instable due to its structure. For the prolonged wet preservation with SoS the formation of DONS-2 is, therefore, preferred. In another preservation experiment, a similar observation was also reported (Paulick et al. 2015b).

Recommendation for effective application of sulfiting agents: Based on the results of the mentioned different preservation experiments, the supplementation of sulfiting agents in DON contaminated cereal grains should be ≥ 2.5 g/kg to ensure a desirable DON reduction. Accordingly, the sulfiting agents´ expenditure necessary to reduce the initial DON concentration by approximately 80% should be at least 72 mg SoS/mg DON and 114 mg SBS/mg DON (Table 1). Moreover, due to chemical reactions of sulfites during preservation conditions, the SO_2 formation is predominant, and thus the sulfiting agents´ expenditure for DON reduction should be considered as the amount of SO_2 equivalents. Accordingly, the ratio of converted SO_2 equivalent to DON should be at least 36 mg and 58 mg/mg DON for SoS and SBS, respectively. The difference between the SBS or SoS needed for a corresponding DON reduction might be explained by occurring chemical reactions whereby 1 mmol SBS is equal to 2 mmol SO_2 equivalent, whereas 1 mmol SoS is equal to 1 mmol SO_2 equivalent. Moreover, it needs to be stressed that numerous chemical reactions can also affect the efficacy of sulfites as preservation agents; for example, the oxidation to sulfates, which leads to the formation of hydroxysulfonates (Rose 1993). Moreover, the chemical structures such as the disulfite bond in proteins, sugars and aldehydes can also react with sulfites and thus reduce the sulfite equivalents needed for DON reduction and for prevention of yeast and bacteria growth (King et al. 1981; Gibson et al. 1988).

Other influential factors concerning the efficacy of the reaction between sulfiting agents and DON are moisture content and storage period. It has been suggested that treatment with SoS required higher moisture amount and longer storage time for DON reduction than SBS (Schwartz-Zimmermann et al. 2014b). At 14% moisture content SoS treatment appeared less effective with <50% DON reduction even after a longer storage period (Table 1). At a higher moisture concentration of 30%, the 50% DON reduction was achieved after one day storage for both SoS and SBS treatments. Due to the presence of propionic acid the needed moisture for 50% DON reduction was lower with 20% moisture concentration. Moreover, the DON reduction was increased up to 85% after storage for 8 weeks. These observations are further

supported by other large scale preservation experiments with 20% moisture content (Figure 1; Paulick et al. 2015b; Paulick et al. 2018; Tran et al. 2018; Bahrenthien et al. 2020).

Regarding the hydrothermal treatment, Young et al. (1987) reported that an increase of moisture contents from 10% to 60% improved the DON reduction from 43% to 79% at similar SO_2 equivalent addition of 23 mg/mg DON. Moreover, a higher SO_2-equivalent expenditure was needed for a nearly complete DON reduction (Table 1; Young et al. 1987; Dänicke et al. 2005; Rempe et al. 2013).

Taken together, for a successful DON reduction a SO_2-equivalent expenditure with at least 36 mg/mg DON should be considered when sulfiting agents are supplemented with the aim of DON reduction in DON-contaminated cereal grains. Furthermore, a moisture content ≥ 20% is required to remain an effective DON reduction as well as the corresponding DONS formation. The addition of propionic acid is not only essential to avoid the growth of microbial spoilage but also necessary to stabilize the formed DONS as well as to contribute to the moisture content required for at least 50% DON reduction (Schwartz-Zimmermann et al. 2014b). Moreover, a storage time of at least 56 days is needed for a maximum DON reduction level, as it was shown in the preservation experiments (Table 1).

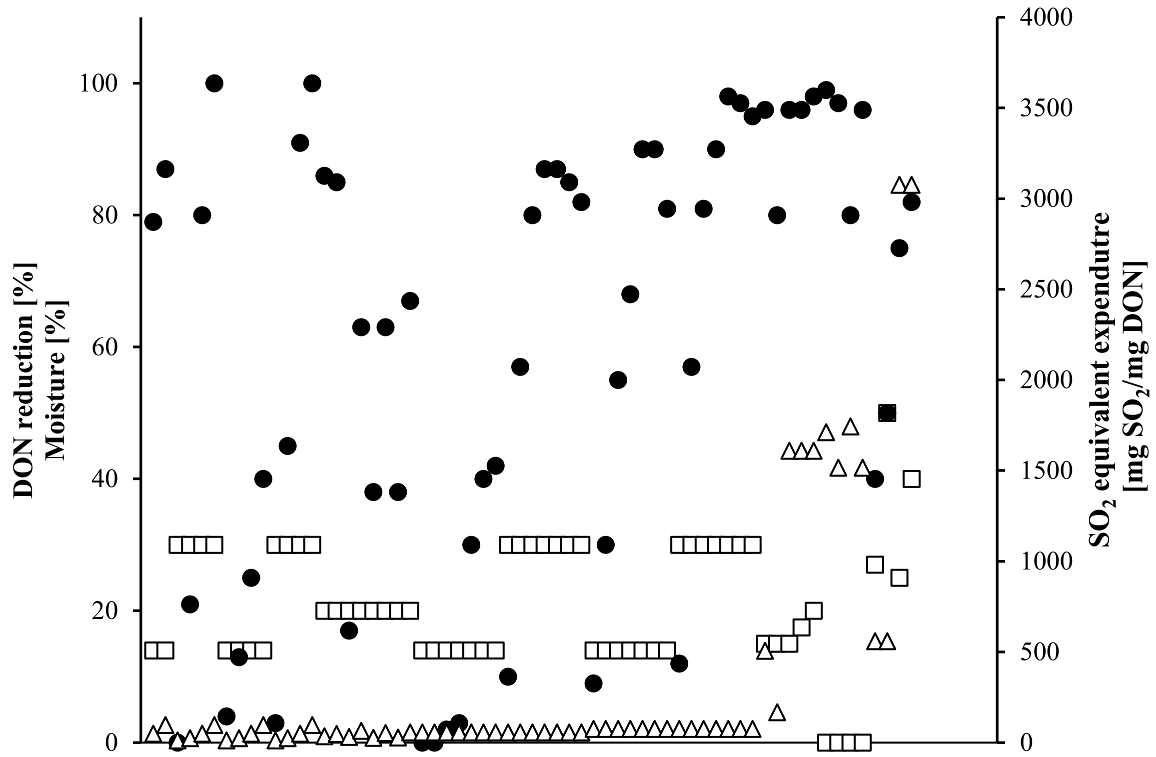


Figure 1. Influence of moisture (□) and SO_2 equivalent expenditure (Δ) on DON reduction (•) in different DON-contaminated cereal grains (Data are collected from various studies and shown in Table 1)

Table 1. Calculation for the needed sulfiting agents´ expenditure as well as the SO_2-equivalent expenditure for 1mg DON reduction

Feed matrix	Substance	Dose (mg substance/kg)	SO_2 - equivalents (mg/kg)	Moisture (%)	Initial DON (mg/kg)	DON Reduction (%)	Substance expenditure (mg substance/mg DON)	SO_2 - equivalent expenditure (mg SO_2/mg DON)	Storage (d)	Temperature (°C)	Duration (min)	References
Storage experiments												
MK	SoS	1250	635	14	51.6	41	24	12	79			Paulick et al. 2015a
MK	SoS	2500	1270	14	51.6	65	48	25	79			
MK	SoS	5000	2540	14	51.6	79	97	49	79			
MK	SoS	10000	5079	14	51.6	87	194	98	79			
MK	SoS	1250	635	30	51.6	0	24	12	79			
MK	SoS	2500	1270	30	51.6	21	48	25	79			
MK	SoS	5000	2540	30	51.6	80	97	49	79			
MK	SoS	10000	5079	30	51.6	100	194	98	79			
MM	SoS	1250	635	14	51.6	4	24	12	79			
MM	SoS	2500	1270	14	51.6	13	48	25	79			
MM	SoS	5000	2540	14	51.6	25	97	49	79			
MM	SoS	10000	5079	14	51.6	40	193.8	98	79			
MM	SoS	1250	635	30	51.6	3	24.2	12	79			
MM	SoS	2500	1270	30	51.6	45	48.4	25	79			
MM	SoS	5000	2540	30	51.6	91	97	49	79			
MM	SoS	10000	5079	30	51.6	100	194	98	79			
MK	SoS	5000	2540	20	69.93	86	72	36	79			Paulick et al. 2018
MK	SoS	5000	2540	20	53.6	85	93	47	79			Tran et al. 2018
MK	SoS	2500	1270	20	38.8	17	64	33	63-70			Bahrenthien et al. 2020
MK	SoS	5000	2540	20	38.8	63	129	65	63-70			
MK	SoS	2500	1270	20	46.9	38	53	27	63-70			
MK	SoS	5000	2540	20	46.9	63	107	54	63-70			

MK	SoS	2500	1270	20	44.4	38	56	29	63-70			
MK	SoS	5000	2540	20	44.4	67	113	57	63-70			
MK	SoS	5000	2540	14	44	0	114	58	0.042			Schwartz-Zimmermann et al. 2014b
MK	SoS	5000	2540	14	44	0	114	58	1			
MK	SoS	5000	2540	14	44	2	114	58	3			
MK	SoS	5000	2540	14	44	3	114	58	7			
MK	SoS	5000	2540	14	44	30	114	58	44			
MK	SoS	5000	2540	14	44	40	114	58	58			
MK	SoS	5000	2540	14	44	42	114	58	94			
MK	SoS	5000	2540	30	44	10	114	58	0.042			
MK	SoS	5000	2540	30	44	57	114	58	1			
MK	SoS	5000	2540	30	44	80	114	58	3			
MK	SoS	5000	2540	30	44	87	114	58	7			
MK	SoS	5000	2540	30	44	87	114	58	44			
MK	SoS	5000	2540	30	44	85	114	58	58			
MK	SoS	5000	2540	30	44	82	114	58	94			
MK	SBS	5000	3367	14	44	9	114	77	0.042			
MK	SBS	5000	3367	14	44	30	114	77	1			
MK	SBS	5000	3367	14	44	55	114	77	3			
MK	SBS	5000	3367	14	44	68	114	77	7			
MK	SBS	5000	3367	14	44	90	114	77	44			
MK	SBS	5000	3367	14	44	90	114	77	58			
MK	SBS	5000	3367	14	44	81	114	77	94			
MK	SBS	5000	3367	30	44	12	114	77	0.042			
MK	SBS	5000	3367	30	44	57	114	77	1			
MK	SBS	5000	3367	30	44	81	114	77	3			
MK	SBS	5000	3367	30	44	90	114	77	7			
MK	SBS	5000	3367	30	44	98	114	77	44			
MK	SBS	5000	3367	30	44	97	114	77	58			

MK	SBS	5000	3367	30	44	95	114	77	94			
T	SBS	5000	3367	15	6.63	96	754	508	63			Dänicke et al. 2009
W	SBS	5000	3367	15	20	80	250	168	36			Beyer et al. 2010
W	SBS	5000	3367	15	2.09	96	2392	1611	56			Dänicke et al. 2010
W	SBS	5000	3367	17.5	2.09	96	2392	1611	56			
W	SBS	5000	3367	20	2.09	98	2392	1611	56			
W	SBS	10000	6733	-	3.93	99	2545	1713	42	4		Richter et al. 1996
W	SBS	10000	6733	-	4.44	97	2252	1517	42	4		
W	SBS	10000	6733	-	3.86	80	2591	1744	42	25		
W	SBS	10000	6733	-	4.44	96	2252	1517	42	25		
MM	SHS	10000	6154	27	11	40	909	559	4			Young et al. 1987
MM	SHS	10000	6154	50	11	50	909	559	4			
MM	SHS	20000	12308	25	4	75	5000	3077	4			
MM	SHS	20000	12308	40	4	82	5000	3077	4			
Hydrothermal experiments												
MM	SHS	10000	6154	10	540	34	19	11		120	60	Young et al. 1987
MM	SHS	10000	6154	30	540	41	19	11		120	60	
MM	SHS	10000	6154	60	540	49	19	11		120	60	
MM	SHS	20000	12308	10	540	43	37	23		120	60	
MM	SHS	20000	12308	30	540	61	37	23		120	60	
MM	SHS	20000	12308	60	540	79	37	23		120	60	
MM	SHS	50000	30769	10	540	65	93	57		120	60	
MM	SHS	50000	30769	30	540	87	93	57		120	60	
MM	SHS	50000	30769	60	540	95	93	57		120	60	
WM	SBS	10000	6733	22	7.6	96	1316	886		100	15	Dänicke et al. 2005
MM	SBS*	5000	3367	17	46	99	109	73		80	30	Rempe et al. 2013
MM	SBS*	5000	3367	17	46	99	109	73		80	30	
MM	SBS*	5000	3367	17	46	83	109	73		80	30	
MM	SBS**	5000	3367	17	46	64	109	73		80	30	

MM	SBS**	5000	3367	17	46	74	109	73		80	30	
MM	SBS**	5000	3367	17	46	92	109	73		80	30	

* treatment with methylamine; ** treatment with methylamine and $Ca(OH)_2$; MK: maize kernel; MM: maize meal; T: triticale; W: wheat; WM: wheat meal SoS: sodium sulfite; SBS: sodium metabisulfite; SHS: sodium bisulfite

7.2. Comparative toxic effects of DON, DON-sulfonates and sulfiting agents on animal health

7.2.1. DON and DONS

In the literature, there are several investigations on the toxicity of DON and its derivate DONS including DONS-1, -2 and -3 (Young et al. 1987; Richter et al. 1996; Beyer 2009; Dänicke et al. 2008, 2010; Schwartz-Zimmerman et al. 2014a; Paulick et al. 2015b). In their early study, Young et al. (1987) found that the pure DON induced emesis when 0.096 and 0.160 mg DON/kg body weight was orally administered via soft plastic feeding tubes inserted down the esophagus into the stomach of pigs weighing 30 kg, while the same amount of DONS showed no effect in all dosed pigs. These acute DON effects are in accordance with the reported lowest observed adverse effect level (LOAEL) of other investigations (Young et al. 1983; Pestka et al. 1987; Prelusky and Trenholm 1993). In addition, the acute DON-induced vomiting was also identified when 0.053mg DON/kg body weight was intravenously injected to pigs (Goyarts and Dänicke 2006).

Besides the vomiting effect, the cytotoxicity of DON and DONS was also investigated. There are, however, only a few studies with porcine primary cells (Goyarts et al. 2006; Döll et al. 2009; Dänicke et al. 2010; Tanaru et al. 2010; Diesing et al. 2011). Especially porcine primary blood mononuclear cells (PBMC) appear to be useful for investigating DON and DONS toxicity (Gutleb et al. 2002). Goyart et al. (2006) examined the adverse DON effects on the immune function using different proliferation assays such as BrdU and MTT assays. The IC_{50} (inhibition concentration by 50% compared with untreated control) of DON for PBMC amounted to 0.7 and 1.0 µM in BrdU and MTT assay, respectively. Similar results were reported in another investigation (Dänicke et al. 2010b), too. With regard to other porcine immune cells, analysis of pulmonary alveolar macrophages also showed the same DON effect (Döll et al. 2009a), whereas an investigation on the porcine splenocytes suggested that this cell type responded more sensitively to DON with lower concentration (IC_{50}) of 0.27 µM (Tanaru et al. 2010). In comparison to DON, the DONS showed no adverse effects on the PBMC proliferation until 17 µM DONS concentration in the tested conditions (Dänicke et al. 2010b). In another recent study on the toxicity of DONS derivates, DONS-1, -2 and -3 as compared to DON were further investigated (Schwartz-Zimmerman et al. 2014a). The researchers found that the DONS-1 appeared to be non-toxic in all experimented conditions. The DONS-2 and -3 showed an effect on the ribosomes with the IC_{50} values of 44 and 50 µM, respectively. In comparison with the IC_{50} of DON with 1.7 µM, both DONS-2 and -3 are much less toxic than DON by the factors

29 and 33. Furthermore, the cell viability of IPEC-J2 cells was only reduced to 60% at the highest DONS-2 concentration of 54 µM, while the IC_{60} of DON was 0.7 µM (Schwartz-Zimmermann et al. 2014a). Similarly, Paulick et al. (2018a) found that the IC_{50} of DONS-2 level of 32.71 µM affected the viability of PBMC. Compared to the IC_{50} of DON level of 0.84 µM, DONS-2 was much less toxic than DON by the factor 73. Taken together, the major effects of DON including vomiting and cytotoxicity were obviously reduced or even non-toxic for DONS derivates.

As discussed above, under physiological conditions the formed DONS can be converted back to DON. Thus, the DON and DONS residues in various physiological samples of pigs fed the SBS or SoS treated diets were further examined. Blood plasma or serum appeared to be useful for detecting ingested DON (Dänicke et al. 2008b). After applying SBS in a preservation experiment with triticale kernels contaminated with 2.31 mg DON/kg, the median plasma DON concentration of piglets fed a *Fusarium*-contaminated diet (FUS diet) amounted to 10.5 ng/ml. In the groups fed the *Fusarium*-contaminated SBS-treated diet (FUS-SBS diet), the plasma DONS levels were detected with a median concentration of 15.5 ng/ml, whereas the corresponding DON level was only 2ng/ml. These findings were comparable with the measured DON and DONS levels in the corresponding FUS and FUS-SBS diets (Dänicke et al. 2010a). In the preservation study using SoS, similar observations were found (Paulick et al. 2018a). However, data from plasma samples of the FUS-SoS diet showed only DONS-2 with a median level of 2.28 ng/ml, whereas numbers of DONS-1 and DONS-3 were lower than the indicated limits of detection. The difference between studies might arise from the different used analysis methods. While in the study of Dänicke et al. (2010a) the various DON sulfonates were not discriminated, the experiment by Paulick et al. (2018a) reported individual DON sulfonates. These observations led to the speculation that the formed DONS and also further DONS derivates would be stable under the physiological conditions of the porcine gastro-intestinal tract. Thus, the DONS appear as desirable metabolites for DON reduction. Moreover, it is known that ingested DON can be degraded and transformed into its non-toxic form de-epoxy DON (DOM-1) due to the microbes in the rumen or in the porcine digestive tract (Dänicke et al. 2004a; Pestka and Smolinski 2005). Due to the special distribution of porcine microbes being located almost in the hindgut while the unmetabolized DON resorption occurs already in the upper part of the small intestine, the contribution of this metabolic pathway for DON inactivation is limited (Dänicke et al. 2004a; Eriksen et al. 2002). Furthermore, conjugation with glucuronic acid and sulfation are other metabolic pathways for DON and DOM-1 which

in turn can increase water solubility and thus promote their excretion via bile and urine (Dänicke and Brezina 2013). In addition to blood samples, analytical results from urine and bile samples can be used as further indicators for the diagnosis of exposure to DON and its metabolite DOM-1. Brezina et al. (2014) detected the highest DON residue level in urine of female piglets fed diets with up 4.52 mg DON/kg. A similar result was obviously detected in our study with castrated male piglets fed diets containing 5.36mg DON/kg (Paper I).

7.2.2. Sulfiting agents

Besides the evaluation of the toxicity of DON sulfonates, also possible adverse effects of sulfiting agents need to be evaluated.

7.2.2.1. SBS

Data from piglets fed SBS-treated diets revealed a significant increase of total plasma protein and a trend for increased albumin levels (Dänicke et al. 2008a). Furthermore, the authors also found an alteration of liver functions when using the ^{13}C-methacetin breath test. In another study conducted by the same researcher group, SBS treatment decreased the proliferation of PBMC as indicated by a lower stimulation index (representing the ratio between the absorbance of concanavalin A stimulated and unstimulated PBMC) (Dänicke et al. 2010b).
It has been reported that the toxic effect of sulfite is based on the oxidation of sulfite to sulfite radicals leading to an initiation of lipid peroxidation (Elmas et al. 2005). The malondialdehyde (MDA) levels, known as an indicator of lipid peroxides were significantly increased in rat kidney and liver when 520 mg SBS/kg per day was orally dosed. In a pig study, multifocal renal glomerulosclerosis was found in three out of 16 piglets fed SBS-treated uncontaminated and *Fusarium*-contaminated diets. Additionally, the proportion of sclerotic glomeruli was also increased in pigs fed both SBS treated diets. However, these observations were not supported by any statistical significance (Dänicke at al. 2008a).

7.2.2.2. SoS

Results from biochemical traits revealed no effect of SoS treatment on the liver function and integrity (Paper II). However, an alteration concerning the differential white blood count was observed with a statistically significant interaction between SoS treatment and LPS induced acute phase reaction suggesting possible effects of SoS treatment under inflammatory conditions. In order to evaluate this effect, the phenotypes of lymphocytes, the main function of granulocytes and monocytes such as phagocytic activity and capability to mount an oxidative burst were investigated (Paper III). It was shown that the proportions of $CD8^{low}$ and $CD8^{high}$

cells were altered in the presence of SoS treatment. It has been suggested that the ingested sulfite amount consisting of sulfite salts like SoS was metabolized by the enzyme sulfite oxidase to sulfate and then excreted via urine (Dänicke et al. 2012). However, due to its limited capacity in macrophages and neutrophils, a certain amount of non-metabolized sulfite might still exist in the blood samples and contribute to the alteration of $CD8^{low}$ and $CD8^{high}$ subsets. Interestingly, statistical analysis revealed a significant interaction between SoS treatment and LPS on the MFI level of ROS formation in granulocytes. On closer inspection, SoS treatment increased the number of granulocytes in those pigs fed FUS SoS-treated diets due to an interaction between FUS maize and SoS treatment (Paper II). It is clear that SoS is already being used for food preservation reasons due to its antioxidant and antimicrobial effects and is currently registered as food additive E221 (Nair and More 2003). As a result of the mentioned investigations, it can be assumed that SoS may, furthermore, act as antioxidant in order to prevent the LPS-induced ROS production.

8. CONCLUSIONS

The present preservation experiment with SoS in DON-contaminated maize achieved a desirable DON reduction and inactivation. The inclusion of treated feed in a diet for rearing piglets overcame the negative effects of DON on the growth performance. Moreover, clearly decreased concentrations of DON and its metabolites in blood as well as in various specimens (urine, bile and liquor) indicated an efficient DON inactivation in the maize. The unaltered haematological and biochemical parameters showed no undesirable effects on the animals´ health derivated from the SoS-treated maize or chemical substances in the applied dosages. During the challenged stress situation induced by LPS, furthermore, similar observations were found with the expception of the alteration of neutrophils. Other alterations of several immunological parameters suggested that an interaction between SoS treatment and stress factors like LPS may have occurred and influenced the immune cells. Therefore, further investigations are needed to clarify not only the efficacy of SoS treatement for DON reduction but also the safety of SoS treatment concerning the health and performane of pigs before applying for permission of use as a feed additive for pigs.

9. SUMMARY

Deoxynivalenol (DON) is one of the most common *Fusarium* mycotoxins due to its frequent occurrence with relevant concentrations in cereals. Moreover, DON is known to be stable under the conventional milling processes. Pigs appear to be the most sensitive species to DON effects with a major feed intake depression, inhibition of protein synthesis as well as immunomodulation. As cereal grains such as wheat or maize are the main components in the feedstuffs of pigs, investigations on possibilities to reduce the DON concentrations therein or inactivate its adverse DON effects are needed. Recently, wet-preservation with sodium sulfite (SoS) in DON contaminated maize has proven positive effects due to its high efficacy for DON reduction and easy application, especially at the farm level. In order to evaluate the effectiveness of DON reduction and the effects of the SoS treatment itself a feeding trial with piglets was conducted according to a two by two factorial design. Inoculated *Fusarium* toxin-contaminated (FUS) and control maize (CON) were included at a proportion of 10% into a diet either untreated (-) or treated (+) with SoS. Eighty castrated male piglets (7.57 ± 0.92 kg BW) were assigned to four groups: CON− (control diet, with 0.09 mg DON and <0.01 mg ZEN/kg diet), CON+ (diet CON−, wet-preserved with 5 g SoS/kg maize; containing 0.05 mg DON and <0.01 mg ZEN/kg diet), FUS− (diet with *Fusarium*-contaminated maize; containing 5.36 mg DON and 0.29 mg ZEN/kg diet), and FUS+ (diet FUS−, wet-preserved with 5 g SoS/kg maize; resulting in 0.83 mg DON and 0.27 mg ZEN/kg diet). After 42 days, 10 animals per group were slaughtered for the dissection of organs and collection of chyme, urine and blood for different analyses and 10 pigs per group entered an LPS-challenge to be discussed further along the line. For mycotoxin analysis, urine, bile and *liquor cerebrospinalis* were also sampled. The DON residues were found in all tested samples in pigs fed the FUS diets (untreated and SoS-treated) with highest concentration in urine samples as the main excretory route of this mycotoxin. The metabolite DOM-1 was also detected in almost all samples. An obvious DON reduction with approximately 75% was detectable in the group FUS+, indicating an effective DON reduction process with SoS treatment. ZEN levels were found in all urine, bile and liquor with the highest levels in bile samples, whereas their metabolites were only observed in urine and bile. These mycotoxin residues remained unaffected in the presence of SoS treatment. Analysis of blood samples revealed the influence of DON on the differential white blood counts with an increase of the total count of leukocytes and segmented neutrophils in pigs fed the FUS diets, irrespective of SoS treatment. A similar finding was detectable when the albumin concentration was decreased in the pooled group FUS. Interestingly, the NO production that had decreased

through DON was recovered in the presence of SoS treatment with an increase in the group FUS+ showing an effect of SoS on the redox level.

As sulfiting agents, both SBS and SoS have similar modes of action for DON reduction through the formation of DONS including DONS-1, -2 and -3. Although the factors moisture content, pH value and storage time play a crucial role for the efficacy of SBS and SoS in reaction with DON, also unspecific effects on the physiological samples can be found after SoS or SBS treatment. Moreover, there is evidence for the synergistic toxic effects of DON and LPS on the immune system. Therefore, the feeding effects combined with SoS treatment were investigated in a porcine LPS challenge. After a feeding period of 42 days, pigs of each dietary group were injected intraperitoneally with either 7.5µg LPS/kg BW (CON-/LPS, CON+/LPS, FUS-/LPS, FUS+/LPS; n = 5 per group*LPS) or with 0.9% NaCl as placebo (CON-/NaCl, CON+/NaCl, FUS-/NaCl, FUS+/NaCl; n = 5 per group*NaCl). Within a period of 2 h, starting 15 min before the LPS challenge until 120 min after the injection clinical symptoms were recorded and blood samples were taken once directly before slaughter for further evaluation of TNF-α, haematology, clinical chemistry as well as the redox status. The body temperature and TNF-α levels were obviously increased in the LPS-injected piglets. A further LPS injection effect was found regarding the total leukocyte count with strong reduction until even lower than the physiological reference range, indicating an efficient application model of LPS-induced systemic inflammation. Analysis of different tested parameters showed almost no effect of SoS treatment except for neutrophils, whereby the alteration was more pronounced in pigs fed the FUS+ diet.

In order to further evaluate such alterations, as a part of the present study, the lymphocytes including its subsets were phenotyped and analysed using flow cytometry. Moreover, the main functions of granulocytes and monocytes such as phagocytosis and capacity to mount an oxidative burst were also investigated for assessing its effects on other immune cells. As consequent immune responses to LPS, the activation of monocytes due to alteration of $CD14^{+}$ cell levels was detected in blood samples of LPS-injected pigs. Moreover, an increase in the percentage of basal ROS production in granulocytes as well as in the $CD4^{+}/CD8^{+}$ ratio further supported the success of the LPS challenge model. With regard to SoS treatment, an influence on the basal ROS production of granulocytes was shown due to the reduction of the mean fluorescence intensity in the LPS-injected pigs, suggesting an LPS-modulated effect. Moreover, other effects of SoS treatment on the lymphocyte subsets were found but only in the blood

samples. Especially, both the $CD8^{low}$ and $CD8^{high}$ T-cells seemingly appeared sensitive to the presence of sulfite content in blood.
In conclusion, the wet-preserved SoS treatment demonstrated an effective approach for DON inactivation with strong reduction of DON concentration accompanied by an increase of the formed DONS derivates in the highly *Fusarium*-contaminated maize. In the present feeding trial, there were no negative effects of SoS treatment on the piglet performance and the health-related parameters. Furthermore, the adverse DON effects could be inactivated in the presence of SoS. Additionally, SoS treatment can modulate the negative LPS effects in an experimental LPS-induced inflammation. There are, however, also some indications for immune effects of SoS treatment which are independent of the initial *Fusarium* toxin and the LPS-induced inflammation. Therefore, further investigations with regard to the immune system are needed to prevent harmful effects of SoS treatment on animal health.

10. ZUSAMMENFASSSUNG

Deoxynivalenol (DON) ist eines der am weitesten verbreiteten *Fusarium*-Mykotoxine, welches sehr häufig mit relevanten Konzentrationen in Getreide vorkommt. Außerdem ist DON unter den konventionellen Mahlprozessen als verhältnismäßig stabil bekannt. Schweine scheinen die empfindlichste Tierart gegenüber DON Effekten zu sein, denn sie weisen die größte Reduktion der Futteraufnahme, Inhibition der Proteinsynthese sowie Immunmodulation auf. Getreidekörner wie Weizen oder Mais sind die Hauptkomponenten von Schweinefutter; deswegen werden Untersuchungen über die Möglichkeiten einer Reduktion der darin enthaltenen DON Konzentrationen oder einer Inaktivierung ihrer negativen Effekte benötigt. In jüngster Zeit zeigte die Feuchtkonservierung mit Natriumsulfit (SoS) in DON-kontaminiertem Mais positive Auswirkungen wegen ihrer hohen Effektivität für eine DON Reduktion und der besonders für landwirtschaftliche Betriebe simplen Anwendungsmöglichkeiten. Zur Evaluierung der Effektivität der DON Reduktion und der Effekte der SoS-Behandlung selbst wurde ein Fütterungsversuch nach einem zwei mal zwei faktoriellen Design durchgeführt. Nicht behandelter Mais (CON) und durch Inokulation mit *Fusarium*-Toxin kontaminierter Mais (FUS) wurde zu einem Anteil von 10 % in Futter eingemischt, entweder unbehandelt (-) oder behandelt mit SoS (+). Achtzig kastrierte männliche Absatzferkel (7,57 ± 0,92 kg KG) wurden in 4 Fütterungsgruppen aufgeteilt: CON- (Kontrollfutter mit 0,09 mg DON und <0,01 mg ZEN/kg Futter), CON+ (Kontrollfutter mit feuchtkonservierter Behandlung mittels 5 g SoS/kg Mais; enthaltend 0,05 mg DON und <0,01 mg ZEN/kg Futter), FUS- (Futter mit *Fusarium*-kontaminiertem Mais; enthaltend 5,36 mg DON und 0,29 mg ZEN/kg Futter) und FUS+ (FUS-Futter mit feuchtkonservierter Behandlung mittels 5 g SoS/kg Mais; im Ergebnis 0,83 mg DON und 0,27 mg ZEN/kg Futter). Nach 42 Fütterungstagen wurden 10 Tiere je Gruppe zur Untersuchung der Organe und Gewinnung von Chymus, Harn und Blut für verschiedene Analysen geschlachtet und 10 Tiere wurden einem LPS-Challenge unterzogen, die im Weiteren diskutiert wird. Zur Mykotoxinanalytik wurden ferner Proben aus Harn, Galle und Liquor gesammelt. Rückstände von DON wurden in allen Proben der mit FUS Futter gefütterten Ferkel gefunden, mit der höchsten Konzentration im Harn, dem Hauptexkretionsweg dieses Toxins. Das Stoffwechselprodukt DOM-1 wurde auch in fast allen Proben detektiert. Eine eindeutige DON Reduktion mit ungefähr 75 % wurde in der Gruppe FUS+ gefunden, wodurch sich die SoS-Behandlung als effektiver DON Reduktionprozess erwies. ZEN Konzentrationen wurden in allen Harn-, Galle- und Liquorproben, mit den höchsten Werten in Galleproben, gefunden, während ihre Metaboliten nur in Harn und Galle beobachtet wurden. Diese

Mykotoxinrückstände erschienen unbeeinflusst von der SoS-Behandlung. Die Blutanalytik zeigte einen Einfluss von DON auf das Differentialblutbild mit einem Anstieg der Gesamtzahl von Leukozyten und segmentierten Neutrophilen in den mit FUS Futter gefütterten Schweinen, und zwar unabhängig von der SoS-Behandlung. Ein ähnliches Resultat zeigte sich über den Abfall der Albuminkonzentration in der gepoolten FUS Gruppe. Interessanterweise stieg die durch DON reduzierte NO Produktion nach einer SoS-Behandlung in der Gruppe FUS+ erneut an, wodurch sich ein Effekt von SoS auf das Redox-Niveau zeigte.

Als Sulfite haben sowohl SBS als auch SoS durch die Formation von DONS wie DONS-1, -2 und -3 einen ähnlichen Wirkungsmechanismus auf die DON Reduktion. Obwohl viele Faktoren wie Feuchtigkeit, pH-Wert und Lagerungszeit eine wichtige Rolle für die Effizienz von SBS und SoS in der Reaktion mit DON spielen, können auch unspezifische Effekte auf die physiologischen Proben nach SoS- oder SBS-Behandlung vorkommen. Außerdem gibt es auch Hinweise auf synergistische toxische Auswirkungen zwischen DON und LPS auf das Immunsystem. Aus den genannten Gründen wurden Fütterungseffekte von SoS in einer LPS-Challenge mit Schweinen untersucht. Nach der Fütterungsphase über 42 Tage wurden 10 Tiere je Fütterungsgruppe einem Challenge unterzogen, entweder mit einer intraperitonealen Injektion von 7,5µg LPS/kg KG (CON-/LPS, CON+/LPS, FUS-/LPS, FUS+/LPS; n = 5 je Gruppe*LPS) oder mit 0,9% NaCl als Placebo (CON-/NaCl, CON+/NaCl, FUS-/NaCl, FUS+/NaCl; n = 5 je Gruppe*NaCl). Über einen Zeitraum von 2 Stunden mit Beginn 15 Minuten vor der LPS Challenge bis 120 Minuten nach der Injektion wurden die klinischen Symptome aufgenommen und das Blut einmalig direkt vor der Schlachtung für ausführliche analytische Auswertungen von TNF-α, Hämatologie, klinischer Chemie sowie Redoxstatus beprobt. Körpertemperatur und TNF-α Level waren in den LPS-injizierten Ferkeln eindeutig angestiegen. Ein weiterer Effekt der LPS Injektion wurde bei der Gesamtzahl an Leukozyten gefunden; hier wurde eine deutliche Reduktion sogar noch unter den physiologischen Referenzbereich aufgezeigt und erwies sich als Indikation für die Effizienz des Einsatzes einer LPS-induzierten systemischen Inflammation. Die Analytik weiterer Proben zeigte keine Wirkung der SoS-Behandlung außer bei den Neutrophilen, wobei die Veränderung bei diesen Zelltypen in der Fütterungsgruppe FUS+ ausgeprägter war.

Zur nähergehenden Überprüfung solcher Veränderungen wurden die Lymphozyten einschließlich ihrer Subpopulationen in der vorliegenden Studie weiter phänotypisiert und über Durchflusszytometrie analysiert. Weiterhin wurden die Hauptfunktionen von Granulozyten und Monozyten, wie zum Beispiel die Phagozytose und die Kapazität, oxidativen Burst zu

induzieren, untersucht, um derartige Effekte auf andere Immunzellen betrachten zu können. Als konsequente Immunantwort in Folge der LPS wurde die Aktivierung von Monozyten durch Änderungen bei der Konzentration von $CD14^+$ Zellen in Blutproben der LPS-injizierten Ferkel nachgewiesen. Außerdem wurde ein Anstieg in der prozentualen basalen ROS Produktion von Granulozyten sowie in der $CD4^+/CD8^+$ Ratio beobachtet, und somit ein weiterer Indikator für den Erfolg des LPS-Challenge Models. In Bezug auf die SoS-Behandlung zeigte sich in den LPS-injizierten Ferkeln durch eine Reduktion der durchschnittlichen Fluoreszenzintensität ein Einfluss auf die basale ROS Produktion von Granulozyten und somit ein LPS-modulierter Effekt. Darüber hinaus wurden weitere Effekte des Einsatzes von SoS auf die Subpopulationen der Lymphozyten festgestellt, dies allerdings nur in den Blutproben. Besonderes die $CD8^{low}$ und $CD8^{high}$ T-Zellen schienen sehr sensibel auf Sulfitgehalte im Blut zu reagieren.

Zusammenfassend lässt sich feststellen, dass sich die Feuchtkonservierung mit SoS als effiziente Methode für DON Inaktivierung in stark *Fusarium*-kontaminiertem Mais erwiesen hat, wobei eine deutliche Reduktion von DON Konzentrationen bei gleichzeitiger Erhöhung der Anzahl an entstandenen DONS Derivaten erkennbar war.

Im vorliegenden Fütterungsversuch wurden keine negativen Effekte einer SoS-Behandlung auf die Tierleistung sowie die gesundheitsbezogenen Parameter vorgefunden. Darüber hinaus wurden unerwünschte Effekte von DON durch die SoS-Behandlung inaktiviert. Weiterhin konnte die SoS-Behandlung die negativen Effekte von LPS in einer experimentellen LPS-induzierten Inflammation modulieren. Allerdings sind auch einige Indikatoren für Effekte auf das Immunsystem zu beobachten, welche mehr mit der SoS Behandlung selbst in Zusammenhang stehen und unabhängig von dem initialen *Fusarium*-Toxin bzw. LPS-induzierten Inflammation zu betrachten sind. Deshalb sollten weitere detaillierte Studien, besonders im Hinblick auf das Immunsystem betreffende Effekte durchgeführt werden, um die unbedenklichen Effekte von SoS auf die Tiergesundheit identifizieren zu können.

11. REFERENCES (cited in Introduction, Background and General Discussion)

Aakre D, Flaskerud G, Hellevang K, Lardy G, McMullen M, Ransom J, Sorenson B, Swenson A. 2005. DON (vomitoxin) in wheat – basic questions and answers. NDSU extension service.1302-1305.

Awad WA, Ghareeb K, Bohm J, Zentek J. 2010. Decontamination and inactivation strategies for the Fusarium mycotoxin deoxynivalenol in animal feed and the effectiveness of microbial biodegradation. Food Additives & Contaminants Part A. 27:510-520.

Bata A, Lasztity R. 1999. Inactivation of mycotoxin-contaminated food and feed by microorganisms. Trends in Food Science & Technology. 10:223-228.

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

Beyer M. Produktion, Analytik und Abbau bedeutender Trichothecen-Mykotoxine. 2009. Dissertation, Mathematisch-Naturwissenschaftliche Fakultät der Westfälischen Wilhelms-Universität Münster.

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

Brul S, Coote P. 1999. Preservative agents in foods. Mode of action and microbial resistance mechanisms. International of Journal of Food Microbiology. 50:1–17.

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 metabisulfite ($Na_2S_2O_5$, SBS), on weaned piglets with a special focus on liver function as determined by the ^{13}C-methacetin breath test. Archives of Animal Nutrition. 62:263-286.

Dänicke S, Döll S, Goyarts T, Seeling K, Valenta H, Ueberschär K-H. 2008b. Metabolism, kinetics and occurence of deoxynivalenol (DON) and zearalenone (ZON) in physiological samples of pigs, ruminants and poultry. In: Oswald IP, Taranu I (eds.) Mycotoxins in Farm Animals. Transworld Research Network, Trivandrum. Kerala, India, pp 235–273.

Dänicke S, Pahlow G, Goyarts T, Rohweder D, Wilkerling K, Breves G, Valenta H, Döll S. 2009. Effects of increasing concentrations of sodium metabisulfite ($Na_2S_2O_5$, 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, Beyer M, Breves G, Valenta H, Humpf HU. 2010b. Effects of oral exposure of pigs to deoxynivalenol (DON) sulfonate (DONS) as the non-toxic derivative of DON on tissue residues of DON and de-epoxy-DON and on DONS blood levels. Food Additives & Contaminants Part A. 27:1558-1565.

Dänicke S, Hegewald AK, Kahlert S, Kluess J, Rothkötter HJ, Breves G, Döll S. 2010c. Studies on the toxicity of deoxynivalenol (DON), sodium metabisulfite, DON-sulfonate (DONS) and deepoxy-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, 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 LR, Kahlert S, Kluess J, Döll S, Valenta H, Rothkötter HJ. 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, Döll S. 2010a. 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, 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 H-U. 2010d. Investigations on the kinetics of the concentration of deoxynivalenol (DON) and on spoilage by moulds and yeasts of wheat grain preserved with sodium metabisulfite ($Na_2S_2O_5$, SBS) and propionic acid at various moisture contents. Archives of Animal Nutrition. 64:190-203.

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. 2005. On the effects of a hydrothermal treatment of deoxynivalenol (DON)-contaminated wheat in the presence of sodium metabisulfite ($Na_2S_2O_5$) on don reduction and on piglet performance. Animal Feed Science and Technology. 118:93-108.

Dänicke S, Valenta H, Überschär KH. 2000. Risikoabschätzung und Vermeidungsstrategie bei der Fütterung. Landbauforschung Völkenrode. 216:35-138.

Diesing AK, Nossol C, Panther P, Walk N, Post A, Kluess J, Kreutzmann P, Dänicke S, Rothkötter HJ, Kahlert S. Mycotoxin deoxynivalenol (DON) mediates biphasic cellular response in intestinal porcine epithelial cell lines IPEC-1 and SIPEC-J2. 2010. Toxicology Letters. 200:8–18.

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

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, Schrickx JA, Dänicke S, Fink-Gremmels J. Deoxynivalenol-induced cytotoxicity, cytokines and related genes in unstimulated or lipopolysaccharide stimulated primary porcine macrophages. 2009. Toxicology Letters. 184:97–106.

Dorner JW, Cole RJ. 2002. Effect of application of nontoxigenic strains of *Aspergillus flavus* and *A. parasiticus* on subsequent aflatoxin contamination of peanuts in storage. Journal of Stored Products Research. 38: 329–339.

EFSA. 2004. Opinion of the scientific panel on contaminants in the food chain on a request from the commission related to deoxynivalenol (DON) as undesirable substance in animal feed. EFSA Journal. 73:1-41.

EFSA. 2013. Deoxynivalenol in food and feed: occurrence and exposure. EFSA Journal. 11:3379.

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

Elmas O, Aslan M, Caglar S, Derin N, Agar A, Aliciguzel Y, Yargicoglu P. 2005. The prooxidant effect of sodium metabisulfite in rat liver and kidney. Regulatory Toxicology and Pharmacology. 42:77–82.

Eriksen GS, Pettersson H, Johnsen K, Lindberg JE. 2002. Transformation of trichothecenes in ileal digesta and faeces from pigs. Archives of Animal Nutrition. 56:263-274.

Etienne M, Wache Y. 2008. Biological and physiological effects of deoxynivalenol (DON) in the pig. In *Mycotoxins in farm animals,* 1st ed.; Oswald IP, Taranu I. Eds. Trivandrum, India: Transworld Research Network. 113-130

Feinberg B, McLaughlin CS. 1989. Biochemical mechanism of action of trichothecene mycotoxins. In: Beasley, V.R. (Ed.), Trichothecene Mycotoxicosis: Pathophysiologic Effects, Volume I. CRC Press, Boca Raton, Florida. 27-35.

Gibson DM, Kennelly JJ, Aherne FX, Mathison GW. Efficacy of sulfur-dioxide as a grain preservative. 1988. Animal Feed Science and Technology 19:203–218.

Goyarts T, Dänicke S. 2005. Effects of deoxynivalenol (DON) on growth performance, nutrient digestibility and don metabolism in pigs. Mycotoxin Research. 21:139-142.

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

Gutleb AC, Morrison E, Murk AJ. 2002. Cytotoxicity assays for mycotoxins produced by Fusarium strains: A review. Environmental Toxicology and Pharmacology. 11:309–320.

He JW, Zhou T, Young JC, Boland GJ, Scott PA. 2010. Chemical and biological transformations for inactivation of trichothecene mycotoxins in human and animal food chains: A review. Trends in Food Science & Technology. 21:67-76.

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.

Jard G, Liboz T, Mathieu F, Guyonvarc'h A, Lebrihi A. 2011. Review of mycotoxin reduction in food and feed: From prevention in the field to inactivation by adsorption or transformation. Food Additives & Contaminants Part A. 28:1590-1609.

JECFA. 2001. Deoxynivalenol - safety evaluation of certain mycotoxins in food. Joint fao/who expert committee on food additives, 56th report, safety evaluation of certain mycotoxins in food, WHO food additives series 47, Geneva. 419-556.

Kabak B, Dobson AD, Var I. 2006. Strategies to prevent mycotoxin contamination of food and animal feed: A review. Critical Reviews in Food Science and Nutrition. 46:593-619.

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.

King AD, Ponting JD, Sanshuck DW, Jackson R, Mihara K. 1981. Factors Affecting Death of Yeast by Sulfur-Dioxide. Journal of Food Protection. 44:92–97.

Krska R, Baumgartner S, Josephs R. 2001. The state-of-the-art in the analysis of type-a and -b trichothecene mycotoxins in cereals. Fresenius' Journal of Analytical Chemistry. 371:285-299.

Maier K, Hinze H, Leuschel L. 1986. Mechanism of sulfite action on the energy-metabolism of Saccharomyces cerevisiae. Biochimica et Biophysica Acta. 848:120–130.

Nair B, Elmore AR. 2003. Final report on the safety assessment of sodium sulfite, potassium sulfite, ammonium sulfite, sodium bisulfite, ammonium bisulfite, sodium metabisulfite and potassium metabisulfite. International Journal of Toxicology. 22:63-88.

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, Höppner F, Ellner F, Weinert J. 2017. *Fusarium* diseases of maize associated with mycotoxin contamination of agricultural products intended to be used for food and feed. Mycotoxin Research. 33:167-182. DOI: 10.1007/s12550-017-0277-y.

Pestka JJ, Lin WS, Miller ER. 1987. Emetic activity of the trichothecene 15-acetyldeoxynivalenol in swine. Food and Chemical Toxicology. 25:855–858.

Pestka JJ. 2007. Deoxynivalenol: Toxicity, mechanisms and animal health risks. Animal Feed Science and Technology. 137:283-298.

Pestka JJ. 2010. Deoxynivalenol-induced proinflammatory gene expression: Mechanisms and pathological sequelae. Toxins (Basel). 2:1300-1317.

Pestka JJ, Smolinski AT. 2005. Deoxynivalenol: Toxicology and potential effects on humans. Journal of Toxicology and Environmental Health-Part B. 8:39-69.

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.

Prelusky DB, Trenholm HL. 1993. The efficacy of various classes of anti-emetics in preventing deoxynivalenol-induced vomiting in swine. Journal of Natural Toxins 1:296–302.

Prelusky DB, Gerdes RG, Underhill KL, Rotter BA, Jui PY, Trenholm HL. 1994. Effects of low-level dietary deoxynivafenol on haematological and clinical parameters of the pig. Natural Toxins. 2:97-104.

Prelusky DB, Hartin KE, Trenholm HL, Miller JD. 1988. Pharmacokinetic fate of 14c-labeled deoxynivalenol in swine. Fundamental and Applied Toxicology. 10:276-286.

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.

Rempe I, Kersten S, Valenta H, Dänicke S. 2013a. 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 Research. 29:169-175.

Rempe I, Kersten S, Valenta H, Dänicke S. 2013b. Effects of a Fusarium toxin-contaminated maize treated with sodium metabisulfite, methylamine and calcium hydroxide in diets for female piglets. Archives of Animal Nutrition. 67:314-329.

Rose AH. Sulphur dioxide and other preservatives. 1993. Journal of Wine Research, pp. 43–47.

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

Schwartz-Zimmermann HE, Wiesenberger G, Unbekannt C, Hessenberger S, Schatzmayr D, Berthiller F. 2014a. Reaction of (conjugated) deoxynivalenol with sulphur reagents - novel metabolites, toxicity and application. World Mycotoxin Journal. 7:187-197.

Schwartz-Zimmermann HE, Paulick M, Dänicke S, Schatzmayr D, Berthiller F. 2014b. Determination of deoxynivalenol sulfonates in cereal samples: method development, validation and application. World Mycotoxin Journal 7: 233-245.

Schwartz HE, Hametner C, Slavik V, Greitbauer O, Bichl G, Kunz-Vekiru E, Schatzmayr D, Berthiller F. 2013. Characterization of three deoxynivalenol sulfonates formed by reaction of deoxynivalenol with sulfur reagents. Journal of Agricultural and Food Chemistry. 61:8941-8948.

Shima J, Takase S, Takahashi Y, Iwai Y, Fujimoto H, Yamazaki M, Ochi K. 1997. Novel inactivation of the trichothecene mycotoxin deoxynivalenol by a soil bacterium isolated by enrichment culture. Applied and Environmental Microbiology. 63:3825-3830.

Sudakin DL. 2003. Trichothecenes in the environment: Relevance to human health. Toxicology Letters. 143:97-107.

Taranu I, Marina DE, Burlacu R, Pinton P, Damian V, Oswald IP. 2010. Comparative aspects of in vitro proliferation of human and porcine lymphocytes exposed to mycotoxins. Archives of Animal Nutrition. 64:383-93. DOI: 10.1080/1745039X.2010.492140.

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. 229:7-9.

The Commision of the European Communitites. 2006b. Commission regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. Official Journal of the European Union. 364:5.

The Commission of the European Communities. 2009. Commission Regulation 386/2009/EC amending Regulation (EC) No. 1831/2003 as refers to establishment of a new functional group of feed additives. Official Journal of the European Union. 118:66.

The Commission of the European Communities. 2015. Commission Regulation (EU) 2015/786. of 19 May 2015 defining acceptability criteria for detoxification processes applied to products intended for animal feed as provided for in Directive 2002/32/EC of the European Parliament and of the Council. Official Journal of the European Union. 125:10.

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 Additive 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.

Visconti A, Haidukowski EM, Pascale M, Silvestri M. 2004. Reduction of deoxynivalenol during durum wheat processing and spaghetti cooking. Toxicology Letter. 153:181–189.

Wu Q, Kuca K, Humpf H, Klimova B, Cramer B. 2017. Fate of deoxynivalenol and deoxynivalenol-3-glucoside during cereal-based thermal food processing: a review study. Mycotoxin Research. 33: 79–91.

Young LG, Mcgirr L, Valli VE, Lumsden JH, Lun A. 1983. Vomitoxin in corn fed to young pigs. Journal of Animal Science. 57:655–664.

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

Young JC, Trenholm HL, Friend DW, Prelusky DB. 1987. Inactivation 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.

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EIDESSTATTLICHE ERKLÄRUNG/DECLARATION UNDER OATH

Ich erkläre an Eides Statt, dass ich die Arbeit selbständig und ohne fremde Hilfe verfasst, keine andere als die von mir angegebene Quelle und Hilfsmittel benutzt und die den benutzten Werken wörtlich und inhaltlich entnommen Stellen als solche kenntlich gemacht haben.

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.

Lehrte, 23.06.2022

Anh Tuan Tran

DANKSAGUNG

An dieser Stelle möchte ich die Gelegenheit nutzen, all denen von meinem ganzen Herzen zu danken, die mich bei der Durchführung der Versuche sowie der Erarbeitung dieser Dissertation auf vielfältige Weiser unterstützt haben und ohne deren Hilfe diese Arbeit nicht möglich gewesen wäre:

Herrn Prof. Dr. Dr. Sven Dänicke für die Möglichkeit, diese Arbeit am Institut für Tierernährung in Brauschweig durchführen zu dürfen sowie die konstruktiven Anregungen, die kritische und geduldige Durchsicht meiner Manuskripte, die maßgeblich zum Gelingen dieser Arbeit beitragen haben.

Frau Dr. Jeannette Klüß für die Betreuung des sehr langen Weges zur Dissertation. Sie führte mich immer wieder auf den wissenschaftlichen Weg zurück und stand mir jederzeit mit Rat und Tat zur Seite und trug mit viel Mühe zur Organisation, Motivation und Gestaltung der Publikationen bei.

Frau Dr. Jana Frahm und Frau Dr. Susanne Kersten für die fachliche Unterstützung während der Vorbereitung, Durchführung und Auswertung der Untersuchungen.

Frau Britta Immenroth für die große Hilfsbereitschaft bei den sprachigen Korrekturen.

Der Firma Biomin bin ich für die finanzielle Förderung dieser Arbeit zu großem Dank verpflichtet.

Allen Doktoranden und den Mitarbeitern des Instituts für Tierernährung bin ich sehr dankbar für die Hilfe bei der Durchführung der aufwändigen Versuche.

Vielen Dank, Rolf und Karina, für die unterhaltsame Bürozeit und die vielen wissenschaftlichen und nicht-wissenschaftlichen Austausche.

Besonderer Dank gilt meiner Tochter, meiner Frau sowie meiner ganzen Familie in Vietnam, die mich immer bedingungslos unterstützt und in schwierigen Phasen besonders motiviert haben.