Norwegian University of Life Sciences
Faculty of Veterinary Medicine

Philosophiae Doctor (PhD)
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Deoxynivalenol in pigs: Effects, toxicokinetics, vertical transmission and microbial detoxification

Deoxynivalenol hos gris:
Effekter, toksikokinetikk, vertikal overføring og mikrobiell avgiftning

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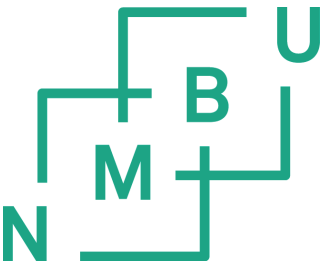
Department of Production Animal Clinical Sciences

Adamstuen (2018)



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“The knowledge of anything, since all things have causes, is not acquired or complete unless it is known by its causes.”

Avicenna (980 –1037)

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Amin Sayyari

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Summary

Mycotoxins are secondary metabolites of fungi that can have a variety of adverse effects on animals and humans. One of the most common mycotoxins produced by *Fusarium* fungi is the trichothecene deoxynivalenol (DON), and this toxin can contaminate wheat, oats, maize, and barley. DON is known to cause significant economic losses in farm animal production, due to impaired growth performance. According to the Norwegian Scientific Committee for Food and Environment (VKM), DON, T-2, and HT-2 are the trichothecenes most frequently found in Norwegian cereal grain. Pigs are more sensitive to DON exposure than other farm animals, possibly due to differences in uptake. Acute exposure of the pigs to high levels of DON (20 mg/kg or above) will typically cause emesis. Chronic dietary exposure of pigs to diets naturally or artificially contaminated with DON has led to decreased feed consumption, visible feed aversion, and reduced weight gain, with the lowest observed adverse effect levels (LOAEL) varying from 0.35 to 2 mg DON/kg feed in different experiments. Transient reduction in feed intake at relatively low contamination levels has been observed in several of these experiments. The exposed animals may sometimes manage to compensate for some or most of the reduction in weight gain later in the growing period. The European Commission Recommendation 2006/576/EC has set the recommended maximum acceptable level for DON to 0.9 mg/kg, while the Norwegian national feed safety authority recommends a lower maximum acceptable level of 0.5 mg DON/kg for pig feed. Despite this national recommendation, Norwegian pig farmers and feed industry have reported observations of temporary reduction in feed consumption and increased stress in the pigs when using feed batches with DON levels close to the Norwegian limit. Therefore, Norwegian pig farmers, feed industry, and authorities are in need of additional knowledge about the occurrence, importance and prevention methods of mycotoxin contamination in pig production.

The risk of toxic effects of mycotoxins can be reduced by different methods and strategies. The supplementation of detoxifying feed additives, such as mycotoxin adsorbents, chemical supplements or active biotransforming agents containing bacteria, fungi or enzymes that can degrade mycotoxins into non -or less toxic metabolites have all been used in feed processing. Some of these methods, such as the addition of adsorbents, are valuable in binding of aflatoxins and several other mycotoxins, but less effective in binding and deactivating of trichothecenes. Addition of the ruminal microbe *Coriobacteriaceae gen. nov. sp. nov.* DSM 11798 (also known as BBSH 797), which can detoxify DON to de-epoxy-DON (DOM-1) has been investigated by European Food Safety Authority (EFSA). This product has been used as

a commercialized method, and has been shown to de-epoxidize DON *in vitro*. It has also been shown to have activity against DON in some *in vivo* studies, but the results of different studies have varied. Thus, the ability of DSM 11798 to detoxify DON in animals under practical feeding conditions still needed clarification.

An objective of the present thesis was to study the effects of DON on pigs in different stages of their life cycle. The thesis is based on two main feeding studies. The first study aimed to investigate the effects of 6 weeks' exposure of growing pigs to DON (up to 5.7 mg/kg) in pelleted feed produced from naturally contaminated oats, with and without the addition of DSM 11798; by analyzing feed uptake, growth performance, clinical parameters, hematology and biochemistry parameters, as well as plasma levels of DON and its metabolites. The second feeding study consists of two main study-parts conducted under a real-word condition in a commercial specific pathogen-free (SPF) high-yield piglet production unit. The first study-part (clinical study) aimed to elucidate the effects of DON (up to 1.7 mg/kg) on sow feed consumption, body weight (BW) changes, litter gain, and reproduction performance in late gestation and during lactation. In addition, the effect of different levels of DON contamination on hematology and biochemistry parameters, skin temperature, litter size, number of stillborn piglets, and production results in the subsequent litter were studied in this field trial. The second study-part aimed to study the DON uptake of the sows and vertical transmission from the sows to their offspring; through the placenta in the last stage of gestation and through colostrum and milk during lactation. Before the start of the study on growing pigs, a toxicokinetic study of uptake and metabolism of pure DON in growing pigs was also performed, in order to get a better basis for the evaluation of the results of the main experiments.

The results of the growing pig study showed that feeding growing pigs with DON-contaminated diets (up to 5.7 mg/kg) resulted in a temporary reduction in feed intake and weight gain, in the highest DON level groups. This effect on growth performance was reduced and the growth was normalized towards the end of the experiment. DON exposure reduced serum protein levels in pigs after 3 weeks of exposure and at the end of the experiment, in a dose-dependent manner. Serum calcium and phosphorous were also reduced in DON-exposed pigs at the end of the experiment. DON did in contrast not affect hematological parameters. No association between gender and DON-related toxicity was found in tested animals. In this study, the addition of DSM 11798 to experimental diets had no influence on the DON-related changes in feed intake and growth performance. Furthermore, the data from the plasma

analyses of DON and its metabolites, especially DOM-1, showed that DSM 11798 was ineffective in detoxifying DON. There were also no differences in plasma concentrations of DON and its metabolites between male and female piglets. Addition of DSM 11798 had also no effect on the DON related changes in serum biochemical parameters. A possible reason for the ineffectiveness of DSM 11798 in this study could be the incorporation of the microbial feed additive into pelleted feed. It should also be mentioned that the stabilizing matrix used by Biomin in the preparation of the DSM 11798 product for our experiments was somewhat different from the standard commercial product.

The results of the first (clinical) part of the feeding study in sows demonstrated that naturally contaminated diets with DON concentrations up to 1.7 mg/kg did have a small but significant effect on feed intake during lactation; but no significant effect on sow BW or performance, including backfat thickness. No effects on production or reproduction performance were detected. The blood parameters were unaffected and the effects on skin temperature were variable. The possibility that SPF status made the sows in this herd more resistant to DON-related toxicity can not be ruled out. This may be an important issue for future studies.

The results from the second part (uptake and transmission study) of the study in sows demonstrated a high degree of variability in the uptake of DON, both among individuals and between different times of sampling, probably due to the variation in feed intake at different stages during gestation, farrowing and lactation. However, the results for glucuronidation rates showed that metabolism of DON was not strongly affected by the different stages in gestation and lactation. A comparison between the data in the sow study and the data from the growing pig study, indicated a somewhat higher glucuronidation rate in the sows, compared with the growing pigs. The transmission data showed that DON is transferred across placenta and through milk, from sows to their full-term, new-born and suckling piglets. However, the results from plasma DON concentrations in sows and piglets and the levels of DON in milk samples showed that the DON transfer is more efficient across placenta than through milk, indicating that piglets are most strongly at risk of DON exposure during the fetal period and in the first days of life.

Taken together, these studies provide a knowledge update on DON and its toxicity in pigs in different stages of the production cycle, under both controlled and realistic, practical conditions. In addition, vertical transmission of DON and exposure of sows and their piglets in late gestation and during lactation were elucidated.

Sammendrag (Norwegian)

Mykotoksiner (muggsoppgifter) er giftstoffer produsert av forskjellige muggsopparter som kan forårsake akutt forgiftning eller langvarige bivirkninger hos dyr og mennesker. En av de vanligste mykotoksinene produsert av *Fusarium*-arter er trichothecenet deoxynivalenol (DON). Dette giftstoffet kan forekomme i hvete, havre, mais og bygg. DON er kjent for å forårsake betydelige økonomiske tap i husdyrproduksjon på grunn av negative effekter på fôropptak og tilvekst. Ifølge Vitenskapskomiteen for mat og miljø (VKM) er trichothecenene DON, T-2 og HT-2 de som oftest finnes i norske kornvarer. Grisen er en art som er mer følsom for mykotoksiner av trichotecene-typen enn andre husdyr, muligens på grunn av forskjeller i opptak. Akutt eksponering av grisene til høye nivåer av DON (20 mg/kg eller høyere) vil typisk forårsake oppkast. Kronisk eksponering av gris for naturlig eller kunstig DON-kontaminert fôr har ført til redusert fôrforbruk, synlig fôraversjon og nedsatt vektutvikling, med de laveste observerte skadelige effektnivåene (LOAEL) varierende fra 0,35 til 2 mg DON/kg i fôr i ulike studier. Forbigående reduksjon i fôropptak ved relativt lave forurensningsnivåer er observert i flere av disse studiene. De eksponerte dyrene kan i noen tilfeller klare å kompensere for noe eller det meste av reduksjonen i tilvekst senere i produksjonsfasen. En anbefaling fra EU-kommisjonen (2006/576/EC) har satt det anbefalte maksimale akseptable nivået for DON i svinefôr til 0,9 mg/kg, mens Mattilsynet i Norge anbefaler en lavere grenseverdi på 0,5 mg DON/kg i fôr til norske griser. Til tross for denne nasjonale anbefalingen har norske griseprodusenter og fôrindustrien rapportert om observasjoner av midlertidig reduksjon i fôrforbruk og økt stress hos grisene ved bruk av fôrpartier med DON-nivåer nær den anbefalte grenseverdien i Norge. Derfor har norske griseprodusenter, fôrindustri og myndigheter behov for ytterligere kunnskap om forekomst, betydning og forebyggende metoder for mykotoksiner i griseproduksjonen.

Risikoen for toksiske effekter av mykotoksiner kan reduseres med forskjellige metoder og strategier. Tilskudd av avgiftende tilsetningsstoffer som mykotoksinadsorbenter, kjemiske tilsetninger eller aktive biotransformasjons-produkter som inneholder bakterier, sopp eller enzymer kjent for å kunne nedbryte mykotoksiner til mindre giftige metabolitter, har blitt brukt i fôrprosessering. Noen av disse metodene, som tilsetning av adsorbenter, har dokumentert effekt i binding av aflatoksiner og flere andre mykotoksiner, men de er mindre effektive i binding og deaktivering av trichotecener. Tilsetning av vommikroben *Coriobacteriaceae gen. nov. sp. nov.* DSM 11798 (også kjent som BBSH 797), som kan

konvertere DON til de-epoxy-DON (DOM-1), har blitt undersøkt av European Food Safety Authority (EFSA). Dette produktet har blitt brukt som en kommersiell metode, og har vist seg å de-epoxidisere DON *in vitro*. Produktet har også vist en viss effekt *in vivo*, men med noe varierende resultater. Dermed er det fortsatt behov for avklaring om evnen til DSM 11798 til å avgifte DON hos dyr, under praktiske fôringsbetingelser.

Denne avhandlingen tar sikte på å etablere kunnskap om effekter av muggsoppgiften deoxynivalenol (DON) på helse, velferd og produktivitet hos griser i ulike stadier av livssyklusen. Avhandlingen er basert på to fôringsstudier. Den første studien er et 6 ukers eksponeringsforsøk, og hadde til hensikt å undersøke effekter av DON (opptil 5,7 mg/kg) i pelletert fôr laget av naturlig kontaminert havre, med og uten tilsatt DSM 11798, på nylig avvente griser. Dette er oppnådd ved å analysere fôropptak, tilvekst, kliniske parametere, hematologi og biokjemiske parametere, samt plasmanivåer av DON og dets metabolitter. Den andre fôringsstudien, som er utført på purker under reelle praktiske forhold i en kommersiell spesifikk-patogenfri (SPF) smågrisproduksjonsbesetning med høye produksjonsresultater, består av to hoveddeler. Den første (kliniske) delen hadde til hensikt å belyse effekten av DON (opptil 1,7 mg / kg) på fôropptak, endringer i kroppsvekt, kulltilvekst og reproduksjonsevne hos purker i de siste faser av drektigheten og under laktasjon. I dette feltforsøket ble effekten av forskjellige nivåer av DON på blodparametere (hematologi og kjemi), hudtemperatur, kullstørrelse, antall dødfødte grisunger og produksjonsresultater i det etterfølgende kullet også studert. Den andre delen av studien på purker (opptaks- og overføringsstudien) hadde som mål å studere DON-opptaket hos purkene og vertikal overføring fra purker til deres avkom; gjennom morkake i den siste fasen av drektigheten og gjennom råmelk og vanlig melk under laktasjonen. Før starten av fôringsforsøkene ble det også utført en toksikokinetikkstudie på avvente smågriser, for å etablere kunnskap om toksikokinetikk av ren DON ved oral og intravenøs eksponering. Resultater fra denne studien har blitt brukt til å få bedre grunnlag for evalueringen av resultatene i hovedeksperimentene (fôringsstudiene).

Resultatene fra smågrisforsøket viste at fôring av avvente smågriser med DON-kontaminert havre (opptil 5,7 mg/kg) førte til en forbigående reduksjon i fôropptaket og tilvekst i de høyeste DON-nivågruppene. Denne effekten ble redusert, og veksten ble normalisert mot slutten av forsøket. En doseavhengig DON-indusert reduksjon i serumproteinnivået ble registeret hos griser etter 3 ukers eksponering og på slutten av forsøket. Serumkalsium og fosfor ble også redusert i DON-eksponerte griser ved slutten av eksperimentet. DON-nivået

påvirket derimot ikke de hematologiske parametrene. Det ble ikke funnet noen sammenheng mellom kjønn og DON-relatert toksisitet i testede dyr.

I denne studien hadde tilsetningen av DSM 11798 til forsøksfôret ingen effekt på DON-relaterte endringer i fôropptak og tilvekst. Videre viste de målte konsentrasjonene av DON og dets metabolitter (særskilt DOM-1) i plasma at DSM 11798 var ineffektiv i detoksifisering av DON. Det var heller ingen kjønnsforskjeller i plasmakonsentrasjonene av DON og dets metabolitter. Tilsetning av DSM 11798 hadde heller ingen effekt på DON-relaterte endringer i serumbiokjemiske parametere. En mulig årsak til manglende effekt av DSM 11798 i denne studien kan være inkorporeringen av det mikrobielle tilsetningsstoffet i pelletert fôr. Det bør også nevnes at den stabiliserende matrisen som ble brukt av Biomin i fremstillingen av DSM 11798-produktet for smågrisstudien var noe annerledes enn det kommersielle produktet.

Resultatene fra den første (kliniske) delstudien i fôringsforsøket på purker viste at naturlig kontaminert fôr med DON-konsentrasjoner opptil 1,7 mg/kg hadde mild, men signifikant effekt på fôropptaket under laktasjonen. DON hadde derimot ikke signifikant effekt på kroppsvekt og andre tilvekstparametre, inkludert målt spekktykkelse. Ingen effekter på produksjon eller reproduksjonsytelse ble oppdaget. Blodparametere var upåvirket og effektene på hudtemperaturen var variable. Muligheten for at SPF-status gjorde purkene i denne besetningen mer robuste mot DON-relatert toksisitet kan ikke utelukkes. Dette kan være et viktig tema for fremtidige studier.

Resultatene fra den andre delstudien (opptaks- og overføringsstudien) i forsøket på purker viste stor variasjon i DON-opptak hos purkene, både blant individer og mellom forskjellige prøvetidspunkt. Dette kan være forårsaket av forskjellene i fôropptak på forskjellige stadier i sen drektighet, under grising og gjennom laktasjonen. Resultatene for glukuronideringsgrad viste imidlertid at DON metabolismen ikke var sterkt påvirket av tidspunktet, under drektighet og laktasjon. Ved sammenligning av dataene i smågrisforsøket og dataene fra purkeforsøket fant vi en aldersrelatert forskjell i glukuronideringsgrad, i form av noe høyere glukuronideringsgrad i purker sammenlignet med smågriser. Overføringsdataene viste videre at DON overføres både gjennom morkake og melk fra purker til nyfødte og diende smågriser. Resultatene fra plasma DON-konsentrasjoner hos purker og grisungene og DON-nivåene i melkeprøver viste imidlertid at DON-overføringen er mer effektiv gjennom morkake enn via melk, noe som indikerer at spegrisene har størst risiko for DON-eksponering som fostre i den siste del av drektigheten og i de første dagene av livet.

Samlet sett gir disse studiene en kunnskapsoppdatering om DON og dets toksisitet hos griser i ulike stadier av produksjonssyklusen, under både kontrollerte og realistiske/praktiske forhold. I tillegg ble vertikal overføring av DON og eksponering av purker og deres smågris i sen drektighet og under laktasjon belyst.

Abbreviations

5-HT – 5-hydroxytryptamine or serotonin

AC – Activated carbon

ADFI –Average daily feed intake

ALP – Alkaline phosphatase

ALT – Alanine aminotransferase

AST – Aspartate amino transferase

BBB – Blood-brain barrier

BCS – Body condition score

BLI – Biolayer interferometry

BUN – Blood urea nitrogen

BW – Bodyweight

CCK – Cholecystokinin

CFU – Colony forming units

COCs – Cumulus-oocyte complexes

CPG – Central pattern generator

CSF – Cerebrospinal fluid

CTZ – Chemoreceptor trigger zone

DAS – Deacetoxyscipenol

DOM-1 – De-epoxy-DON

DON – Deoxynivalenol

EFSA – European Food Safety Authority

ELISA – Enzyme-linked immune-sorbent assay

EN – Electronic nose

ER – Endoplasmic reticulum

FA – Feed additive

FB – Fumonisin

FCR – Feed conversion ratio

FSH – Follicle-stimulating hormone

GC-MS – Gas chromatography mass spectrometry

HPLC – High-performance liquid chromatography

HSCAS – Hydrated sodium calcium aluminosilicate

IARC – International Agency for Research on Cancer

IGF1 – Insulin-like growth factor 1

IGFALS – Insulin-like growth factor acid-labile subunit

IRB – Immuno-rotary biosensor

IUPAC – International union of pure and applied chemistry

LC-HRMS – Liquid chromatography-high resolution mass spectrometry

LC-MS – Liquid chromatography-mass spectrometry

LC-MS/MS – Liquid chromatography-tandem mass spectrometry

LFI – Lateral flow immunoassay

LH – Luteinizing hormone

LOAEL – Lowest observed adverse effect level

MOS – Metal-oxide-semiconductor

NMBU – Norwegian University of Life Sciences

OTA – Ochratoxin

PCV2 – Porcine circovirus type 2

PRRSV – Porcine reproductive and respiratory syndrome virus

PWG – Planar waveguide

PYY – Peptide YY

RDW – Red cell distribution width

ROS – Reactive oxygen species

SOCS – Suppressors of cytokine signaling

SPF – Specific pathogen-free

SPR – Surface plasmon resonance

TDI – Tolerable daily intake

TEER – Transepithelial electrical resistance

TLC – Thin layer chromatography

TRFIA – Dual-label time-resolved fluoroimmunoassay

VKM – Norwegian Scientific Committee for Food and Environment

ZEA – Zearalenone

List of publications

Paper I – *Food Additives & Contaminants: Part A* (2018), 35:7, 1394–1409, DOI: 10.1080/19440049.2018.1461254

Effects and biotransformation of the mycotoxin deoxynivalenol in growing pigs fed with naturally contaminated pelleted grains with and without the addition of *Coriobacteriaceum* DSM 11798

Amin Sayyari, Christiane Kruse Fæste, Ulrik Hansen, Silvio Uhlig, Tore Framstad, Dian Schatzmayr and Tore Sivertsen.

Paper II –*Porcine Health Management* (2018), 4:26, DOI: https://doi.org/10.1186/s40813-018-0102-9

Effects of feeding naturally contaminated deoxynivalenol diets to sows during late gestation and lactation in a high-yield specific pathogen-free herd

Amin Sayyari, Tore Framstad, Anette Kristine Krogenæs, and Tore Sivertsen.

Paper III – Manuscript Submitted to *Toxins*

Transfer of deoxynivalenol (DON) through placenta, colostrum and milk from sows to their offspring during late gestation and lactation.

Amin Sayyari, Silvio Uhlig, Christiane Kruse Fæste, Tore Framstad and Tore Sivertsen.

Paper IV – *Archives of Toxicology* (2018), 92: 2195, DOI: https://doi.org/10.1007/s00204-018-2220-1

Prediction of deoxynivalenol toxicokinetics in humans by in vitro-to-in vivo extrapolation and allometric scaling of in vivo animal data

Christiane Kruse Fæste, Lada Ivanova, Amin Sayyari, Ulrik Hansen, Tore Sivertsen and Silvio Uhlig.

Introduction

Mycotoxins are diverse fungal metabolites that are known to be present in cereals consumed by animals and humans. Approximately 25% of the world's crop production is contaminated by mycotoxins (Pinton & Oswald 2014). The most important toxicogenic fungi are *Aspergillus*, *Fusarium,* and *Penicillium* (Plumlee 2004). They produce a variety of mycotoxins such as aflatoxins, zearalenone (ZEA), trichothecenes, fumonisins (FB_1 and FB_2) and ochratoxin A (OTA) (Plumlee 2004). The trichothecene deoxynivalenol (DON) is known to cause significant economic losses in farm animal production, due to reduced feed intake and growth (Plumlee 2004). Norwegian grain production has over time suffered from a variable, but persistent contamination with *Fusarium* fungi, and consequently experienced levels of trichothecene mycotoxins that some years are problematically high (VKM 2013). Therefore, mycotoxin contamination of feed can be considered as an economic issue for the Norwegian pig farmers (VKM 2013). Despite Norway's low recommended maximum concentration of DON in feed, reports of mycotoxin-related problems in pig farming are common (VKM 2013). Norwegian pig farmers, feed industry, and authorities do therefore request further knowledge on the occurrence, importance and prevention methods of mycotoxin contamination in pig production (Bernhoft A & Sivertsen T 2013; Mattilsynet 2015). Acute DON toxicosis may result in shock-like responses including diarrhoea, vomiting, leukocytosis, hemorrhage and even mortality at very high doses (Pestka 2007). Chronic exposure to DON and other trichothecenes can lead to a variety of dose-related clinical signs, such as feed refusal, reduced weight gain, poor nutritional efficiency, neuroendocrine changes and immune modulation (Pestka 2007).

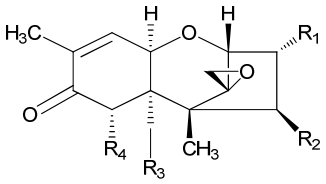
Fusarium mycotoxins

The most important toxicogenic fungi which infect cereal grain in northern Europe during the growing season, belong to the genus *Fusarium* (Barug et al. 2006). *Fusarium culmorum* and *Fusarium graminearum* have been known as two of the most common and important *Fusarium* spp. isolated from cereals in Norway. The frequencies of registered contaminations with *F.culmorum* decreased and the frequencies of *F.graminearum* increased during 2010–2013 in Norway (VKM 2013). The most common *Fusarium* mycotoxin groups are trichothecenes, ZEA, and fumonisins. Within the group of trichothecenes, DON, T-2, and

deacetoxyscipenol (DAS) have the highest clinical importance in swine (Zimmerman et al. 2012).

Deoxynivalenol (DON)

DON, also known as vomitoxin, belongs chemically to the trichothecene family (**Figure 1**). DON is water-soluble, extremely heat-resistant and stable in temperatures up to 350 °C. **Table 1** shows the physicochemical properties of DON (Sobrova et al. 2010). This mycotoxin is produced mainly by *F. graminearum* and *F. culmorum,* and is the most commonly detected trichothecene worldwide (Sobrova et al. 2010). Occurrence of high DON levels have also been reported in Norwegian oat grains in recent years (VKM 2013). This may possibly be related to global warming and changes in weather conditions (Hjelkrem et al. 2017; Medina et al. 2017). The high levels of DON in Norwegian grains are especially important in pigs, as they are the most DON-sensitive domestic animal, and eat cereal-based feed as their main diet. The lowest observed adverse effect levels (LOAEL) that could result in reduced average daily feed intake (ADFI) varies considerably between studies (range from 0.35 to 2 mg/kg feed) (VKM 2013). The risk of performance and welfare effects in pigs due to DON exposure is also influenced by the recipes of cereals used in pig feed production. Because the highest DON levels are usually found in oats, the risk will increase with higher amount of oats in the swine diets (VKM 2013). The effects of DON on animals (with a special focus on pigs) and humans will be mentioned in separate chapters in this thesis.



	R_1	R_2	R_3	R_4
4-deoxynivalenol (DON)	OH	H	OH	OH
3-acetyl-DON	*O*Acetyl	H	OH	OH
15-acetyl-DON	OH	H	*O*Acetyl	OH

Figure 1. Chemical structure of deoxynivalenol and acetylated derivatives.

Table 1. Physicochemical properties of deoxynivalenol.

Property	Information
Name	Deoxynivalenol (DON), vomitoxin
IUPAC name	12,13-epoxy-3α,7α,15-trihydroxytrichothec-9-en-8on
Molecular formula	$C_{15}H_{20}O_6$
Molar mass	296.32 g/mol
Physical state	Colourless fine needles
Boiling Point (°C)	543.9±50.0
Melting Point (°C)	151–153
Flash Point (°C)	206.9±2.5
Vapour Pressure (Torr)	4.26×10^{-14} 25 °C
Soluble in:	Polar organic solvents (e.g., aqueous methanol, ethanol, chloroform, acetonitrile, and ethyl acetate) and water

Zearalenone (ZEA)

ZEA is described chemically as a resorcyclic acid lactone produced mainly by *F. graminearum* (Döll & Dänicke 2011) (**Figure 2**). ZEA survives storage, milling and feed processing and is stable at high temperatures (Döll & Dänicke 2011). Some physicochemical properties of ZEA are presented in **Table 2** (Döll & Dänicke 2011). The chemical structure of ZEA is similar to 17β-Estradiol. This mycotoxin can therefore bind competitively to estrogen receptors in target organs (Zimmerman et al. 2012). Pigs are considered as especially sensitive to ZEA due to hyperestrogenic effects, which may result in urogenital and reproductive problems, depending on dosage and age of the exposed pigs (Zimmerman et al. 2012). Concentration levels between 1 and 5 mg/kg cause vulvovaginitis, tenesmus and in some cases rectal prolapse in prepuberal gilts (Zimmerman et al. 2012). In mature sows, feeding ZEA-contaminated feed with 3–10 mg ZEA/kg may lead to anestrus (Zimmerman et al. 2012). Clinical signs such as reduced conception rate, increased number of repeat breeders, decreased litter size, and increased number of stillbirths are reported as a hyperestrogenic syndrome in swine (Zimmerman et al. 2012). Vertical transmission of ZEA and its metabolites are also reported and may contribute to estrogenic effects on vulva, teats, uterine

and ovary in female piglets (Zimmerman et al. 2012). ZEA has no effects on mature boars, however, young boars exposed to ZEA may show reduced libido and decreased testicular size (Zimmerman et al. 2012).

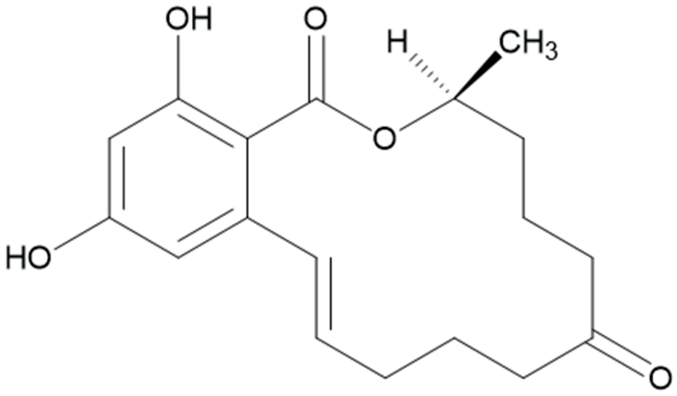


Figure 2. Chemical structure of zearalenone.

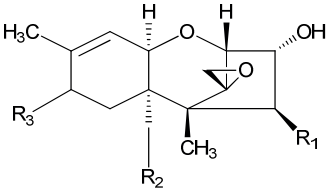
Table 2. Physicochemical properties of zearalenone.

Property	Information
Name	Zearalenone (ZEA)
IUPAC name	6-(10-hydroxy-6-oxo-trans-1-undecenyl)-β-resorcyclic acid lactone
Molecular formula	$C_{18}H_{22}O_5$
Molar mass	318.369 g/mol
Physical state	Colourless fine needles
Melting Point (°C)	164–165
Soluble in:	Aqueous alkali and various organic solvents

T-2 and HT-2 toxins

T-2 and HT-2 are co-occurring trichothecene mycotoxins, primarily produced by *F. langsethiae* in the field (VKM 2013). T-2 is stable in different environments, and can tolerate light and high temperature; however, strong acid and alkaline conditions can deactivate this

toxin effectively (Li et al. 2011). Chemical structure of these toxins is presented in **Figure 3**. The contamination of grains such as maize, wheat, barley and oats with T-2 and HT-2 is widespread. In Norway, however, they are found most frequently in oats (VKM 2013). In animals, T-2 is metabolized rapidly into other products, primarily HT-2 (Li et al. 2011). Therefore, it may be difficult to distinguish the clinical effects of T-2 from those of HT-2. T-2 and HT-2 are known as the most acute toxic mycotoxins among the trichothecenes. These toxins inhibit protein, DNA, and RNA synthesis, induce apoptosis, and can affect membranes and lipid peroxidation (VKM 2013). The LOAEL for T-2 is set to 0.5 mg/kg. Exposure of pigs to high levels of T-2 can cause skin irritation, lesion, necrosis, emesis, feed refusal, weight loss, diarrhea, lethargy, hemorrhage, profound lymphoid depletion, necrosis, damage of cartilaginous tissues and death (Li et al. 2011; Zimmerman et al. 2012).



	R_1	R_2	R_3
T-2 toxin	*O*Acetyl	*O*Acetyl	*O*Isovaleryl
HT-2 toxin	OH	*O*Acetyl	*O*Isovaleryl

Figure 3. Chemical structures of T-2 and HT-2 toxins.

Mycotoxin co-occurrence and combined effects

Mycotoxinogenic fungi produce normally more than one mycotoxin, and the raw materials in the feed have usually been infected by a variable number of fungal species (Streit et al. 2012). Therefore, exposure of animals to a combination of mycotoxins are possible. DON and ZEA are produced by the same fungi and can often occur simultaneously in the feed (Döll & Dänicke 2011). Co-occurrence of mycotoxins, which could lead to a range of variable effects on animal health and performance, ranging from synergistic or additive interactions to antagonistic effects, is an emerging issue, (Streit et al. 2012). An overview of studies on mycotoxin co-occurrence in feed from EU countries and UK shows that animal feed and feed raw materials have been infected by different mycotoxins, and co-occurrences of two or more mycotoxins are reported in most of the studies (Streit et al. 2012). Swine compound feedstuffs produced in Portugal were contaminated by DON/ZEA as the most frequently co-occurring mycotoxins, however, simultaneous contamination of swine feed with OTA/ZEA and

OTA/DON was also reported (Almeida et al. 2011). Analysis of different swine feed materials revealed that 75% of the feed samples were contaminated with more than one mycotoxin (Ma et al. 2018; Monbaliu et al. 2010).

Due to the importance of co-occurrence and combined effects of mycotoxins, a range of *in vitro* and *in vivo* studies has investigated the combined effects of mycotoxins (Grenier & Oswald 2011). Among the feeding studies on pigs, co-occurrence of *Fusarium* mycotoxins was investigated more than other possible mycotoxin co-occurrences in contaminated swine diets. Simultaneous contamination of swine feed with DON and ZEA may result in additive, synergistic effects, however, these effects may also depend on the ratio of these fusariotoxins to each other (Döll & Dänicke 2011).

The combined effects of mycotoxins on swine immune response are investigated in both *in vivo* and *in vitro* studies. Pigs fed diets contaminated with DON and FB had an additive interaction effect in suppression of cytokines production (Bracarense et al. 2012).

Molecular and cellular effects of DON

DON can inhibit protein synthesis by binding to ribosomes. This induces a process known as "ribotoxic stress response" via activation of constitutive ribosome-associated kinases and the endoplasmic reticulum (ER) stress response (Pestka 2010). DON induces DNA damage, chromosome aberrations and chromatid fragmentation (Sobrova et al. 2010). DON can also induce production of reactive oxygen species (ROS) which can lead to oxidative stress and cellular damage (Payros et al. 2016). The other cellular effects of DON are pro-apoptotic effects in a variety of cells such as hematopoietic and intestinal epithelial cell lines, macrophages, monocytes and hepatocytes (Payros et al. 2016). In spite of these cellular and molecular effects, DON was not recognized as a potential carcinogen by the International Agency for Research on Cancer (IARC) and has therefore been categorized as Group 3, "not classifiable as to their carcinogenicity to humans" (Sobrova et al. 2010).

Effects of DON on swine health and welfare

DON can affect all animal species evaluated up to now, with the following rank order of sensitivity: pigs > mice > rats > poultry ≈ruminants (Pestka 2007). The variations in toxicokinetics of DON among different species could be a reason for this differential sensitivity (Pestka 2007). The clinical DON-related symptoms in pigs may be acute or chronic, depending on the course of exposure and dose levels. Under practical agricultural conditions, the risk of acute health effects in swine is reported to be low; however, chronic

adverse health effects have been estimated to be higher; in pigs exposed to DON and its acetyl derivatives (Knutsen et al. 2017). The most common chronic adverse effects of DON in pigs are reduced feed intake and BW gain (Knutsen et al. 2017). Vomiting is considered as a critical acute effect of DON, with a LOAEL of 2.8 mg/kg feed (Knutsen et al. 2017). Impaired immune response, reproductive, neurological, hematological, and molecular effects are also reported from *in vivo* or *in vitro* studies (Sobrova et al. 2010).

Emesis, anorexia and growth effects

Emesis is a reflex and considered as a protective mechanism against food or feed poisoning. However, severe emesis can lead to nausea, disruption in the optimal uptake of nutrients, and electrolyte imbalance (Wu et al. 2013). As mentioned, acute exposure of pigs to high levels of DON do typically result in emesis. A rapid onset of vomiting has been shown to occur after feeding pigs with contaminated diets with 19.7 mg DON/kg (Forsyth et al. 1977). The underlying mechanisms of DON-induced emesis are not widely understood. However, the rapid induction of emesis also after parenteral administration of DON indicates a systemic component (Forsyth et al. 1977). The central pattern generator (CPG) located in the medulla oblongata of the hindbrain coordinates emetic response, which is integrated by neurotransmitters, hormones, and visceral afferent neurons (Wu et al. 2013). Two pathways could trigger the emesis. The first mechanism is related to the action of peripheral blood- and cerebrospinal fluid (CSF) emetic stimuli such as hormones and neurotransmitters at the area postrema (AP) located in the medulla. The AP has been thought to be the primary chemoreceptor trigger zone (CTZ) that is involved in initiating emesis (Prelusky & Trenholm 1993; Wu et al. 2013). The lack of a specific blood-brain barrier (BBB) in AP leads to sensation of emetic stimuli in blood and CSF. This results in activation of the CPG and consequently emesis. In the second mechanism, emetic mediators such as 5-hydroxytryptamine (5-HT or serotonin) are involved (Wu et al. 2013). The other hormonal components that might induce emesis are satiety hormones peptide YY (PYY) and cholecystokinin (CCK) (Wu et al. 2013). DON-exposure led to increased levels of PYY, 5-HT and/or CCK in animals (Flannery et al. 2012; Ruonan et al. 2018; Wu et al. 2013). Interestingly, in animals exposed to DON or T-2 toxin emesis co-occurred with increases in levels of PYY and 5-HT (Wu et al. 2013; Wu, W. et al. 2015). This finding suggested that both PYY and 5-HT might be considered as prominent mediators of DON-induced emesis.

A variety of mechanisms and mediators, including growth and satiety hormones, immune response, neuroendocrine pathway or a central neuronal signaling may thus be involved in

DON-induced emesis, anorectic effects and consequently poor growth performance (**Figure 4**).

As mentioned before, DON increased circulatory levels of neurotransmitter 5-HT and gut satiety hormones, PYY and CCK. These hormones play a key role in DON-induced anorexia. Exposure of mice (Girardet et al. 2011) and piglets (Bracarense et al. 2012) to DON increased production of IL-1ß, IL-6, and TNF-α, proinflammatory cytokines that may contribute to sickness behavior, including anorexia. In addition, proinflammatory cytokines induce expression of hepatic suppressors of cytokine signaling (SOCS), which inhibit growth factor signaling pathway due to a reduction in insulin-like growth factor acid-labile subunit (IGFALS) insulin-like growth factor 1 (IGF1) (Amuzie & Pestka 2010).

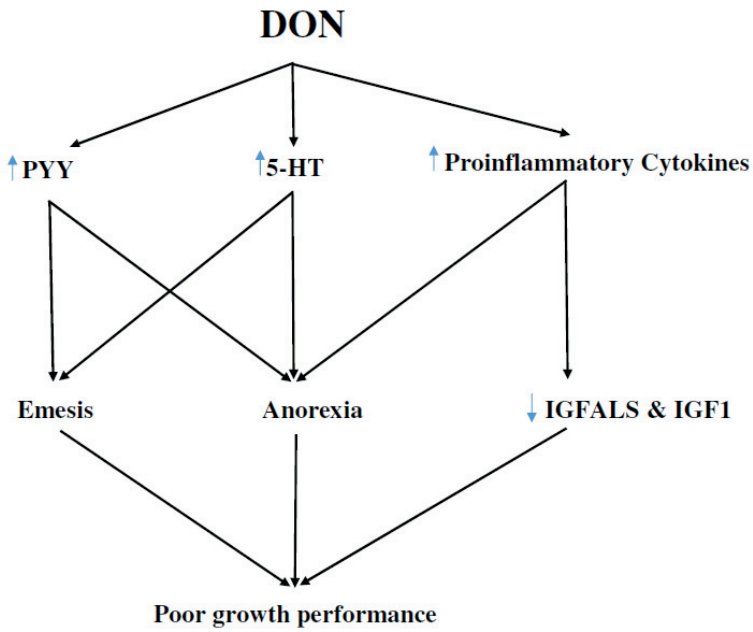


Figure 4. Proposed mechanisms for DON-related growth impairment.

Intestinal toxicity

The effects of DON on epithelial cells of the gastrointestinal tract as one of the first targeted tissues for DON toxicity have been in focus in the last years (Payros et al. 2016). It is reported

that DON can induce intestinal lesions in jejunum and ileum in pigs (Pinton & Oswald 2014). This effect can lead to histomorphological changes in the pig intestine, including decreased villus height and increased crypt depth even at low levels of DON contamination. (0.9 mg/kg feed) (Alizadeh et al. 2015). Other morphological changes such as multifocal atrophy, villus fusion, apical villus necrosis and edema of lamina propria were also observed in pigs fed 2.8 mg DON/kg (Rotter et al. 1994).

The other important effect of DON on pig intestine is alternations in intestinal barrier functions since DON induces a reduction in transepithelial electrical resistance (TEER), which is described as an important indicator of the barrier function integrity (Pierron et al. 2016b). A dose-dependent reduction effect of DON on TEER, indicating impaired intestinal barrier function and consequently increased cellular permeability, was reported in pigs (Halawa et al. 2012).

DON (3 mg/kg feed) can affect the physical mucosal defense by reducing the number of goblet cells (Bracarense et al. 2012). These cells synthesize and secrete mucin, which is involved in normal intestinal barrier function. DON-induced alternation in the physical gut mucosal barrier can result in intestinal tissue damages and increase the risk for invasion of pathogens and increased uptake of DON itself through the intestinal epithelium (Ghareeb et al. 2015).

The ability of DON to affect the absorption of nutrients (such as amino acids and sugars) are reported in both *in vivo* and *ex vivo* studies, however the exact mechanism of action of DON is not completely known (Pinton & Oswald 2014). One possible explanation is that DON can inhibit the expression of key nutrient transporter proteins in the enterocytes (Maresca et al. 2002).

The gut microbiota as a major factor for animal health is also affected by exposure to DON (Ghareeb et al. 2015). Although information that can prove the effects of DON on gut microbiota in pigs is scarce, there is some evidence that feeding pigs with naturally contaminated diets with DON (2.8 mg/kg) could modify the gut microbiota and might disrupt the balance of intestinal bacteria communities (Wache et al. 2009).

DON can modulate local immunity in pig intestine by induction of a proinflammatory response. Feeding the piglets with contaminated diets with DON (3 mg/kg) resulted in DON-induced intestinal expression of IL-1ß, IL-6, and TNF-α (Bracarense et al. 2012).

Taken together, the effect of the DON on intestinal function can result in time- and dose-dependent changes that might impair swine health and performance. Although poor growth performance and severe health issues are observed at relatively high levels of DON contamination, the exposure of animals to low and moderate levels can result in cytotoxic effects that consequently lead to impairment of health and performance (Ghareeb et al. 2015). A variety of mechanisms may be involved in DON-induced intestinal toxicity and consequently poor health and growth performance (**Figure 5).**

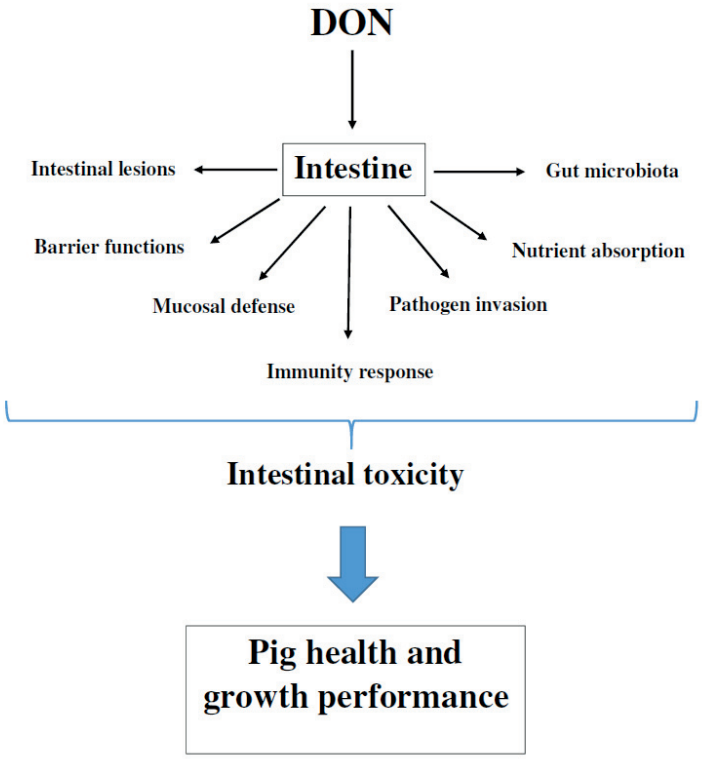


Figure 5. Proposed mechanisms for DON-related intestinal effects and toxicity.

Immunotoxicity

After crossing the epithelial barrier, DON targets immune cells. Depending on the dose and the duration of exposure, DON can modulate immune responses in different ways (**Figure 6**). At high concentration, DON suppresses immune function and at low concentration, DON exhibits immunostimulatory activity (Payros et al. 2016). Acute exposure of mice with high levels of DON results in dramatic leukocyte apoptosis, which is responsible for immunosuppressive effects of DON exposure (Pestka et al. 2004). On the other hand, DON at low doses stimulates the immune system by increasing the expression of cytokines and elevation of IgA levels in serum (Pestka 2003).

Measuring immunoglobulins and cytokines as important regulators of both humoral and cell immunity in order to study the effects of DON on immune function have been in focus in the last decades.

An increased cytokine production by macrophages and T cells followed by exposure to DON can mediate increased secretion of IgA (Pestka et al. 2004). Pigs fed diets contaminated with high levels of DON (8 mg/kg) had lower IgG and IgM levels in serum (Reddy et al. 2018). The authors did also report DON-related immunosuppression due to a reduction in the expression of inflammatory cytokines (Reddy et al. 2018).

As described under intestinal toxicity, DON also induces the expression of proinflammatory cytokines, which contribute to intestinal inflammation.

Altogether, DON-related immunotoxic effects may contribute to susceptibility of pigs to infectious diseases, reactivation of chronic infection and impaired vaccination efficacy (Pierron et al. 2016a).

DON in interaction with porcine circovirus type 2 (PCV2) led to higher viral replication of the virus in pigs fed diets contained 2.5 mg DON/kg (Savard et al. 2015b). DON-induced decreased immune response to porcine reproductive and respiratory syndrome virus (PRRSV) was also observed in pigs fed diets contaminated with 2.5 mg DON/kg (Savard et al. 2014). DON at low and realistic contamination levels in co-occurrence with *Salmonella Typhimurium* has a synergistic effect and can enhance inflammatory reaction by an increased production of proinflammatory cytokines in pigs (Vandenbroucke et al. 2011).

At doses that do not affect global immune response, DON can alter vaccine immune response in pigs (Pierron et al. 2016a). Feeding pigs with diets contaminated with DON (2.5 to 3.5

mg/kg) inhibited the vaccination efficacy of PRRSV modified live vaccine (Savard et al. 2015a).

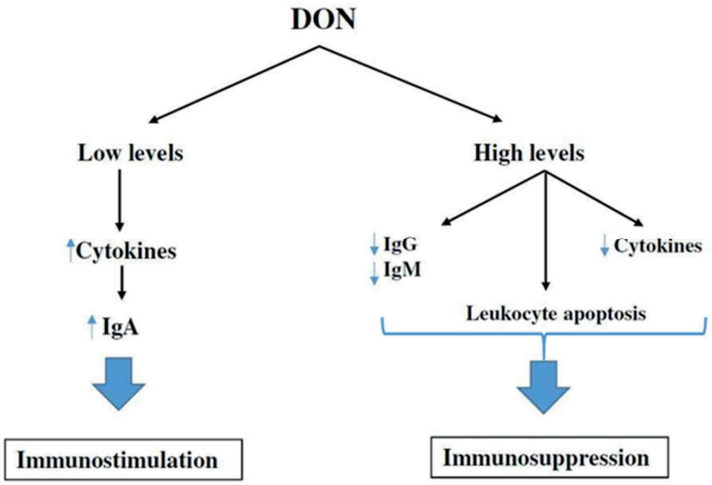


Figure 6. Proposed mechanisms for DON-related immunotoxicity.

Effects on hematological and biochemical parameters

Results from experiments where effects of DON on swine blood parameters were studied are variable. The most frequently reported changes in blood parameters of pigs challenged with DON are decreased serum proteins, albumin, alpha-globulin and urea (Zimmerman et al. 2012). The other reported changes in biochemistry and hematology, including increased serum thyroxin, alternations in serum cortisol levels, decreased hematocrit, hypocalcaemia, hypophosphatemia and appearance of segmented neutrophils, are inconsistent (Zimmerman et al. 2012).

Pigs fed diets contaminated with 3.5 mg DON/kg had lower serum protein, albumin, calcium, and phosphorus (Bergsjø et al. 1993). The only hematological effect of DON on pigs receiving a high level of DON (8 mg/kg feed) was increased RDW (Reddy et al. 2018). Decreased RBC and HGB in pigs upon DON exposure have also been shown in some previous studies (Reddy et al. 2018; Rotter et al. 1994). Serum albumin, creatinine, and glucose were unchanged by

feeding pigs with 3−12 mg DON/kg feed (Wu, L. et al. 2015). In the same study, higher blood urea nitrogen (BUN), alkaline phosphatase (ALP), alanine aminotransferase (ALT) and aspartate amino transferase (AST) were observed in pigs fed high levels of DON (6-12 mg/kg).

The mechanism of action of DON related changes in blood parameters is still unknown. As described these changes in blood parameters were mostly reported in experiments where pigs were fed high levels of DON. Some researcher suggested that the reduction in serum protein levels might be a result of the inhibitory effect of DON on protein synthesis (Rotter et al. 1995). It is also suggested that DON has time- and dose-dependent hepatotoxic effects (Bracarense et al. 2016), which can result in liver injury and consequently reduced protein production and increased serum AST and ALT.

The changes in serum electrolyte levels could be linked to reduced feed intake or DON-induced changes in epithelial morphology, permeability, absorption and ion transporters (Alizadeh et al. 2015; Maresca et al. 2002). However, the exact mechanism is still not understood.

Some of the authors who studied the effects of DON on serum proteins and other blood parameters suggested that some of these changes might be indirectly related to reduced feed intake and poor growth performance. However, a direct effect of DON on platelets, white and red blood cells are also reported in long-term DON-exposure experiments in rodent and farm animals (Payros et al. 2016).

Neurotoxicity and behavioural effects

Effects of DON on CNS has been mentioned previously, under the discussion of emesis and anorexia. In this section, some other DON-related neurotoxic effects and how these effects could be linked to behavioural changes will be described.

A close positive correlation between DON plasma concentrations and DON concentrations in CSF following oral exposure of pigs to DON was reported (Prelusky et al. 1990). In the same study, DON was detected in swine CSF after less than 2.5 min following intravenous administration. It is also reported that DON can rapidly reach CNS by crossing BBB in pigs (Behrens et al. 2015). When DON has reached CNS, it can affect glial cell viability and functions (Razafimanjato et al. 2011), effects that could lead to modification of brain homeostasis and neurological disorders (Bonnet et al. 2012; Kim & de Vellis 2005).

In addition to the proinflammatory effects of DON that could result in anorexia and reduced feed intake, upregulation of proinflammatory cytokines such as IL-1ß, IL-6, and TNF-α might elicit depression and irritability (Bay-Richter et al. 2011). This proinflammatory effect could hypothetically be associated with some welfare issues such as tail biting in pigs (Caplen et al. 2016).

We are only aware of one experiment which demonstrated the behavioural effects of DON and fumonisin (Al-Hazmi & Waggas 2013). In this study, DON-exposed mice showed an increase in the number of bites and aggressive behavior (Al-Hazmi & Waggas 2013). Because of some inconsistencies in the reported doses and results, this study was not assessed by EFSA (Knutsen et al. 2017).

The expression of c-Fos proteins as a marker of neuronal activity was also used in the investigation of DON-induced responses (Bonnet et al. 2012). DON-induced c-Fos immunoreactivity was observed in several central structures, including hypothalamic nuclei and amygdala in pigs (Gaige et al. 2013). It is worth mentioning that the amygdala was excessively activated in animals which showed aggressive behavior patterns (Haller et al. 2006; Poletto et al. 2010).

Reprotoxicity

The *in vivo*, *in vitro* and *ex vivo* effects of DON on swine reproductive organs and performance have been in focus in the last years. DON intoxication could lead to reduced reproductive performance in animals directly by impairing oocyte maturation and embryo development and indirectly by reducing feed consumption (Yu et al. 2017). However, no studies have indicated that DON at more typical levels of naturally contaminated feed is a reproductive toxin in pigs (Mostrom & Raisbeck 2007).

The effects of DON during oocyte maturation and development were investigated in an *in vitro* study using cumulus-oocyte complexes (COCs) (Schoevers et al. 2010). In the same study, the authors showed that DON can impair oocyte development and fertilization by interfering directly with microtubule dynamics during meiosis and by disturbing oocyte maturation. The quality of oocytes has been reduced by feeding the gilts with increasing levels of DON from 0.21 to 9.57 mg/kg (Alm et al. 2006). It is suggested that DON does not have a direct interaction with the steroid hormone receptors (Ndossi et al. 2012). However, the effects of DON on cell viability, steroidogenesis, and gene expression may contribute to endocrine disruptions (Ndossi et al. 2012). Cultured porcine endometrial cells were also

affected by exposure to DON (Tiemann et al. 2003). In an *ex vivo* study, the effects of DON on ovarian morphology were studied using ovarian samples collected from mature, non-pregnant sows (Gerez et al. 2017). The ovarian explants exposed to DON had an increase in histological lesion scores and a reduction in the number of follicles (Gerez et al. 2017).

There is some evidence that DON transfers from pregnant sows to the fetus through the placenta (Goyarts et al. 2007a). The vertical transmission of DON will be described separately and in detail in a later section of the thesis. In this section, the possible DON-induced fetus abnormalities will be mentioned.

Exposure to DON can cause bone malformation in the fetus (Yu et al. 2017). DON-induced bone malformation might be associated with inhibition of protein synthesis and alternations in the expression of genes related to bone development (Yu et al. 2017). It is also suggested that DON can induce abnormalities in early growth stage following hypoxia. One possible explanation for this mechanism is that DON-exposure may result in direct cell damages in red blood cells that can trigger eryptosis (suicidal erythrocyte death) (Yu et al. 2017).

Exposure of male rats to 2.5 to 5 mg/kg/day DON via gastric intubation resulted in dose-dependent reproductive effects (Sprando et al. 2005). In the 5 mg/kg/day DON-treatment group, decreased sperm counts, serum testosterone levels, and increased serum concentrations of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) were reported (Sprando et al. 2005). Increase in the number of rats with testicular germ cell degeneration, failure of sperm release and abnormal germ cell development were also observed at both 2.5 and 5 mg DON/kg dosages (Sprando et al. 2005). To the best of author's knowledge, the effects of DON on the reproductive function of male pigs has not been described to date.

Reproductive performance of sows fed naturally contaminated diets with DON was also recorded in *in vivo* feeding experiments. Feeding lactating sows with 5 mg DON/kg reduced litter weight gain compared to control sows (Jakovac-Strajn et al. 2009). In the same study, the farrowing length was longer in sows fed DON-contaminated feed (Jakovac-Strajn et al. 2009). However, other reproductive parameters such as litter size and total weaned piglets were unaffected in other published reports (Chavez 1984; Diaz-Llano & Smith 2006; Diaz-Llano & Smith 2007; Friend et al. 1986a; Gutzwiller 2010; Herkelman et al. 2017). The weaning-to-service interval as an important reproductive factor in modern piglet production units is also registered in sows exposed to DON. A tendency to have a longer weaning-to-

service interval in sows fed diets contaminated with 5.5 mg DON/kg is recorded (Diaz-Llano & Smith 2007).

Taken together, although DON can demonstrate some direct toxic effects on swine reproduction cells and organs, the main clinical reproductive effects are probably related to indirect effects such as reduced feed intake, resulting in reduced growth in young sows and increased loss of bodyweight in sows during lactation .

Sex and age in DON-related responses

Some studies in swine have shown that males are more sensitive than females when feed consumption and growth performance have been in focus (Andretta et al. 2012; Cote et al. 1985). Feeding 5-wk-old castrated males and female piglets with DON-contaminated diets containing 3.1 and 5.8 mg DON/kg showed that castrated males had a greater suppression of weight gain than females receiving the same diets (Cote et al. 1985). A meta-analytic study of mycotoxins in pig feed showed also that DON-contaminated feed resulted in a 20% reduction in feed intake of male pigs, compared to only 3% reduction in this parameter in the females (Andretta et al. 2012). In the same investigation, DON suppressed weight gain to a higher degree in male pigs compared with females; with growth reductions of 34% and 2%, respectively (Andretta et al. 2012).

Sex-related reduced feed intake and weight gain in rodents were evaluated by measuring markers, including proinflammatory cytokines (*i.e.*, IL-1β, IL-6, and TNF-α) and satiety hormones that are linked to DON-induced anorexia (Clark et al. 2015). Male mice were more sensitive than female mice to DON exposure due to a decreased renal clearance of the toxin and an increased IL-6 response in males (Clark et al. 2015; Pestka et al. 2017). However, the other parameters changed similarly in both female and males.

Another factor that can be involved in differences in severity of DON-related response is age. A higher sensitivity to DON is observed in younger pigs, showing a greater reduction in weight gain compared with older animals, and this may be associated with a reduced capacity of DON metabolism and detoxification in liver (Andretta et al. 2012). Weanling mice had higher plasma and tissue concentration of DON compared with adults receiving identical doses of DON (Pestka & Amuzie 2008). In the same investigation, the levels of proinflammatory cytokines, including IL-1β, IL-6, and TNF-α were higher in young mice compared to adults.

DON and its toxicity in humans

Cereal-based products can be considered as a main source of DON-exposure for human (Knutsen et al. 2017). Based on biomonitoring surveys, more than 90% of the European population has measurable DON concentrations in urine (Carlo et al. 2015). The tolerable daily intake (TDI) of 1 µg/kg BW in humans is established for DON and its acetylated metabolites by EFSA (Knutsen et al. 2017). Small children are especially at risk for exposure to doses that exceed the TDI (Sundheim et al. 2017). Outbreaks of acute gastroenteritis characterised by nausea, vomiting, diarrhoea, abdominal pain, headaches, dizziness, fever and, in severe cases, bloody stool due to ingestion of DON-contaminated food have been reported repeatedly (Knutsen et al. 2017). However, there is a lack of data related to clinical effects of chronic exposure to DON in humans (Knutsen et al. 2017). Therefore, reduced BW and reduction in weight gain as the most common DON-related chronic effects in experimental animals were identified as the most relevant effect to characterize the chronic hazard for humans (Knutsen et al. 2017).

Taken together, concerns about DON-related adverse effects on human health are mainly based on the results from animal studies, and most of the effects that are reported are similar to those that are established and reported in animal experiments.

Toxicokinetics of DON

Toxicokinetics explains how the body handles a toxin and is commonly described by the processes of absorption, distribution, metabolism, and elimination/excretion.

The absorption of DON in animals can be affected by different parameters such as species, age, and sex (Payros et al. 2016). Following oral exposure, DON is absorbed rapidly and appears in blood within 15 to 30 minutes in most mammals (Payros et al. 2016). However, the oral absorption rate of DON varies from 7% in ruminants to 25 % in rodents, and goes up to 89 % and 54% after chronic and acute exposure in pig, respectively (Goyarts & Dänicke 2006; Payros et al. 2016). As mentioned, higher levels of DON in tissue and blood were detected in young mice compared with adult mice (Pestka & Amuzie 2008). The absorption of DON in pigs takes place mainly in the proximal part of the small intestine (Dänicke et al. 2004a).

The distribution of DON to body tissues, including plasma, muscle, abdominal fat, stomach, intestines, liver, kidney, heart, brain, lung, skin, spleen, testes, ovary and adrenals of experimental animals is relatively rapid and transient (Wan et al. 2014). Following oral

administration of 0.5 and 2.5 mg DON/kg/day to rats, the highest concentrations 6 h after administration were found in the gastrointestinal tract (Wan et al. 2014). The following rank order of DON tissue concentrations was observed at 6 h post-exposure in rats receiving 2.5 mg DON/kg: large intestine > small intestine > stomach > liver > kidney >lung > spleen > testes > heart > skin > adrenals > ovary > plasma > muscle > brain > abdominal fat (Wan et al. 2014). Following intravenous administration of 250 and 750 µg DON/kg to pigs, the following rank order of DON tissue distribution was observed at 30 min post-administration for the 750 µg dose: mesenteric lymph nodes > muscle > kidney > stomach > cecum > liver > rectum > ileum > spleen > duodenum > plasma > colon > bile > jejunum (Deng et al. 2015). In the same experiment, the concentrations of DON in tissues of pigs receiving the 250 µg/kg dose of DON exhibited the following rank order: kidney > mesenteric lymph nodes > muscle > stomach > jejunum > colon > plasma > spleen > bile > rectum > cecum > liver >ileum > duodenum (Deng et al. 2015). Comparing the results from these two studies is difficult, due to different routes of exposure.

DON metabolism refers to the mechanisms which are involved in converting the original toxin to various degradation derivatives (Dänicke & Brezina 2013). There is no evidence for bio-activation of DON to more toxic compounds, nor of oxidation to less toxic compounds through phase I metabolism (Payros et al. 2016). However, some pathways for phase II metabolism, including conjugations to glucuronic acid, sulfate or sulfonate have been reported for DON in animals and humans (Payros et al. 2016). Glucuronidation as the major conjugation reaction is widely studied and considered as a major pathway of DON metabolism (Payros et al. 2016). Different species can reduce DON toxicity by different glucuronidation activities (Uhlig et al. 2013). The dominant metabolite in analyzed urine samples of the pigs was DON-15-GlcAc followed by DON-3-GlcAc (Schwartz-Zimmermann et al. 2017). In addition, a novel DON-derived glucuronide Iso-DON-3-GlcAc was detected in pig urine samples (Schwartz-Zimmermann et al. 2017).

Microbial biotransformation of DON into de-epoxy-DON (DOM-1), the most prominent microbial metabolite of DON, has been reported *in vivo* in the digestive tract of both human and several animal species (Payros et al. 2016). There are several bacteria in the digestive tract of animals which can transfer DON into DOM-1. For instance, mixtures of microorganisms from the rumen in dairy cattle, from the gut in chickens and from digestive microbial culture in fish have been shown to reduce DON into DOM-1 (Payros et al. 2016). More specifically, the anaerobic bacterial strain DSM 11798 (also known as BBSH 797)

isolated from rumen fluid is able to biotransform the epoxide group of trichothecenes, including DON into a diene (Fuchs et al. 2002; Grenier et al. 2013). Microbial biotransformation will be discussed further under detoxification of DON in feed.

A rapid clearance of DON is demonstrated in several animal species and the main excretion route of DON is through urine (Goyarts & Dänicke 2006). The other routes of elimination of DON are faeces, and to a very low degree via expiration (Payros et al. 2016). The half-lives of DON in pigs after intravenous and oral administration were 1.5 and 3 h, respectively. (Saint-Cyr et al. 2015). The half-lives of DON in sheep after intravenous and oral administration were 1.2 and 3.2 h (Prelusky et al. 1986). Thus, animal species, dose and route of administration are all important for the estimation of plasma elimination half-life of DON (Dänicke & Brezina 2013).

Vertical transmission of DON

Vertical transmission of DON refers to the transfer of DON from the sows to the fetus via the placenta and to the piglets via colostrum and milk (**Figure 7**). Following maternal DON exposure, embryo toxicity, fetal malformation and developmental disorders have been observed and are evidences for a vertical transmission of DON (Debouck et al. 2001; Tiemann et al. 2008a). Knowledge on this transmission is important to understand the exposure of the fetus during pregnancy and of the new-born piglets.

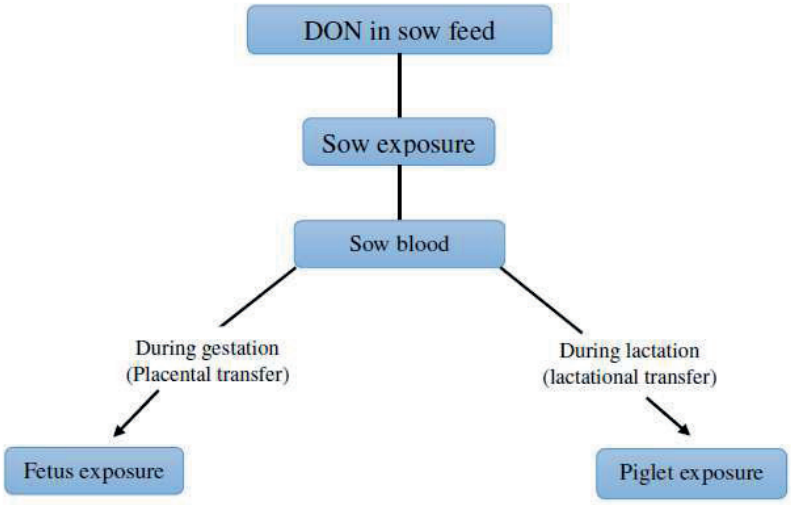


Figure 7. Exposure of fetus and piglets to DON following sow exposure during gestation and lactation.

Placental transfer

The placental transfer of DON may be related to the type of placenta, which is species-dependent (Goyarts et al. 2007a). Due to biological differences between rodents, which have a placenta hemochorialis and pigs, which have a placenta epitheliochorialis, the efficiency of placental mycotoxin transfer might be different (Dänicke et al. 2007). Another factor that may influence the transplacental transfer of DON is the time of exposure in different stages of the gestation period (Goyarts et al. 2007a). Transfer of DON through placenta has been reported in both *in vitro* using cell lines and *ex vivo* using human placenta (Mose et al. 2012; Nielsen et al. 2011). The *in vivo* studies on transfer of DON via placenta are not many. Two *in vivo* studies in swine demonstrated the transfer of DON from the sow to the foeti during days 35–70 of gestation (Goyarts et al. 2007a) and to the piglets during the last third of gestation (Dänicke et al. 2007). In both studies, the foeti and the piglets were delivered by Caesarean section. These authors concluded that the swine foeti and full-term piglets are exposed to DON following exposure of sows to DON-contaminated diets (Dänicke et al. 2007; Goyarts et al. 2007a). Following feeding the pregnant sows in late gestation with contaminated diets containing 9.57 mg DON/kg feed, 9 ng/kg DON was detected in sows' serum and

approximately similar concentration (8 ng/kg) was found in piglet's serum (values are given as medians) (Dänicke et al. 2007).

Lactational (colostrum and milk) transfer

The transfer rate of toxic compounds (including mycotoxins and their metabolites) to milk can be dependent on the anatomical structures of the blood-milk barrier, the polarization, lipophilicity and molecular structure of the substances, degree of the binding to plasma proteins and the differences between the pH of plasma and milk (Fink-Gremmels 2008). The transfer of mycotoxins including DON from feed to bovine milk is studied and reviewed previously (Fink-Gremmels 2008; Flores-Flores & Gonzalez-Penas 2017). However, we are not aware of any previous study on the transfer of DON from contaminated feed to sow milk and the consequent lactational transfer to the piglets. Milk contains high levels of fat and protein. This may contribute to matrix effects that can interfere with the determination of mycotoxins (Winkler et al. 2015). The fat and protein levels in sow milk are higher than in cow milk (Quesnel et al. 2015). This can make the detection of trace compounds such as mycotoxins more complicated in sow milk, compared with cow milk. Colostrum refers to the first milk after parturition and has a composition different from normal milk. Higher protein levels in colostrum compared to normal milk and the less functional blood-milk barrier during early lactation may possibly contribute to differences between the carry-over rate of DON from plasma to milk and colostrum (Wall et al. 2015).

Analytical techniques for DON detection

The methods based on principles of immunoassay, chromatography, and mass spectrometry are mentioned as the available analytical methods for detection of DON and its derivatives (Ran et al. 2013). Although the same analytical methods can be used to determine mycotoxins in different matrices, there are different sample preparation methods to extract the analytes of interest from food, environmental and biological matrices (Capriotti et al. 2012).

The analytical methods can be classified into two main categories; 1) fast screening methods and 2) chromatography or mass spectrometry-based quantitative methods (Ran et al. 2013). Fast screening methods include thin layer chromatography (TLC), enzyme-linked immune-sorbent assay (ELISA), dual-label time-resolved fluoroimmunoassay (TRFIA), multiplex flow cytometric microsphere immunoassay, immunochip, immuno-rotary biosensor (IRB), lateral flow immunoassay (LFI), planar waveguide (PWG), surface plasmon resonance (SPR), biolayer interferometry (BLI) and electronic nose (EN) with metal-oxide-semiconductor

(MOS). Chromatography or mass spectrometry-based quantitative methods include high-performance liquid chromatography (HPLC), gas chromatography-mass spectrometry (GC-MS) and liquid chromatography-mass spectrometry or liquid chromatography-tandem mass spectrometry (LC-MS or LC-MS/MS) (Ran et al. 2013).

A number of immunoassay techniques as fast screening method and LC-MS/MS for quantitative analysis are considered the most accepted methods, because of high sensitivity and rapid performance. LC-MS/MS may also be used to monitor DON, its derivatives and the other co-occurring mycotoxins simultaneously (Ran et al. 2013). Multi-mycotoxin LC-MS/MS analysis is an important and valuable method to monitor a large number of mycotoxins as well as "masked" mycotoxins; mycotoxin conjugates that may be produced by plants as part of defense mechanism (Streit et al. 2013). LC-MS/MS is also the method of choice for detecting mycotoxins in biological samples (Brezina et al. 2014).

Countermeasures

In the last decade, a variety of strategies and approaches have been developed in order to prevent mycotoxin contamination, achieve decontamination of mycotoxins in food and feed, and/or inhibit mycotoxin absorption in the gastrointestinal tract (Kabak et al. 2006).

Prevention of DON contamination

The risk of feed contamination with DON and other trichothecenes can be reduced by different approaches, which can be divided into pre-harvest, harvest and post-harvest management and control strategies (Aldred & Magan 2004).

At the pre-harvest stage, development of resistant varieties and use of appropriate fungicides in combination were shown to reduce DON production (Aldred & Magan 2004). The full-dose application of fungicides that target *Fusarium* species at mid-flowering has shown to be the most effective inhibitor of DON production (Aldred & Magan 2004). Deep plowing that can change the surface environment and remove the residual fungal material from the surface and crop rotation that can break down the production of *Fusarium* infectious material could be mentioned as appropriate field management strategies (Aldred & Magan 2004). Another pre-harvest control method is the use of biological control agents that can reduce DON production by competing with *Fusarium* pathogens and lead to decreased sporulation of *Fusarium* species (Aldred & Magan 2004).

Providing appropriate storage conditions, use of preservatives and sorting the feed batches before feed processing are the important keys at the post-harvest stage. Felleskjøpet as the

leading Norwegian agricultural cooperative has a well-established control and analysis system, to ensure good hygienic quality of grains for swine feed (**Figure 8**) (Ljøkel et al. 2013). After receiving the grain batches, a fast screening test (Rida-Quick) is performed to map the quality of the grains. These analyses provide the basis for classifying the grains in high and low DON contents. The grains containing high DON levels will not be approved for swine feed. In addition, a high number of ELISA analyses are performed to estimate DON levels more exactly; in order to sort the grains of different quality to the right factories and animal species. Felleskjøpet has also well established strategies to control storage key points such as humidity and temperature (Ljøkel et al. 2013).

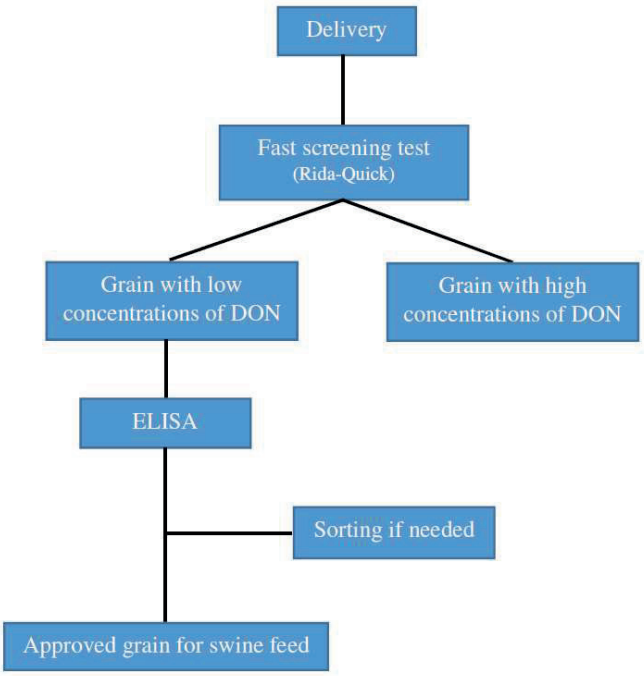


Figure 8. Overview of Felleskjøpet's system to ensure acceptable DON levels in grains for swine feed.

Detoxification of DON in feed

It is not always possible to prevent mycotoxin contamination by pre-harvest, harvest and post-harvest stages. Therefore, it is important to develop other methods to reduce or eliminate mycotoxins before the use of grains for feed purposes (Kabak et al. 2006). Different approaches based on the use of chemical agents as adsorbents and feed additives containing enzymes and biological agents have been used to prevent mycotoxin toxicity. However, some of these methods, such as the addition of adsorbents, are less effective in binding and reducing the bioavailability of trichothecene toxins, due to the high water solubility of these compounds (Kabak et al. 2006).

Addition of non-nutritional adsorbents

The adsorbing abilities of some adsorbents, including activated carbon (AC), hydrated sodium calcium aluminosilicate (HSCAS) and sepiolite were investigated in previous *in vitro* studies (Kabak et al. 2006). None of these adsorbents were sufficiently effective in their adsorbing ability to reduce the bioavailability of DON (Kabak et al. 2006). However, some types of AC showed a relevant adsorbing ability to bind DON (Galvano et al. 1998). It is also worth mentioning that some of these adsorbents such as AC are relatively unspecific adsorbents, so adsorption of some essential nutrients can not be ruled out when we use these components (Awad et al. 2010).

Chemical

The use of sodium bisulfite and ozone gas have shown to be effective in reducing or degrading DON (Awad et al. 2010). However, the chemical methods are not recommended due to the production of toxic metabolites and reduction of the nutritional value of treated food and feed (Awad et al. 2010).

Biological

There are few approved and effective strategies which can limit the DON-related adverse effects of contaminated grain on livestock. One of the most promising approaches is the use of feed additives containing microorganisms, which have been shown to be effective in detoxification of trichothecene mycotoxins (Awad et al. 2010). Different chemical reactions including acetylation, deacetylation, oxidation, de-epoxidation, epimerization, and glycosylation can be involved in the microbial biotransformation of trichothecene mycotoxins (McCormick 2013). However, only oxidation and de-epoxidation are clearly documented as types of bacterial degradation of DON (McCormick 2013). A soil microbe, *Agrobacterium–*

Rhizobium (strain E3-39), can convert DON to 3-keto-DON by oxidation reaction (Shima et al. 1997). Bacteria from fish and chicken intestine are able to convert DON to DOM-1 (McCormick 2013). Under aerobic conditions, a mixture of enterobacteria, including *Serratia*, *Clostridium*, *Citrobacter*, and *Enterococcus*, isolated from soil can also de-epoxidase DON to DOM-1 (Islam et al. 2012).

Rumen and intestinal microbes have been shown to detoxify DON to the less toxic DOM-1. Final metabolism products from de-epoxidation of DON such as DOM-3-GlcAc are the dominant DON metabolites in cow urine (Schwartz-Zimmermann et al. 2017). The occurrence of detoxifying bacteria in bovine rumen is thought to be responsible for the relative tolerance of ruminants to DON contamination. A new genus (*Gen. nov.)* in the family of *Coriobacteriaceae* (DSM 11798), isolated from bovine rumen, has been shown to have de-epoxidase activity and is able to degrade DON to DOM-1 at a minimum dose of 1.7×10^8 CFU/kg complete feed (EFSA 2013). BBSH 797, containing the bacterial strain DSM 11798, is a commercial feed additive produced by Biomin®. The efficacy of this product has been confirmed *in vitro* under anaerobic conditions and *ex vivo* in swine gut (Fuchs et al. 2002; Schatzmayr et al. 2006). EFSA has also assessed the safety and efficacy of this feed additive *in vivo*, based on published and unpublished reports (EFSA 2005; EFSA 2013).

Aims and objectives of the thesis

The overall aim of this thesis is to evaluate the effects of DON on health, welfare and productivity of pigs during different life stages.

The specific objectives were to:

- Study the efficacy of the bacterial strain *Coriobacteriaceum* DSM 11798 (the active ingredient in Biomin® BBSH 797) as a DON-detoxifying agent in growing pigs fed naturally contaminated diets with DON (**Paper I**).
- Evaluate the correlation between actual uptake, metabolism of DON and clinical effects, including blood parameters, as well as uptake and metabolism of DON in growing pigs, and in gestating and lactating sows (**Papers I and II**).
- Evaluate the effects of DON on reproduction performance in gestating and lactating sows (**Paper II**).
- Evaluate vertical transmission from the sows fed contaminated diets with DON to their offspring through the placenta in the last stage of gestation and colostrum and milk during lactation (**Paper III**).
- Provide knowledge about toxicokinetics of DON by oral and intravenous exposure of piglets to DON. These results have been used by Dr. Christiane Fæste et al. in a study on prediction of DON toxicokinetics in humans, and published in relation to that (**Paper IV**).

Materials and Methods

This thesis is based on the data from three experiments, where the first experiment was a controlled and short-term toxicokinetic experiment in piglets, the second one was a controlled feeding experiment in growing pigs, and the last experiment was a feeding field trial in gestating and lactating sows.

Ethical considerations

The animal experiments in this thesis were conducted in accordance with Norwegian regulations for animal testing (FOR-2015-06-18-761), which comply with EU Directive 2010/63/EU. The Norwegian Animal Research Authority approved the study and all experimental procedures in papers **I** and **IV**. A detailed report was also evaluated and confirmed approvable by the Norwegian Animal Research Authority after completion of the study described in papers **II** and **III**. ARRIVE guidelines and three Rs (replacement, reduction and refinement) for animal studies were followed in design and performing of experiments involved in this thesis. The kinetics study and feeding studies in growing pigs and sows were not classified as painful procedure. The only procedure that could contribute to cause pain was blood sampling. However, the use of appropriate sampling methods performed by trained veterinarians reduced the pain and stress related to blood sampling to a minimum.

Animals and housing

Paper **I** is based on the data from a 42-day experiment conducted in two rounds due to logistical limitations. The first round was done in July and August 2014, and the second round in September and October 2014. Forty-eight crossbred (Landrace-Yorkshire/Duroc-Duroc) weanling pigs (24 in each round) with a mean initial weight of 11 ± 1.5 kg were individually housed in floor pens in environmentally controlled rooms in the production animal facility of the Faculty of Veterinary Medicine at NMBU (**Figure 9**). The pigs were divided into 8 feeding groups at the beginning of each round (three animals in each group). We equalized the number of females and castrated males within groups. The temperature of the animal facility was 25 °C and 23 °C in the first and second round, respectively. Routine anthelmintic treatment was given and the animals were allowed seven days acclimatization to environment and diet before the start of the study. In the acclimatization period, the pigs were fed the control-diet supplied for the study. Automatic feeders and drinking cups were used to provide *ad libitum* access to water and feed throughout the experiment. At the study end, the pigs were

euthanized and organs of interest were collected for further pathological and histopathological analysis.

Figure 9. Animal facility used in growing pig study (Paper **I**) (photo: Ulrik Hansen).

Papers **II** and **III** is based on the data from a study that was conducted in a commercial specific pathogen-free (SPF) piglet production unit. This experiment was performed during the period from December 2015 to February 2016. In a 53-day experiment, 47 Norwegian crossbred (Landrace × Yorkshire) sows in a farrowing group were distributed on the base of parity numbers, in order to have an even distribution of parity within groups. Two sows (one from the control group and one in the group receiving feed with the highest DON level) were removed from the trial. The sow from the control group died during the lactation period due to a penetrating gastric ulcer, while the sow in the highest dose group was excluded due to complete refusal of the contaminated feed. The final study therefore included 45 sows (Paper **II** and DON-uptake study in Paper **III**), divided into three groups: (1) control (DON < 0.2 mg/kg) (n = 15); (2) DON level 1 (1.4 mg DON/kg) (n = 15); and (3) DON level 2 (1.7 mg DON/kg) (n = 15). In paper **III**, four sows from the control group, six sows from the DON level 1 group and six sows from DON level 2 group were selected for the transfer study. The sows were selected randomly, excluding sows that started farrowing during the night. The

new-born piglets were caught before they received colostrum. The first five liveborn and healthy piglets from each of these sows were selected (Paper **III**, Figure 4). The selected piglets were ear marked with plastic tags, and gender was recorded. The analyses of DON concentrations in sow plasma indicated that a mistake in the feeding of one sow in the DON level 2 group had occurred. The plasma concentrations of DON and its metabolites in this sow were below the LOD at 5 of 8 sampling points. This sow and its piglets was therefore excluded from all calculations and statistical analysis. Thus, the final dataset included 44 sows (15 controls, 15 at DON level 1, and 14 at DON level 2) in paper **II** and in the DON-uptake study; and 15 sows (4 controls, 6 at DON level 1, and 5 at DON level 2) in the transfer study (Paper **III**, Figure 4). From approximately 3 weeks before expected farrowing until weaning, the sows were placed individually and without fixation in standard farrowing pens. Each pen (7.0 m^2) had a piglet creep area (1.3 m^2), a solid concrete floor and a slatted draining floor at one end of the pen (2.3 m^2). The pens had individual feeding station for sows and both sows and piglets had *ad libitum* access to water. The sows were fed twice daily until 1 week postpartum, when the feeding frequency was increased to 3 times daily, and 4 times daily from 2 weeks postpartum to weaning. In addition, all sows were offered 0.2 kg of hay daily. Farrowing was allowed to occur naturally, and under constant surveillance by the farmer and the research group. Commercial husbandry procedures were performed within 48 h after birth, including teeth grinding and a 270 mg oral iron supplementation, and thereafter a creep feed mixed with iron fortified peat was offered to piglets on the concrete floor of the piglet creep area until 4 days before weaning. During five to ten weeks after farrowing, surgical castration was performed by a local veterinarian, using local anesthesia and analgesia. Necessary cross-fostering of piglets was performed within or across treatments within 48 h after farrowing, and recorded exactly. All litters were weaned at the same day. The average lactation length was 33.3 days (range 29-38).

The *in vivo* piglet experiment in paper **IV** is based on the data from a short-term toxicokinetics study with 12 Norwegian-crossbred (Landrace × Yorkshire) piglets, six of each sex, with approximately 9–16 kg BW. The piglets were obtained from a commercial breeder. The animals were allowed to acclimatize for 7 days in individual boxes at animal facility that is mentioned in paper **I**. The piglets had *ad libidum* access to water with drinking cups and to conventional piglet feed via automatic feeders until 12 h before study start and 2 h after the application of DON. At the start of the experiment, the piglets were weighed and ear marked.

At the study end, the pigs were euthanized and organs of interest were collected for further analysis.

Experimental DON exposure

In paper **I**, after the 1-week acclimatization period, each group was fed pelleted feed with different levels of naturally DON-contaminated oats. Eight diets were formulated to provide the following treatments: (1) control-diet (DON < 0.2 mg/kg), (2) Control-diet supplemented with a feed additive (FA) containing the trichothecene-degrading bacteria DSM 11798, (3) low-contaminated-diet (DON = 0.9 mg/kg), (4) low-contaminated-diet (DON = 1.0 mg/kg) with FA, (5) medium-contaminated-diet (DON = 2.2 mg/kg), (6) medium-contaminated-diet (DON = 2.5 mg/kg) with FA, (7) high-contaminated-diet (DON= 5.0 mg/kg), (8) high-contaminated-diet (DON = 5.7 mg/kg) with FA. The pelleted diets were produced at the Center for Feed Technology (Fôrtek) of Norwegian University of Life Sciences (NMBU), keeping the pelleting temperature close to 80 °C.

In papers **II** and **III**, the sows were fed with diets with different levels of naturally DON-contaminated oats from arrival in the farrowing unit until weaning of the piglets. The following treatment groups were provided: (1) control-diet (DON < 0.2 mg/kg), (2) DON-contaminated diet level 1 (DON = 1.4 mg/kg), and (3) DON-contaminated diet level 2 (DON = 1.7 mg/kg).

In paper **IV**, the piglets were divided in 2 treatment groups of six piglets of both sexes. The piglets in the first group were exposed to DON intravenously by injection of 0.08 mg/kg BW DON in sterile physiological saline solution (0.2 mg DON/ml) via peripheral venous catheter in the ear vein. The other group received 0.125 mg/kg BW DON in water (0.1 mg DON/ml) orally by gavage via an infusion tube behind the tongue.

The different levels of DON in papers **I**, **II** and **III** were provided by blending the naturally contaminated oats harvested in southern Sweden in 2013 with low contaminated oats harvested in Norway in 2013 and 2014 in paper **I** and papers **II** and **III**, respectively. In paper **IV**, DON solution was obtained from Sigma-Aldrich Norway (Oslo, Norway).

The analysis of experimental diets

In papers **I**, **II** and **III**, the experimental feed were analyzed at Norwegian Veterinary Institute for DON, DON-3-Glc, 3-ac-DON, and 15-ac-DON with a liquid chromatography-high resolution mass spectrometry (LC-HRMS) method that is previously validated (Ivanova et al.

2017). In addition, a semi-quantitative multi-toxin screening method at the Centre for Analytical Chemistry at IFA Tulln, Austria was used for analyzing a range of other mycotoxins in the experimental diets in paper **I**.

The number of CFU of DSM 11798 in paper **I** were controlled by Biomin Research Center (Tulln, Austria) in the finished FA and in the pelleted diets, using a validated enumeration method based on Koch's pour plate method: Serial dilutions of a test portion were prepared in a suitable diluent under anaerobic conditions. 1 ml aliquots of three suitable dilutions were transferred to Petri dishes and mixed with an agar medium. After incubation under strictly anaerobic conditions, colonies were counted and CFU of the original sample calculated. The test was performed in triplicate. An amount of viable bacteria considered to be sufficient were confirmed, both before and after the pelleting process.

Data collection

In papers **I**, **III** and **IV**, our research group performed the data collection. In paper **II**, data collection was a combination of data registered by our research group and by the farmer. Some variables were registered by the farmer and reported directly to the research group. Recorded data that were registered or reported by the farmer were the date of insemination, daily feed consumption, culling reasons, number of sows returning to heat at 5 days after weaning, number of conceptions at first subsequent service and number of piglets born alive in subsequent litter.

Daily feed intake

In paper **I**, feed consumption was measured individually and continuously by weighing each of the automated feeding stations, every time new feed was added and otherwise three times a week (**Figure 10**). In papers **II** and **III**, the daily feed consumption of the sows was recorded by a plastic dispenser that showed the amount of feed intake at each feeding (**Figure 11**). In this experiment, daily feed consumption was referred to feed disappearance from the dispenser. Adjusting of the feeder for feed spillage or consumption by piglets was not performed.

Figure 10. A pig is eating from the automated feeding station (Paper **I**) (photo: Ulrik Hansen).

Figure 11. The plastic dispensers used for recording feed consumption (Paper **II**) (photo: Tore Sivertsen).

Body weight

In paper **I**, the body weight (BW) was measured individually twice during the acclimatization period and thereafter twice weekly until the end of experiment. In papers **II** and **III**, the sows were weighed upon arrival to farrowing unit and after 10 days, during the late gestation period. The sows and litters were further weighed at birth (day 1: within 12–36 h after parturition), on days 7 and 21 of lactation and at weaning (**Figures 12** and **13**). The piglets included in the transfer study in paper **III** were also weighed individually, at the same occasions (the first weighing done at 48 ± 12 h after parturition).

Figure 12. Weighing of sow (Papers **II** and **III**) (photo: Tore Sivertsen)

Figure 13. Weighing of litter (Paper **II**) (photo: Tore Sivertsen)

Body condition score

In paper **II**, all sows were evaluated for body condition and given a body condition score (BCS) upon arrival in the farrowing unit, after 10 days, 12–36 h after parturition, on days 7 and 21 during lactation, and at weaning. BCSs were performed by veterinarians in the research group, using the scale developed by Animalia and Norsvin (Norsvin, 2007). The scales were ranging from 1 to 5, with half-point gradations: 1 is emaciated, 2 is thin, 3 is good condition, 4 is overweight, and 5 is obese.

Backfat measurements

In paper **II**, The ultrasound backfat measuring were used to measure the backfat depth (in mm) over the second-to-last rib of the sows. Backfat measurement was performed twice after farrowing: 1–7 days postpartum and at weaning.

Litter and reproductive performance

In paper **II**, gestation length, duration of farrowing, number of total born, born alive, and stillborn piglets were registered for each sow on the day of parturition. Stillborn piglets in

each litter were registered at the end of farrowing and the litter size was recorded at birth, on days 7 and 21 during the lactation period and at weaning. The number of the sows that returned to heat after 5 days was recorded after weaning. In addition, the number of conceptions at first service, and the number of piglets born alive in the subsequent parity were registered.

Skin temperature

In paper **II**, skin temperature of the sows were monitored as an indicator of the clinical and physiological effects of DON. The reason that the rectal temperature was not used is that the handling and restraint increase stress, and can affect the core and surface temperatures. Using a thermal imaging camera (FLIR i7, FLIR System, Inc., Wilsonville, OR, USA), the skin temperatures from the sow's ear (**Figure 14**) were registered upon arrival in the farrowing unit, 10 days after arrival, within 12–36 h after farrowing, on day 7 and 21 during the lactation period and at weaning.

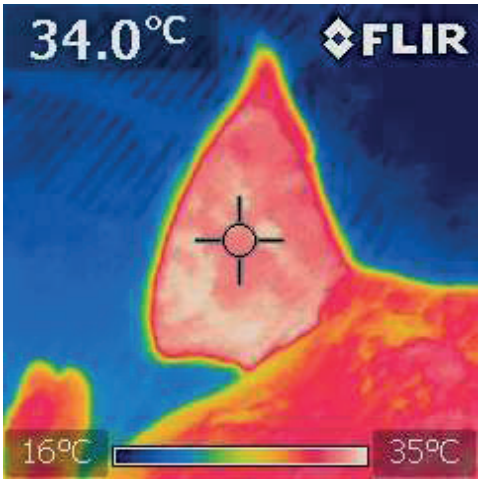


Figure 14. The thermal image taken by FLIR i7 camera shows the skin temperature (Paper **II**).

Clinical Chemistry and Hematology

In paper **I**, blood was sampled from the jugular vein of the pigs using 5 ml vacutainer tubes. Blood samples for clinical evaluations were taken on day −3 (week 0) in the acclimatization period and thereafter on day 4 (week 1), day 18 (week 3), and day 35 (week 5) in the active experiment. Blood for measuring hematological parameters was collected in 5-ml tubes with EDTA as anticoagulant. The tubes were kept refrigerated and delivered to the Central Laboratory of the Faculty of Veterinary Medicine at NMBU. Blood for analysing serum chemistry and biochemistry was collected in 5-ml tubes with gel and clot activating factor. To ensure complete clotting, the tubes were kept at room temperature for 1–2 hours. Serum was then separated by centrifugation at 1500 × *g* for 10 minutes at room temperature (approximately 20 °C), stored in 2-ml cryogenic vials, and delivered to the Central Laboratory.

In paper **II**, blood samples from the sows were collected from the milk vein (*v. subcutanea abdominis*), using 9 ml heparin tubes for chemistry and 5-ml EDTA tubes for hematology. Blood samples were obtained upon arrival to the farrowing unit, 10 days after arrival, within 12–36 h after parturition, and at weaning. The EDTA tubes were kept refrigerated and delivered to the Central Laboratory of the Faculty of Veterinary Medicine at NMBU. Plasma for analysing plasma chemistry and biochemistry was prepared by centrifugation at 1500 ×*g* for 10 minutes at room temperature (approximately 20 °C), stored in 2-ml cryogenic vials, and delivered to the Central Laboratory.

All analyses of hematology and clinical chemistry in papers **I** and **II** were performed by the Central Laboratory. The hematological analyses were done using ADVIA® 2120 Hematology System ADVIA® Multispecies software (Siemens Healthcare Diagnostics, Siemens AG, Erlangen, Germany), with the settings for swine species. The clinical biochemical analyses were done using ADVIA 1800® Clinical Chemistry System (Siemens Healthcare Diagnostics, Siemens AG, Germany), and serum protein electrophoresis was performed on Sebia CapillarysTM 2 (Sebia, Norcross, GA, USA). The biochemical analysis included aspartate aminotransferase (AST), alkaline phosphatase (ALP), gamma-glutamyl transpeptidase (GGT), glutamate dehydrogenase (GLDH), creatine kinase (CK), C-reactive protein (CRP), total serum protein, total serum globulins, urea, creatinine, total bilirubin, cholesterol, glucose, inorganic phosphate, and calcium.

Clinical chemistry and hematology were not included in papers **III** and **IV**.

Plasma concentrations of DON and its metabolites in plasma

In paper **I**, blood samples from the pigs for measuring plasma concentrations of DON and its metabolites were collected once at day −3 in the acclimatisation period, and thereafter three times a day (08:00, 11:00 and 16:00) on days 1, 4, 11, 18, 25, 32 and 35 in the active experiment.

In paper **III**, blood samples from all sows included in the DON-uptake study were taken from the milk vein (*v. subcutanea abdominis*) using 9 ml Lithium-Heparin tubes. The samples were collected upon arrival in the farrowing unit, after 10 days, on day 21 in lactation, and at weaning. In the same paper, the additional blood samples of the sows included in the transfer study were taken from the milk vein, using 9 ml heparin tubes; during farrowing, within 12–36 h after parturition, on day 7 and 21 in the lactation period, and at weaning. Blood samples from the piglets in the DON-transmission study were collected from the jugular vein (*v. jugularis*), using 2.7 ml S-Monovette® Lithium-Heparin tubes (Sarstedt AG & Co. KG, Nümbrecht , Germany) immediately after birth (before uptake of colostrum), within 12–36 h after parturition, on day 7 and 21 in the lactation period and at weaning.

In paper **IV**, blood was collected from the jugular vein using a 21G cannula and heparinised 3 ml vacutainers at 0, 0.042, 0.083, 0.17, 0.33, 0.67, 1, 1.5, 2, 4, 6, 8, and 24 h after intravenous administration of DON. In the same paper, the blood was collected at 0, 0.083, 0.17, 0.33, 0.67, 1, 1.5, 2, 4, 6, 8, and 24 h after oral administration of DON.

In papers **I**, **III** and **IV**, the blood samples were stored refrigerated until plasma as soon as possible was separated by centrifugation at 1500 × *g* for 10 min at room temperature (approximately 20°C) and stored frozen in 2-ml cryogenic vials at − 20 °C until analysis. DON and its metabolites DON-3-GlcA (ng/ml), DON-15-GlcA (ng/ml) and DOM-1 (ng/ml) were then analyzed by LC–HRMS.

Concentrations of DON and its metabolites in colostrum and milk

In paper **III**, the colostrum was collected shortly after the onset of parturition, transient milk on day 1 after parturition, and mature milk on day 7 and 21 in the lactation period and at weaning. The milk samples were prepared by Immunoaffinity columns (*aokinImmunoClean*) for the quantification of DON. The columns were supplied by aokin AG (Campus Berlin-Buch, Robert-Rössle-Straße 10, Berlin, Germany). After preparation of the samples, the residue was transferred to HPLC vials prior to LC-HRMS analysis. DON and its metabolites DON-15-GlcA (ng/ml) and DOM-1 (ng/ml) were analyzed in milk samples.

Statistical Analysis

Statistical analysis in this thesis was performed in JMP®, Version 10 (SAS Institute Inc., Cary, NC, U.S.A.). The level of significance was set to 0.05 in all models, and results with *p*-values between 0.05 and 0.1 were considered significant trends. If not otherwise specified, all results are expressed as the mean ± standard deviation (SD). The normality of distribution of the different parameters was determined by residual and predicted values plot, normal-percentile plots and Shapiro-Wilk test. If the *p*-value for Shapiro-Wilk test was larger than 0.05, the data were considered normal. Data that were not normally distributed were transformed or analyzed by models such as Wilcoxon rank-sum and Kruskal-Wallis tests, which are suited for non-normal distributions. The same tests were performed when the data failed the assumptions of the analysis of variance (ANOVA). If the data generated from the application of ANOVA were significantly different, the post-hoc, Tukey-Kramer honest significant difference (HSD) test was used for multiple comparisons and distinction of significant differences. The Steel-Dwass test was used for multiple comparisons of the data generated from the application of Kruskal-Wallis test in paper **II**. Levene's test was used to check the assumption of homogeneity of variances. In all models, backward elimination was used to remove variables and interaction between variables that were not statistically significant or showing no evidence of statistical trends. In paper **I**, The data were considered as a completely randomized block design with eight treatments in six blocks. Each pig was considered as a random effect and represented an experimental unit for the variables tested. The DON-levels and FA were considered as independent variables. ADFI, ADG, feed conversion ratio (FCR), hematological and biochemical parameters, and plasma concentrations of DON and its metabolites were defined as dependent variables. Performance and blood parameters were subjected to repeated-measure ANOVA according to a three-factorial design. In paper **II**, the data were considered as a completely randomized block design with three treatments in 15 blocks. Each sow and its litter were considered as a random effect and represented an experimental unit for tested variables. The experimental diets were the independent variables. In paper **II**, Growth performance parameters, skin temperature, and hematological and biochemical parameters were defined as dependent variables. In paper **III**, neither mycotoxin residues measured in collected plasma samples from the sows fed experimental diets and in the plasma of their piglets (pooled per sow) nor milk-to-plasma (M/P) ratios were normally distributed, and they were therefore tested by the non-parametric

Wilcoxon Each Pair test ($p < 0.05$). In papers **I** and **III**, concentrations below the LOD were represented by the LOD divided by the square root of 2 in the statistical calculations.

Repeated measures

In paper **I**, performance, hematological and biochemical data were analyzed by repeated measures analysis using a mixed model. The interactions that were not statistically significant were removed from the models by backward elimination.

In paper **III**, piglet growth performance was analyzed by repeated measures analysis using a mixed model. The interactions that were not statistically significant were removed from the models by backward elimination.

Mixed effects model without the repeated statement

In paper **II**, statistical differences between the treatment groups for changes in sow weight, feed consumption, weight loss, litter weight, skin temperature, hematological and biochemical data, total backfat, and total litter gain were analyzed using a mixed effects model with treatment, parity, and their interaction as fixed effects and individuals nested to treatments as a random effect. When appropriate, number of piglets born alive, number of weaned piglets, and lactation length were included in the models as covariates. Covariates and interactions that were not statistically significant were removed from the models by backward elimination.

Log-linear regression

In paper **II**, count data were analyzed using log-linear regression models. Total number born, number born alive, and number of liveborn piglets in subsequent parity were examined by log-linear regression with normal distribution. A log-linear analysis with a Poisson distribution for the number of weaned piglets and with a negative binomial distribution for gestation length and duration of farrowing were used.

Correlation coefficient

In paper **III**, correlation between the DON concentrations in the plasma and milk samples of sows and the piglet plasma DON concentrations were analyzed by a non-parametric Spearman's rank-order correlation test for non-normally distributed data and by Pearson correlation test for normally distributed data ($p < 0.05$). Piglet samples were pooled per sow.

Correlations between the plasma DON concentrations and total serum protein and serum calcium in growing pigs and sows were also analyzed. The results are presented in "Results and discussion" of this thesis.

Simple linear regression

Beside the results reported in the papers, a simple linear resression analysis was used to find the relationship between plasma DON concentrations and total serum protein, DON intake and plasma DON concentrations, and DON intake and plasma DOM-1 concentrations in growing pig and sow studies, based on calculations over the entire exposure period.

T-test

In paper **III**, the glucuronidation rate is defined as the sum of plasma DON-3-GlcA + DON-15-GlcA concentrations divided by total DON (sum of plasma DON + DON-3-GlcA + DON-15-GlcA concentrations). These values regardless of DON levels were normally distributed and analyzed by t-test ($p < 0.05$).

Summary of the papers: main results

Paper I

Effects and biotransformation of the mycotoxin deoxynivalenol in growing pigs fed with naturally contaminated pelleted grains with and without the addition of *Coriobacteriaceum* DSM 11798

In this feeding study, we aimed to investigate the efficacy of a feed additive containing the bacterial strain *Coriobacteriaceum* DSM 11798 in detoxifying DON and evaluate the effects of contaminated diets with different concentrations of DON on growth performance, blood parameters, as well as uptake and metabolism of DON in growing pigs. During the first 7 days of exposure, pigs in the highest dose group (DON = 5.35 mg/kg) showed a 20–28% reduction in feed intake and a 24–34% reduction in weight gain compared with pigs in the control (DON < 0.2 mg/kg) and low-dose (DON = 0.96 mg/kg) groups. These differences were levelled out by study completion. Towards the end of the experiment, dose-dependent reductions in serum albumin, globulin and total serum protein were noted in the groups fed with DON-contaminated feed compared with the controls. The addition of DSM 11798 had no effect on the DON-related clinical effects or on the plasma concentrations of DON. The ineffectiveness of the feed additive in the present study could be a consequence of its use in pelleted feed, which might have hindered its rapid release, accessibility or detoxification efficiency in the pig's gastrointestinal tract.

Paper II

Effects of feeding naturally contaminated deoxynivalenol diets to sows during late gestation and lactation in a high-yield specific pathogen-free herd

The aim of this study was to investigate the effects of feeding grains that are naturally contaminated with moderate, realistic levels of DON on sows during late gestation and lactation, under field conditions in a commercial, high-yield specific pathogen-free piglet production unit. In this study, sows that were fed DON levels 1 (1.4 mg/kg) and 2 (1.7 mg/kg) diets showed a 4-10% reduction in feed consumption during lactation, compared with sows in the control group. Statistically, sows fed the DON level 2 diet had significantly lower ADFI ($6.12 \pm 0.41\ kg$) than the control group ($6.76 \pm 0.56\ kg$) during lactation. The interaction between parity and DON treatment did also have a statistically significant effect on feed consumption during lactation. However, BW or backfat thickness were not significantly affected by the DON-contaminated diets. Similarly, there were effects neither on production or reproduction performance, nor on blood parameters in the sows. Variable effects on skin temperature were also detected. In conclusion, naturally contaminated diets with realistic, moderately increased DON levels (up to 1.7 mg DON/kg feed), fed during late gestation and lactation in a modern high-yield piglet production farm, had limited effects on sow health and production.

Paper III

Transfer of deoxynivalenol (DON) through placenta, colostrum and milk from sows to their offspring during late gestation and lactation.

This study was aimed at investigating the DON uptake in sows fed naturally contaminated diets, DON transfer across placenta during late gestation and transfer of DON to the milk and consequently to the piglets during lactation. Forty-five crossbred (Norwegian Landrace × Yorkshire) sows were fed from 93 ± 1 days of gestation until weaning of the piglets with feed made from naturally contaminated oats, with DON at three concentration levels: (1) control (DON < 0.2 mg/kg), (2) DON level 1 (1.4 mg DON/kg), and (3) DON level 2 (1.7 mg DON/kg). The transfer of DON to the piglets was evaluated in 15 of the sows, with repeated samples of blood and milk from the sows, and repeated blood samples from five of the piglets of each sow. Piglet/sow plasma ratios (mean of concentrations of DON in the plasma of the piglets divided by that of the sow) and milk/plasma (M/P) ratio (DON concentrations in sow milk divided by DON concentration in sow plasma) were calculated as estimates of the degree

of transfer. Average piglet/sow plasma ratios for all sows eating DON-contaminated feed were 2.14, 2.30, 0.08, 0.16 and 0.20 at birth, within 12–36 h after parturition, on day 7 and 21 in lactation and at weaning, respectively. M/P ratios were 0.92, 1.11, 0.94, 1.21 and 0.90 at birth, within 12–36 h after parturition, on day 7 and 21 in lactation and at weaning, respectively. These results indicate that DON is efficiently transferred across placenta and through milk. However, the low piglet/sow plasma ratios at mid-lactation to weaning indicate that the piglets were most strongly exposed to DON risk in early life, despite the high M/P ratios and efficient lactational transfer over the entire lactation.

Paper IV

Prediction of deoxynivalenol toxicokinetics in humans by in vitro-to-in vivo extrapolation and allometric scaling of in vivo animal data

In this paper, *in vitro*-to-*in vivo* extrapolation of depletion constants in hepatic microsomes from different species and allometric scaling of reported *in vivo* animal parameters were used to predict the plasma clearance [0.24 L/(h × kg)] and volume of distribution (1.24 L/kg) for DON in humans. The determined human toxicokinetic parameters were then used to calculate the bioavailability (50–90%), maximum concentration, and total exposure in plasma, and urinary concentrations under consideration of typical DON levels in grain-based food products. The results were compared to data from biomonitoring studies in human populations.

In the parts of this paper included in the present thesis[1], we performed an *in vivo* toxicokinetic study with oral and intravenous administration of DON in pigs to establish benchmark parameters for the *in vitro* extrapolation approach. Toxicokinetic parameters were determined *in vivo* in piglets after iv and po administration of DON. The half-lives of DON in plasma were 2.6 h and 3.8 h after iv and po administration, respectively. Plasma clearance and volume of distribution were 0.21 L/(h × kg) and 0.7 L/kg, respectively. The maximal plasma concentration after po administration was reached after 3.5 h, and absolute bioavailability was determined as 53%.

[1] Determination of *in vivo* toxicokinetic parameters in piglets; results shown in paper IV, Figs 3a and 3b and Table 2.

Results and discussion

After an unusually wet weather during the 1970s, DON was discovered after outbreaks of swine feed refusal in the central USA (Akins-Lewenthal 2014). Ten years after the discovery of DON the world-wide occurrence of this toxin had become well established, and researchers have published an increasing number of publications on DON year-by-year (Yoshizawa 2013). Studies on DON have been designed to elucidate the physiological function, toxicokinetics and preventive methods. However, some aspects of DON-related toxicity are still without definitive answer. Because of high research activity and novel analytical techniques, knowledge of the effects of DON has increased rapidly in the last decade. Both *in vivo* and *in vitro* studies have been conducted to provide useful information. Although *in vitro* studies allow us to have a better control on the physicochemical and physiological environment, and provide a more constant toxin concentration in the exposure period, the complexity of living organisms requires research on *in vivo* models to mimic clinical effects, toxicokinetics and vertical transmission (Escrivá et al. 2015). However, the IVIVE methods used in paper **IV** do also show that the prediction of *in vivo* kinetic data from *in vitro* biotransformation experiments is possible.

Clinical effects of DON with focus on growth performance

The most common chronic adverse effects of DON in pigs are reduced feed intake and BW gain (Knutsen et al. 2017). Other clinical adverse effects of DON such as vomiting, diarrhea, suppressed immune function, poor reproductive performance and neurological disorders are also frequently recorded, but have not been described as common effects in practical husbandry (Sobrova et al. 2010). The CONTAM panel of EFSA suggested a LOAEL of 2.8 mg DON/kg feed for vomiting (Knutsen et al. 2017). A wide range of NOAEL and LOAEL (0.35 to 2 mg DON/kg) for reduced feed intake and BW gain was reported in previous investigations, but EFSA concluded on an overall NOAEL of 0.7 (0.6−0.09) mg DON/kg feed (Knutsen et al. 2017).

In the feeding studies reported in this thesis, there were no severe DON-related clinical effects, such as vomiting, diarrhea, or other pathological conditions, neither at contamination levels up to 5.7 mg DON/kg feed in the feeding study on growing pigs (Paper **I**) nor at more moderate levels (up to 1.7 mg DON/kg) in gestating and lactating sows (Paper **II**). A single event of marginal emesis occurred in several of the exposed piglets shortly after po and iv

application of pure DON, but this was considered insignificant for the results of the toxicokinetic study (Paper **IV**). Reductions in feed intake and reduced BW gain in the first week of the exposure were the main adverse clinical effects recorded in growing pigs (Paper **I**). In sows (Paper **II**), a significantly lower feed consumption during lactation was recorded in the highest DON level group compared to controls, while changes in sow BW were not significantly affected by the DON treatment. The piglets of the sows in the DON-treated groups did also grow normally (Papers **II** and **III**). The transient effect on growth performance in the first week of exposure recorded in the growing pig study (Paper **I**) could not be assessed in the sow study (Paper **II**), since the feeding was restricted (up to 4 kg/day) during the first days of experiment in late gestation. A high individual variation in responsiveness to DON, as well as variations with age and stage in production, was found in our feeding studies. Another research group that conducted feeding experiments on both non-pregnant young gilts and pregnant sows found that the younger gilts got used to the contaminated diets within 21 days of feeding, while pregnant sows fed the same DON-contaminated diets over the entire experiment showed a 38% reduction in feed intake (Dänicke et al. 2007).

In the present work, the LOAEL was 5.35 mg DON/kg for reduced feed intake and BW gain in growing pigs (Paper **I**). Approximately the same levels were recorded in some previous studies (Friend et al. 1986b; Swamy et al. 2002). The LOAEL was 1.7 mg DON/kg for reduced feed intake in sows during lactation (Paper **II**). To the best of the authors' knowledge, this LOAEL is lower than those reported in any previous sow studies. The variation in DON-related adverse effects between those reported in previous studies and in the present thesis can in part be related to the variations in toxicokinetics of DON in different stages of the production cycle (will be discussed later in the thesis), to different sources of contaminated feed, and to co-occurrence of other mycotoxins.

DON can reduce reproductive performance directly by affecting oocyte maturation and embryo development and indirectly by reducing feed consumption. These effects are reported both *in vivo*, *in vitro* and *ex vivo* in different animal models, including pigs (Yu et al. 2017). In our study in sows (Paper **II),** investigated reproductive variables such as gestation length, duration of farrowing, total number of piglets born, number born alive, number of weaned piglets, number of sows that returned to heat at 5 days post-weaning, number of conceptions at first service and number of liveborn piglets per sow in the subsequent litter were not affected by feeding sows with DON, although some reduction in feed intake was recorded during lactation. Unaffected litter size and number of total weaned piglets reported in paper

II are supported by some previous studies (Chavez 1984; Diaz-Llano & Smith 2006; Diaz-Llano & Smith 2007; Friend et al. 1986a; Gutzwiller 2010; Herkelman et al. 2017). However, reduced litter weight and longer farrowing length were reported in sows fed 5 mg DON/kg (Jakovac-Strajn et al. 2009). In the studies of Diaz-Llano and Smith (2006; Diaz-Llano & Smith 2007), feeding gestating and lactating gilts with diets containing 5.5 mg DON/ kg resulted in longer estrus intervals and increased the incidence of stillbirth.

The other clinical factors that were investigated in sows in this thesis are skin temperature and backfat thickness (Paper **II**). Skin temperature was recorded as an indicator of the clinical and physiological effects, and backfat thickness of the sows during lactation was measured as an additional indicator of feed efficiency. Feeding weaning pigs with diets containing 3 mg DON/kg resulted in lower skin temperature than in the control pigs in some previous studies (Prelusky et al. 1994; Rotter et al. 1994). In the current work, sows receiving the DON level 2 diet (1.7 mg DON/kg) had a significantly lower skin temperature than the control sows within 12–36 h after farrowing (Paper **II**). This finding may be accidental, since significant differences in skin temperature between contaminated groups and controls were not recorded at most other times of assessment. In general, plasma DON concentrations of the sows (Paper **III**) and skin temperature (Paper **II**) were not correlated. However, sows might have been more sensitive to the effects of DON on skin temperature around farrowing (Terøy et al. 2014).

In this study, feeding the sows with DON contaminated diets did not have a statistically significant effect on changes in backfat thickness, although the average losses in backfat thickness were numerically higher in contaminated groups than in the control sows (Paper **II**). In another reported study, a significantly increased backfat loss in sows fed diets containing 3 mg DON/kg during lactation was found (Herkelman et al. 2017).

Effects of DON on blood parameters

DON might affect hematological and biochemical parameters directly by alternation in cell's viability and functions (Payros et al. 2016) or indirectly by reduced feed intake and impaired absorption (Alizadeh et al. 2015; Maresca et al. 2002). Paper **I** demonstrated that total serum protein, globulin, and albumin were lower in growing pigs fed contaminated diets than in the controls; after 3 weeks of exposure and at the end of the feeding experiment. However, these variables were not significantly affected in the sow study (Paper **II**). DON did not significantly affect other serum biochemical parameters in growing pigs (Paper **I**) and

gestating and lactating sows (Paper **II**). Our results from paper **II** concurred with a previous study on gilts fed 5.5 mg DON/kg in late gestation (Diaz-Llano & Smith 2006) and are in contrast with the results from two previous studies that showed DON-related reduction of serum urea in sows during late gestation and lactation (Diaz-Llano & Smith 2007; Diaz-Llano et al. 2010).

Our results in paper **I** are supported by some previous studies, which found significant reductions in total serum protein and/or globulin in pigs fed diets containing 3–4 mg DON/kg (Bergsjø et al. 1993; Rotter et al. 1995). However, a number of other previous studies, which also investigated the effects of DON on biochemical parameters, did not show any significant effect of dietary exposure to DON on blood protein concentrations in pigs (Dänicke et al. 2004b; Goyarts et al. 2006; Wu, L. et al. 2015). The reduction in serum protein levels observed in paper **I** and in a proportion of previous feeding experiments may be a consequence of poor protein synthesis in the liver related to reduced feed intake in DON-exposed pigs (Rotter et al. 1995). In paper **I**, however, the DON-related reduction in serum protein levels was detected in the last weeks of the experiment, when feed intake and weight gain were normalized, indicating that this reduction was an independent, direct effect of DON. As another evaluation of a possible DON-induced effect on serum proteins we have calculated correlations between detected DON in plasma samples and total serum protein concentrations. At the end of experiment (week 5) in paper **I**, a negative correlation between plasma DON concentrations and total serum protein concentrations was determined in pigs fed contaminated diets. The negative correlation was stronger in pigs fed medium-DON ($r_s = -0.45$, $p = 0.13$) and high-DON ($r_s = -0.60$, $p = 0.05$) compared with pigs fed low-DON ($r_s = -0.26$, $p = 0.41$). Despite the fact that contaminated diets had no effect on total serum protein levels of sows in paper **II**, the results from the measurements at the end of experiment showed that plasma DON concentrations and total serum protein concentrations were negatively correlated in sows fed DON level 1 ($r_s = -0.25$, $p = 0.36$) and DON level 2 ($r_s = -0.27$, $p = 0.37$). Simple linear regression based on the calculation over the entire exposure period showed a significant relationship between plasma DON concentrations and total serum protein in growing pigs (**Figure 15**) and sows (**Figure 16**) fed with contaminated diets. These findings may in our opinion be seen as additional evidence for DON-induced serum protein reduction as an independent, direct effect.

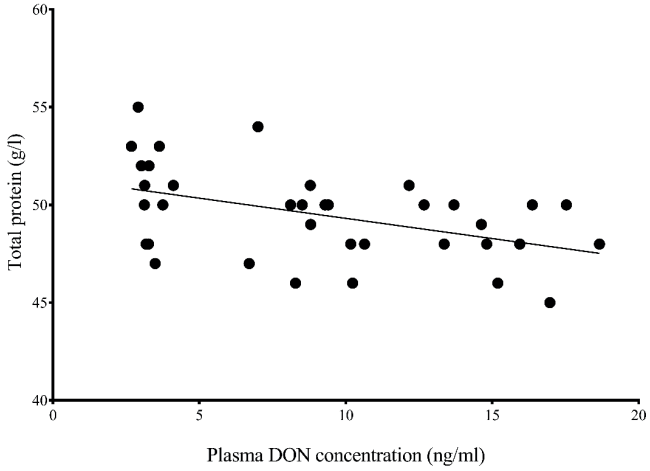


Figure 15. Relationship between individual plasma concentrations of DON and total serum protein concentrations in pigs (*n*=36) fed contaminated diets calculated for the whole experiment. ($y = 51.12-0.18 * x$; $r^2 = 0.18$, $p < 0.05$)

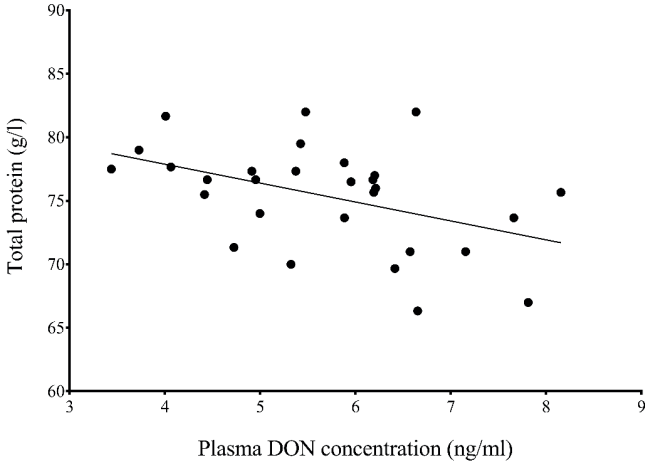


Figure 16. Relationship between individual plasma concentrations of DON and total serum protein concentrations in all the sows (*n*=29) fed contaminated diets calculated for the whole experiment. ($y = 83.84-1.48 * x$; $r^2 = 0.20$, $p < 0.05$).

Exposure of pigs to higher levels of DON (up to 12 mg/kg) has been shown to increase liver enzymes such as ALP, AST and ALT (Wu, L. et al. 2015). The increase in ALP indicates an

abnormal excretion of liver metabolites due to DON-induced systemic toxicity, and the increase in ALT and AST reflects a DON-related injury mechanism in liver (Wu, L. et al. 2015). However, neither ALP nor AST levels were significantly affected by dietary exposure of growing pigs (Paper **I**) and sows (Paper **II**) to DON. This indicates that the levels of DON in the diets used in our studies did not have a pathological effect on liver function that could be observed by clinical-pathological parameters.

In the last week of experiment, a significant reduction in serum calcium in pigs fed with 5.35 mg DON/kg (Paper **I**) were recorded. At the end of experiment (week 5), plasma DON concentrations and serum calcium concentrations were statistically negatively correlated ($r_s = -0.66$, $p < 0.05$) in pigs fed high-DON. This is in line with results from some other experiments (Bergsjø et al. 1993; Prelusky et al. 1994; Rotter et al. 1995). A similar, and statistically significant negative correlation ($r_s = -0.78$, $p < 0.001$) between plasma DON concentrations and serum calcium was found in sows fed DON level 2 at the end of the experiment (Paper **II**). We found no other relationships between plasma DON concentrations and other analyzed biochemical parameters in papers **I** and **II**.

Decreased RBC and HGB, and increased RDW in DON-exposed animals have been reported related in some previous studies (Reddy et al. 2018; Rotter et al. 1994). In this work, hematological parameters were not changed observably by DON-contaminated diets, neither in growing pigs (Paper **I**) nor in sows (Paper **II**).

Uptake and metabolism of DON

The data from paper **I** and **III** gives information on the uptake and metabolism of DON, in growing pigs and in sows. The term “toxicokinetics” of DON is not used in these papers, as the data are not suited for elucidating toxicokinetical details. However, *in vivo* toxicokinetic parameters of DON in weaner pigs has been determined in paper **IV**. Thus, the present discussion will focus on uptake and metabolism of DON from contaminated feed; with the data from paper **IV** as supporting material for the discussion.

The plasma analyses in paper **I** showed that DON from contaminated diets was rapidly absorbed, and was detected at the mean levels of 11.27 ± 1.20 (mean ± SEM) and 15.24 ± 1.34 (mean ± SEM) at 11:00 and 16:00 on the first day of exposure, respectively (**Figure 17**). This is in line with the rapid absorption after oral exposure to pure DON, showing a maximal plasma concentration after 3.5 hours (Paper **IV**). Our results are also supported by a previous

study that showed a rapid absorption following oral exposure of pigs to a diet contaminated naturally with DON (Goyarts & Dänicke 2006).

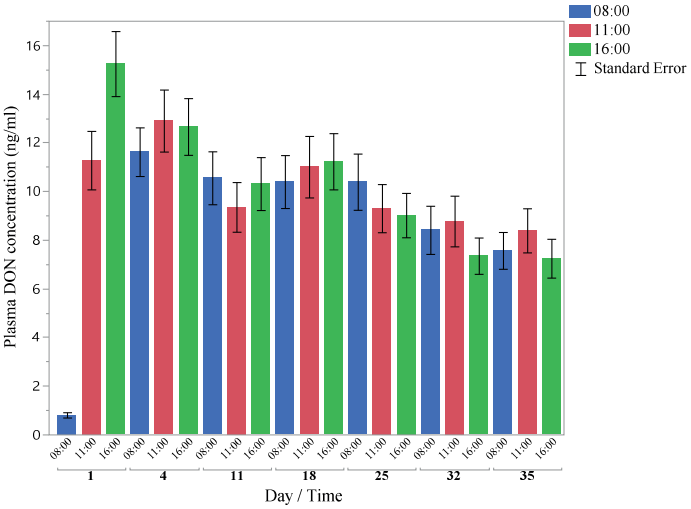


Figure 17. Plasma concentrations of DON in growing pigs over the course of experiment. The columns are the mean of plasma concentrations of all pigs (*n*=36) fed contaminated diets measured at three different time points per day.

Considering the mean feed intake and weights including gain, the approximate average DON doses (µg/kg BW/day) in the toxin-exposed groups were calculated for each week of the study period in growing pigs (Paper **I**) and for late gestation and lactation in sows (Paper **III**). In paper **I**, the estimated doses for each DON exposed group were almost constant throughout the study, due to a practically parallel increase in BW and feed intake (Paper **I**, Table 3). This resulted in nearly stable plasma concentrations in the pigs at each DON level during the experiment (Paper **I**, Figure 2a). The plasma concentrations of DON and its metabolites in paper **I** were more constant and the individual variations were lower than in the sow experiment in paper **III,** probably due to lower individual variations in feed intake in growing pigs. In the sow study, feed intake and BW were strongly affected by the time of recording, as the sows were followed from late gestation to the end of lactation. Thus, the higher feed intake and lower BW during lactation compared to late gestation resulted in considerably

higher exposure rates (DON doses ~2 times higher) in the sows during lactation than in late gestation.

As seen from Figure 2a in paper **I,** the plasma concentrations of DON detected in the growing pigs were highly dose-dependent. The same was true for the major metabolites of DON (Figure 2b, c, and d). In the sow experiment, DON, DON-3-GlcA and DON-15-GlcA were also detected in a dose-dependent pattern in sows fed DON level 1 and DON level 2, both at 10 days after arrival and on day 21 in lactation (Paper **III**, Figure 1). At weaning, however, the dose dependency was not present, possibly because of particularly high variation in feed intake at this point.

The transfer of DON from the diet to sow plasma may also be illustrated by calculation of the plasma/diet ratio (plasma DON concentrations divided by the dietary DON concentrations). Calculated for the entire experimental period, the average plasma/diet ratio was 0.0036 (range: 0.0024 – 0.0048) in growing pigs fed contaminated diets (based on the data of Paper **I**). This value was confirmed by the results from paper **III**, where the average plasma/diet ratio was 0.0037 (range: 0.0024 – 0.0048) in sows fed with contaminated diets. These findings in papers **I** and **III** are in general agreement with the study by Döll et al. (2008), who recorded a serum/diet ratio (carry-over factor) of 0.0035 (range: 0.0000 – 0.0062) in serum of pigs fed pelleted diets with 1.13 mg DON/kg, and the study by Goyarts et al. (2007b) who recorded a serum/diet ratio of 0.0034 (range: 0.0019 – 0.0040) in serum of the pigs fed diets contaminated with 6.68 mg DON/kg. Interestingly, the large differences in DON concentrations in the diets in paper **I** (up to 5.7 mg DON/kg), paper **II** (up to 1.7 mg DON/kg), the study by Döll et al. (2008) (1.13 mg DON/kg) and Goyarts et al.(2007b) (6.68 mg DON/kg), have not lead to notable differences in the registered plasma/diet ratios (carry-over factors). However, differences in feeding practice between fattening pigs and sows need to be considered in interpreting and comparing the diet ratio results. This may result in different DON exposures when related to body weight (Dänicke & Brezina 2013).

In the growing pig study, the analyses of plasma samples taken at different hours showed that the DON concentrations were relatively constant throughout the day, likely due to the *ad libitum* access to feed in this study. This was true both for each dose group (Paper **I**), and when calculated for all contaminated groups together (**Figure 17**). In the sow study, blood samples for DON analysis were not taken at fixed hours. The time between feed intakes may also have varied more strongly in the sows, particularly in the lactation period. This may have

contributed to the larger individual variation in recorded DON plasma concentrations. DON concentrations in plasma can be changed rapidly, depending on the time between last meal and the sample collections (Dänicke & Brezina 2013).

Calculated for the entire exposure period, a linear relationship (**Figure 18**) was found between DON exposure of growing pigs and plasma DON concentrations (based on the data of Paper **I**). Similar relationship between DON doses and plasma DON concentrations was also recorded in the sows (**Figure 19**) (based on the data of Paper **III**). These findings are in line with the review by Dänicke and Brezina (2013) who demonstrated non-linear relationships between DON exposure of fattening pigs and DON concentration in blood.

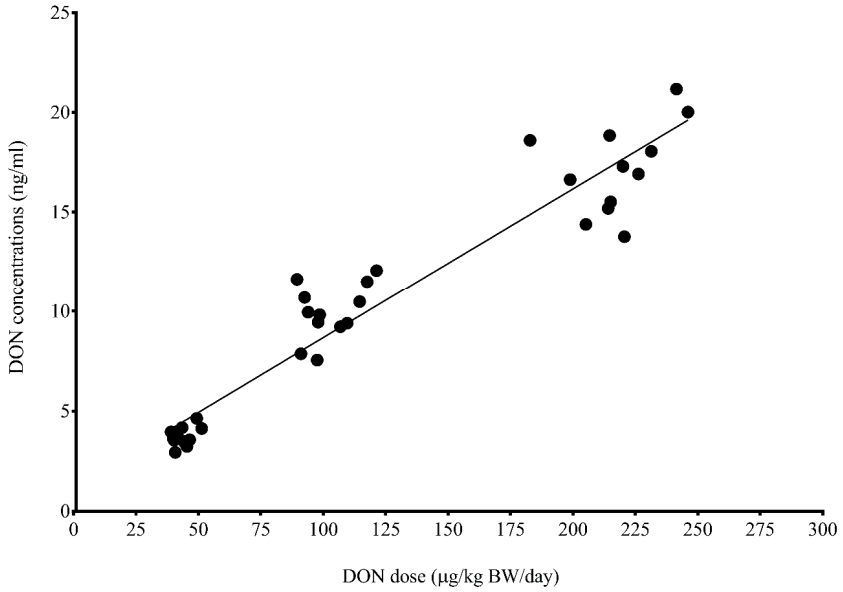


Figure 18. Relationship between individual DON dose and DON concentration in plasma of growing pigs fed contaminated diets; low-DON (n=12) (left side), medium-DON (n=12) (at the middle) or high-DON (n=12) (right side). ($y = 1.27 + 0.07 * x$; $r^2 = 0.92$)

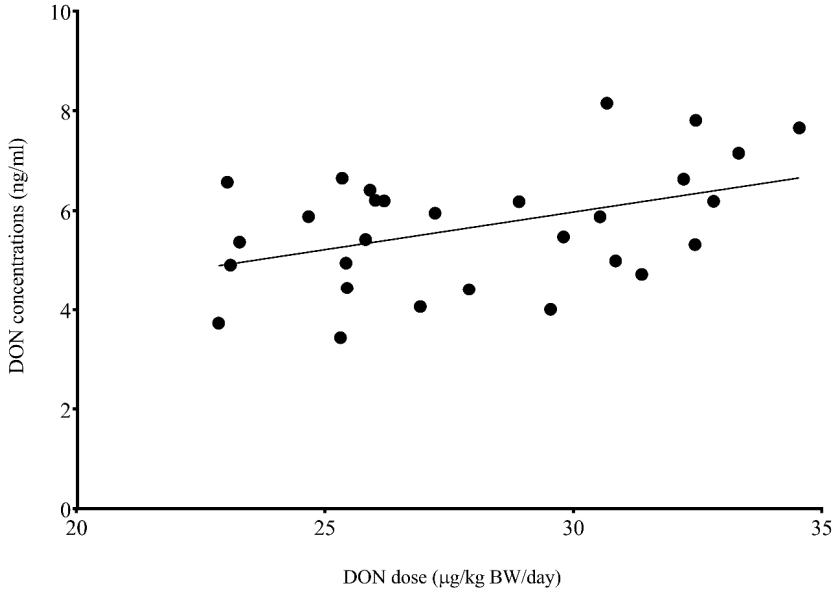


Figure 19. Relationship between individual DON dose and DON concentration in plasma of sows (*n*=29) fed contaminated diets. ($y = 1.46 + 0.15 * x$; $r^2 = 0.19$)

The term "metabolism" of DON refers to the converting of the native toxin to various degradation derivatives. This occurs both in the gut, as mediated by microbes, and in other organs such as intestinal mucosa, liver, and kidneys that are the major players in phase II metabolism (Dänicke & Brezina 2013). This thesis has included determination of plasma concentrations of the microbially metabolized product DOM-1 and the conjugated forms DON-3-GlcA and DON-15-GlcA that are products of the phase II metabolism; both in growing pigs (Paper **I**) and in sows (Paper **III**)

In paper **I**, DOM-1 was detected at very low levels in plasma of only five pigs fed high-DON at 16:00 on the first day of exposure (**Figure 20**). On day 4, however, DOM-1 was detected in almost all the pigs fed contaminated diets (**Figure 20**, and Paper **I**, Figure 2b). The detection of DOM-1 already on the first day of exposure in some individuals in paper **I** is in contrast with the study by Goyarts et al. (2006), who was able to detect DOM-1 in urine or faecal samples of the pigs receiving DON-contaminated diet only for a period longer than 4 weeks. It is important to mention that the type of physiological samples, plasma in paper **I** and urine

or faeces in the cited study, could be responsible for the differences in time of detection. DOM-1 was not detected in the plasma samples of the sows fed with DON-contaminated diets (Paper **III**). Previous studies showed that DOM-1 was not detected in serum and liver samples of the sows fed with contaminated diets (4.42 and 9.57 mg DON/kg), however, this metabolite was found in other analyzed specimens (Dänicke et al. 2007; Goyarts et al. 2007a). The DOM-1 detected in the plasma of the pigs (Paper **I**) could be related to both microbial de-epoxidation of DON in the intestine and to a possible formation in the liver (Dänicke & Brezina 2013). The detected amounts could also originate either from biliary excretion of DON and subsequent de-epoxidation and absorption of the de-epoxydated product, or could be associated with entero-hepatic cycling of DOM-1 (Dänicke & Brezina 2013). In the growing pig study, the proportion of DOM-1 in plasma was approximately 10% of plasma DON concentrations and ~1% of DON doses. However, DOM-1 concentrations were clearly dependent on DON dose (Paper **I**, Figure 2b). Calculated for the entire exposure period, there was a linear relationship between DON doses and plasma DOM-1 concentrations in all pigs eating contaminated diets (**Figure 21**). This is in line with the results from a previous study, where linear relationships were recorded between DON intake and DOM-1 in different physiological samples (Dänicke et al. 2005).

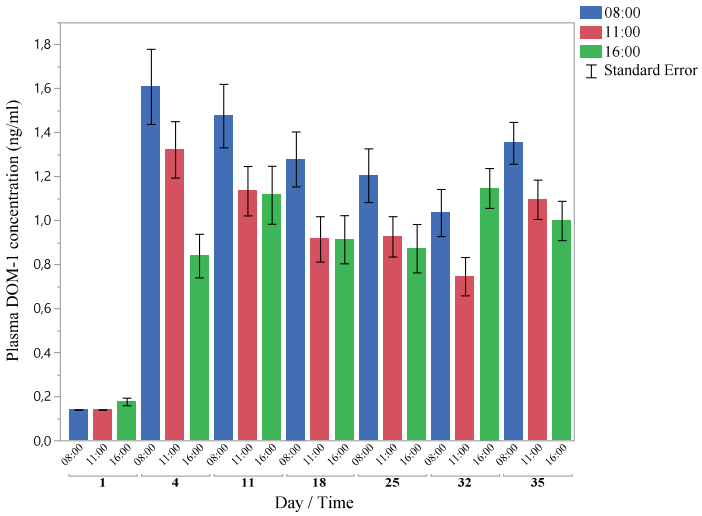


Figure 20. Plasma concentrations of DOM-1 in growing pigs (*n*=36) over the course of experiment. The columns are the mean of plasma concentrations of all pigs fed contaminated diets measured at three different time points per day.

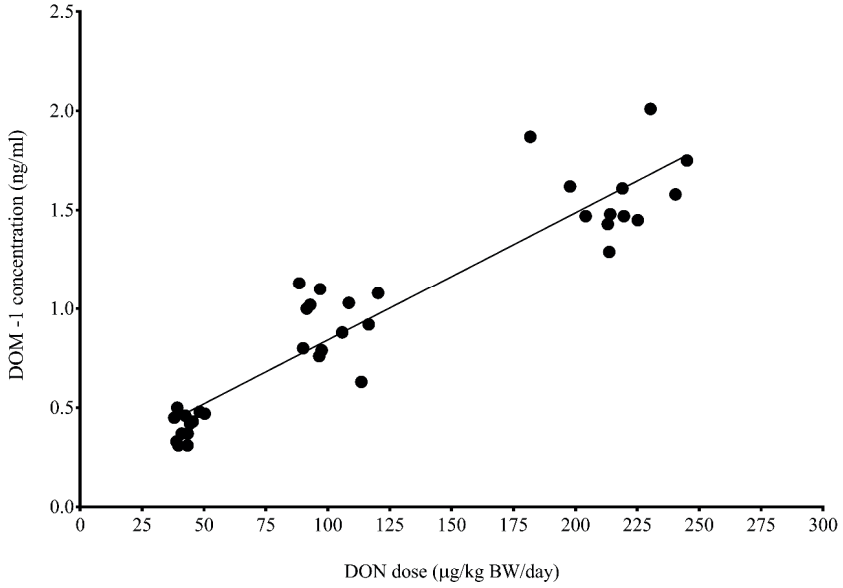


Figure 21. Relationship between individual DON dose and DOM-1 concentration in plasma, of growing pigs fed contaminated diets low-DON (*n*=12) (left side), medium-DON (*n*=12) (at the middle) or high-DON (*n*=12) (right side). ($y = 1.27 + 0.07 * x$; $r^2 = 0.92$)

Considering this fact that DON is almost completely absorbed in the proximal small intestine, while de-epoxidation activity is mainly located at the distal part of the gastrointestinal tract, it could be concluded that de-epoxidation ability is not efficient to protect the pig again DON-related toxicity (Sundstol Eriksen & Pettersson 2003). The effect of DSM 11798 used in paper **I** on de-epoxidation of DON will be discussed separately.

DON-3-GlcA and DON-15-GlcA, the products of glucuronide conjugation, were recorded as the dominant metabolites of DON in pigs, both in papers **I** and **III** (Schwartz-Zimmermann et al. 2017).

The detection of DON-glucuronides already at 11:00 on the first day of exposure in the plasma samples of the growing pigs receiving contaminated diets (**Figures 22** and **23**) indicates the fast metabolism of DON in pigs (Paper **I**). This is supported by the toxicokinetic study in paper **IV,** where the DON metabolites DON-3-GlcA and DON-15-GlcA, were detected shortly after oral and intravenous administration of DON. A similar, fast metabolism of DON

is also described in humans (Warth et al. 2013). Paper **III** showed that DON-glucuronides were also detected at all assessment points during the exposure period in the plasma of the sows fed contaminated diets.

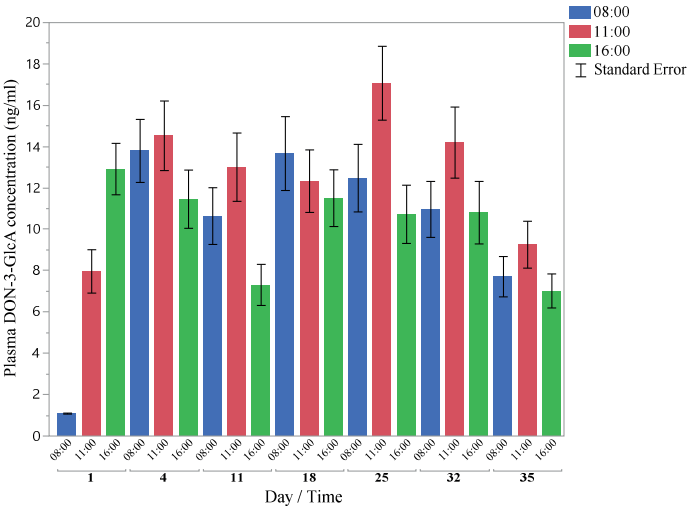


Figure 22. Plasma concentrations of DON-3-GlcA in growing pigs over the course of experiment. The columns are the mean of plasma concentrations of all pigs (*n*=36) fed contaminated diets measured at three different time points per day.

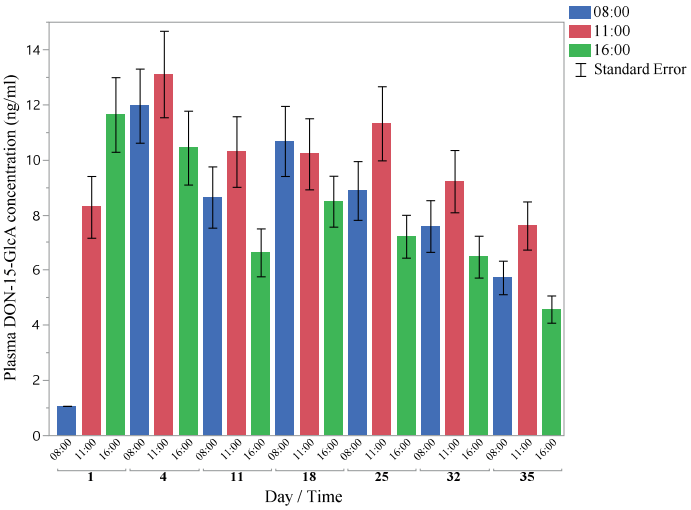


Figure 23. Plasma concentrations of DON-15-GlcA in growing pigs over the course of experiment. The columns are the mean of plasma concentrations of all pigs (*n*=36) fed contaminated diets measured at three different time points per day.

In paper **III**, the DON glucuronidation rate in pigs is defined as the sum of plasma DON-3-GlcA + DON-15-GlcA concentrations divided by total DON (sum of plasma DON + DON-3-GlcA + DON-15-GlcA concentrations). Differences in glucuronidation rate value will be discussed in a later chapter.

Placental and lactational transfer of DON

Paper **III** documents the placental and lactational transfer of DON from sows to their piglets. In this study, placental transfer of DON was investigated *in vivo* by measuring DON in the plasma samples of the piglets collected immediately postpartum, before they received any colostrum. High concentrations of DON in piglet plasma were recorded at birth (Paper **III,** Table 2a). The ratio between the concentrations of DON in piglet and sow plasma samples was calculated, and designated "piglet/sow plasma ratio". The ratios at birth were surprisingly high in both DON-fed groups. Calculated for both DON-fed groups, the mean ratio was 2.14 (median: 1.80) (Paper **III**, Table 2b). In a previous study, the median piglet/sow serum transfer ratio of full-term piglets of the sows exposed to DON during the last third of gestation was 0.75, which means that DON ingested by the sow was present in the circulation of the piglets to nearly the same extent as in the sows (Dänicke et al. 2007). The high calculated

transfer ratio at birth in our study might be interpreted as an up-concentration of DON in the piglets. Another, and in our opinion more likely explanation is related to the feed intake of the sows during farrowing. The piglets selected for sampling and inclusion in the transfer study were generally the first five that were born in each litter, and they were all sampled immediately after birth. However, the blood samples from the sows in farrowing were often taken after we had finished the work with these first piglets. During active farrowing, the sows did generally eat very little. As paper **IV** demonstrated, the plasma half-life of DON in swine is short (p.o. $t½$ = 3.8 h in weaner pigs). The reduced DON plasma levels recorded in the samples of sows taken at farrowing (Paper **III**, Table 2a) may therefore be a result of the reduced feed intake during active farrowing. In contrast, the plasma DON levels in the selected piglets may to a higher degree reflect the levels in the sow plasma and the placenta early in the farrowing process. It is also possible that the high concentrations of DON recorded in the new-born piglets may relate to a slower clearance of the toxin in the piglets during birth, but the toxicokinetics of piglets in this situation is unknown. The sows in the study were fed the experimental diets from arrival. Therefore, they were probably to some degree close to a steady state situation regarding DON exposure in the last part of gestation, as long as they had normal appetite. Based on this assumption, the plasma concentrations of DON found in sows 10 days after arrival, with the reduction in feed allowance approximately 2 days before expected farrowing taken into account, were used alternatively as an estimate of sow plasma DON at the start of farrowing. If this estimate is used, the calculation gives a mean piglet/sow plasma ratio at birth for all DON-exposed sows and piglets in the transfer study close to unity, a value that is closer to that reported in the study of Dänicke et al. (2007). In any case, our findings do in our opinion confirm the efficient placental transfer of DON recorded by previous studies.

In addition to the piglet/sow plasma ratio, we have determined the sow-piglet correlation between DON concentrations in plasma of each sow with the mean concentrations in their sampled piglets, at different times of sampling. We found that the plasma concentrations of DON in sows and their piglets were strongly correlated at birth ($r = 0.88$, $p > 0.001$) (Paper **III**). This may be seen as supporting evidence for an efficient placental transfer of DON.

As mentioned, the efficient placental transfer of DON demonstrated in the current thesis is supported by two previous *in vivo* feeding studies on exposure of pregnant sows in mid-gestation (Goyarts et al. 2007a) and during late gestation (Dänicke et al. 2007). *In vitro* and

ex vivo studies, using human cell lines and placenta have also indicated an efficient placental transfer of DON in humans (Nielsen et al. 2011).

In pigs, the median piglet/sow serum ratios were found to be 0.23 at mid-gestation (Goyarts et al. 2007a), and 0.75 at the last third of gestation (Dänicke et al. 2007). As discussed in the previous paragraphs, the transfer ratio observed in the present study (Paper **III**) was even higher. This may suggest that the placental transfer of DON is dependent on the time of exposure in the gestation period. Physical structures of the placenta are changed throughout the gestation period. As gestation advances, the placental folds become more extensive (Vallet et al. 2014). Thus, the foetal-maternal interactive surface area during late gestation is wider than the surface during mid-gestation.

In comparing our results with the two previous studies mentioned, it is also important to note that there are some other important differences between the studies, which may have contributed to the variation in results. The levels of DON contamination in the experimental feed in the previous studies were considerably higher (4.42 and 9.57 mg DON/kg) than in the present study (up to 1.7 mg DON/kg). Furthermore, DON was the dominant mycotoxin in the experimental diets in the present study, while in both of the cited studies, diets did also contain zearalenone (ZEA). Other differences compared to the present experiment were parity number (age) of the sows, delivery method and litter size. In the two cited studies of (Goyarts et al. 2007a) and (Dänicke et al. 2007), only primiparous sows were examined, average litter size was approximately 13 and the piglets were delivered by Caesarean section; whereas in our experiment, both primiparous and multiparous sows were included, the average litter size was approximately 16 and farrowing was allowed to occur naturally (Paper **III)**. The difference in delivery method is obviously of importance in the comparison of the results. However, litter size may also have some influence on the results. It has been suggested that increased litter size can lead to increased uterine blood flow, which may result in increased transport capacity of the placenta (Vallet et al. 2014).

In paper **III**, the plasma DON concentrations in the piglets measured through different stages of lactation do also demonstrate the transfer of DON from the diet through the sow milk to the suckling piglets. The piglet/sow plasma ratios within 12–36 h after parturition, at day 7, day 21 and at weaning were used to illustrate the lactational transfer of DON throughout lactation. In addition, the milk/plasma ratio (M/P ratio) in sows was calculated to illustrate the transfer of DON from sow plasma to sow milk. This ratio was defined as M/P = DON

concentration in sow milk divided by DON concentration in maternal plasma. The transfer of DON from sow plasma to sow milk was also evaluated by calculation of the correlation between milk and plasma DON concentrations in each sow.

DON was detected in the plasma samples of the piglets during the whole lactation period (Paper **III,** Table 2a). The highest DON level during the lactation period was detected within 12–36 h after partition. Thereafter, the levels of DON in piglet plasma fell strongly during mid-lactation. Towards weaning, DON in piglet plasma again rose somewhat (Paper **III**, Table 2a and Figure S2).

The mean piglet/sow plasma ratios were also highest within 12–36 h after parturition (2.30). A significant reduction in the transfer ratios was detected with the mean values of 0.08, 0.16 and 0.20 on day 7, day 21 and at weaning, respectively. The plasma DON concentrations in sows and the piglets on day 7 were used as a representative indicator for lactational transfer. The concentrations of DON in sows and piglet plasma were not correlated on day 7 in lactation.

DON was detected in all the milk samples collected in different stages of lactation in the transfer study (Paper **III,** Table 4*).* Milk/plasma (M/P) ratios for all sows fed DON-contaminated feed were approximately at the same level at birth (0.92), within 12–36 h after parturition (1.11), on day 7 (0.94) and 21 (1.21) in lactation and at weaning (0.90) (Paper **III,** Figure 3). The transfer of DON from sow plasma to sow milk can also be evaluated by a calculation of the correlations between DON levels in milk samples and plasma samples taken at the same day, in each sow. A strong correlation between plasma concentrations and milk concentrations of DON was found at all points of assessment, in the sows fed DON-contaminated diets (Paper **III**). The strong differences in transfer rates from sows to piglets between day 1 after parturition and the rest of lactation can therefore not be explained by changes in M/P ratios in the sows.

One possible explanation for the high piglet plasma levels and consequently high piglet/sow plasma ratios at 12–36 h after parturition is that the uptake of DON via colostrum might be more efficient in new-born piglets, compared to piglets later in lactation. It is well known that macromolecules and other large molecules pass more easily across the intestinal barrier to the blood of the new-born piglets in the first hours of life. Thereafter, this transmission decreases rapidly in suckling piglets, due to closure of the intestinal epithelium, which is finalized after 18–36 h postpartum (Svendsen et al. 2005). Another possibility is that some of the DON

present in piglet plasma 12–36 h after birth was a remnant of the DON transferred via placenta and recorded in piglet plasma at birth. This would indicate a slower metabolism and excretion of DON in new-born piglets than in older animals.

DON-glucuronides and DOM-1 were not detected in the plasma of the piglets in any of the assessment points (Paper **III**). DON-15-GlcA was detected in more than half of the milk samples collected in different stages in lactation, while DOM-1 was detected in only 11% of the samples. DON-3-GlcA was not measured in milk samples because of practical limitations in the analytical recovery of this metabolite. The absence of DON glucuronides in piglet plasma in the samples taken at birth and the first day post-partum is interesting. As mentioned, DON concentrations in piglet plasma at these points of assessment were substantial. As shown in other parts of this thesis, DON glucuronides at concentrations not far below the concentrations of DON were found both in the toxicokinetic study in weaner pigs (Paper **IV**, Table 2), in growing pigs (Paper **I**) and in sows (Paper **III**). The lack of glucuronides in the piglet plasma in the new-born piglets may therefore indicate that the DON-glucuronides are not transferred as easily across placenta as DON itself, and that the DON glucuronidation capacity is also smaller in full-term piglet fetuses and new-born piglets than in older piglets.

In the piglet plasma samples taken later in lactation, DON concentrations were also low. Therefore, analytical limitations can not be excluded as explanation for the lack of observation of DON metabolites. Chemical analysis of these metabolites in small samples of plasma and milk is not straightforward. As mentioned in a previous section, DOM-1 was not detected in sow plasma samples while it is detected in some milk samples. It is also important to remember that the toxin metabolites investigated in this study were measured in two different physiological matrices, with two different preparation methods.

Sex- and age-related toxicity of DON

There is no consensus on how sex and age affect DON-induced toxicity. However, some previous studies showed that DON-induced toxicity might be both sex- or age-dependent. Castrated male piglets had a lower BW gain than female piglets in a feeding study where all piglets were fed the same DON-contaminated diets (Cote et al. 1985). Moreover, the DON-induced reduction in feed intake was 17% higher in male pigs compared with females, in another reported study (Andretta et al. 2012). The results of the present work did not confirm any sex-dependency in the time-limited impaired growth performance in growing pigs, nor in their plasma DON levels (Paper **I**). The glucuronidation rates in castrated male pigs fed

contaminated diets were also not significantly different from those in female pigs in paper **I**. This finding is in accordance with a previous study that showed that the increased sensitivity of male mice to DON did not relate to differences in toxin metabolism, but to increased urinary excretion ability of the toxin (Pestka et al. 2017). The urinary excretion of DON is not assessed in the present work.

The data from papers **I** and **III** may also be used to evaluate whether the DON metabolism is affected by age in swine. The amount of glucuronides in plasma were registered in both feeding studies (Papers **I** and **III**), and the glucuronidation rate was defined and calculated for sows in paper **III,** as an indicator for DON metabolism. By calculation of average glucuronidation rates in the whole of these two experiments it was found that sows had 8% (p <0.01) higher glucuronidation rates than growing pigs, indicating a certain degree of age-dependency of DON metabolism in swine. A somewhat lower capacity of younger pigs to metabolize DON and forming glucuronides metabolites than in mature sows is the most probable explanation for this finding. In theory, the higher levels of DON contamination (up to 5.7 mg/kg) in the growing pig study (Paper **I**) might result in impaired enteric and/or hepatic glucuronidation mechanism by damaging enterocytes and/or hepatocytes. However, changes in serum hepatic enzyme levels that might indicate hepatic damage were not found in paper **I**. A previous short-term study in mice showed that weanling mice exposed to DON had higher plasma DON concentration and greater induction of proinflammatory cytokines than adult mice, findings that indicate that younger mice are more susceptible than adult mice to DON-related effects (Pestka & Amuzie 2008). The authors of that cited study suggested that these age-related differences might be more pronounced for DON uptake than for clearance.

Biotransformation of DON using DSM 11798

Good feed management practices on the farm and preventive strategies in feed processing have been developed to control mycotoxicosis in farm animals, and are the most important in practical pig production. However, despite good agricultural practices, contamination by mycotoxins can not be avoided altogether. Therefore, several additional approaches have been used to prevent mycotoxin exposure and toxicity. Among them, the inclusion of feed additives based on microbial transformation is of particular interest for the control of trichotecenes. A new genus (*Gen. nov.)* in the family of *Coriobacteriaceae* (DSM 11798), isolated from bovine rumen was the first to be developed and has been assessed by EFSA as a feed additive microorganism for biotransforming DON to DOM-1 in pigs (EFSA 2013). The biotransformation activity of DSM 11798 has been shown *in vitro* under anaerobic

condition and *ex vivo* by using simulated swine gut (Fuchs et al. 2000; Fuchs et al. 2002; Schatzmayr et al. 2006). The ameliorative clinical effects provided by this microorganism on performance and effective decrease of DON concentrations in serum has been reported in previous *in vivo* studies (Cheng et al. 2006; EFSA 2013; Plank et al. 2009; Starkl et al. 2015).

In paper **I**, the efficacy of DSM 11798 addition was investigated by analyzing feed uptake, growth performance, clinical parameters and plasma levels of DON and its metabolites in growing pigs. As mentioned, a moderate and time-limited reduction in feed intake and BW was recorded in the growing pigs fed contaminated diets (Paper **I**). However, addition of DSM 11798 to experimental diets did not show any significant effect on growth performance results (Paper **I**, Table 2, and **Figure 24**). Our finding is not in accordance with some of the previously reported studies, which showed that the addition of this microorganism had a positive effect on the performance of the pigs fed DON-contaminated diets (EFSA 2005; Plank et al. 2009). In three of six performance studies reported by EFSA (2005), the mean final BW of the pigs were significantly higher in the groups fed experimental diets with addition of DSM 11798/BBSH 797, compared to those fed similar diets without the feed additive.

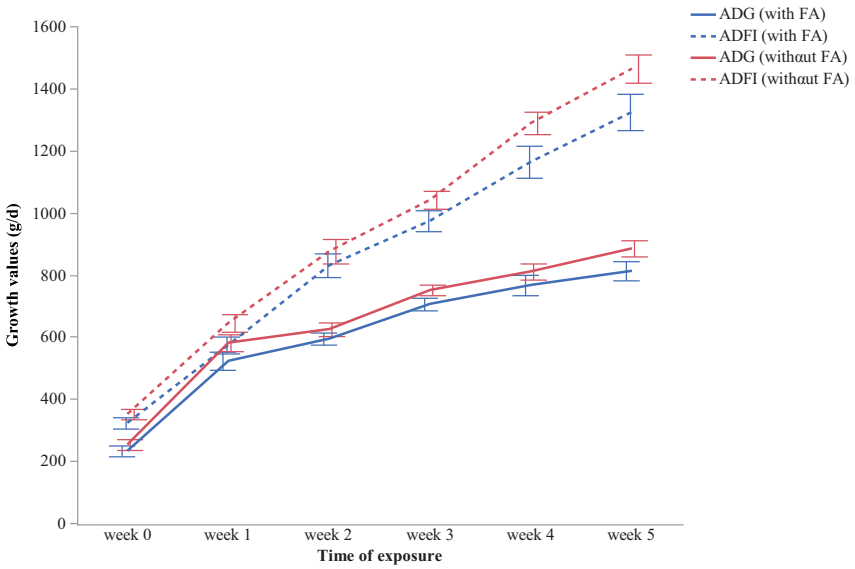


Figure 24. Effect of addition of DSM 11798 (FA) on growth performance parameters. ADG (g/d) and ADFI (g/d) of pigs over the course of experiment according to addition of DSM 11798 (n = 24 in each group, pooled data for the main effect" with or without FA"). Each error bar shows the standard error of the mean (SEM).

Likewise, the changes in serum chemical parameters, especially reduced total serum protein, in the pigs fed contaminated diets were not improved by addition of DSM 11798 in our growing pig study (Paper **I**, and **Figure 25**). This finding is in contrast to a previous study by Grenier et al. (2013) who reported that reduced serum albumin concentrations at the end of trial in the pigs fed contaminated diets with 3 mg DON/kg was prevented by addition of feed additive consisting of BBSH797 (Grenier et al. 2013).

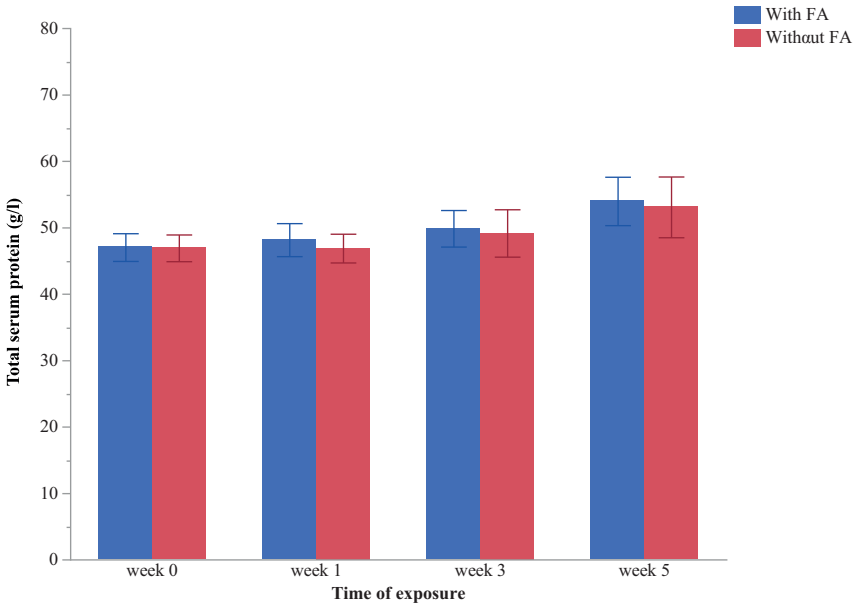


Figure 25. Effect of addition of DSM 11798 (FA) on total serum protein concentration. Total serum protein (g/l) of pigs over the course of experiment according to addition of DSM 11798 (n = 18 (only pigs fed contaminated diets are included) in each group, pooled data for the main effect" with or without FA"). Each error bar shows the standard deviation (SD).

Based on the recommendations from EFSA (2013), experimental feeding studies that combine both clinical parameters such as growth performance variables with the measurement of uptake and/or excretion of DON and its metabolites are of value for testing the efficacy of detoxifying agents in detoxifying DON-contaminated feedstuffs. Thus, the study in paper **I** did also include the data from plasma analyses of DON and its metabolites, especially that of DOM-1, to investigate the efficacy of DSM 11798. The conversion of DON to DOM-1 by this microorganism was expected to result in a fall in plasma DON concentrations and increased DOM-1 levels in plasma (Dänicke et al. 2004b; Fuchs et al. 2002). Two short-term studies reported by EFSA (2013) demonstrated biotransformation of DON to DOM-1 in pigs exposed to DON by oral administration (with and without additive) and by naturally contaminated diets (with and without additive). In these studies, DOM-1 was detected in the groups receiving DON plus feed additive, indicating the efficacy of DSM 11798 to detoxify DON. This is not supported by our results, which showed that plasma DON and DOM-1

concentrations were not significantly affected by addition of DSM 11798 (Paper **I**, Figures 2a and 2b; and **Figure 26**).

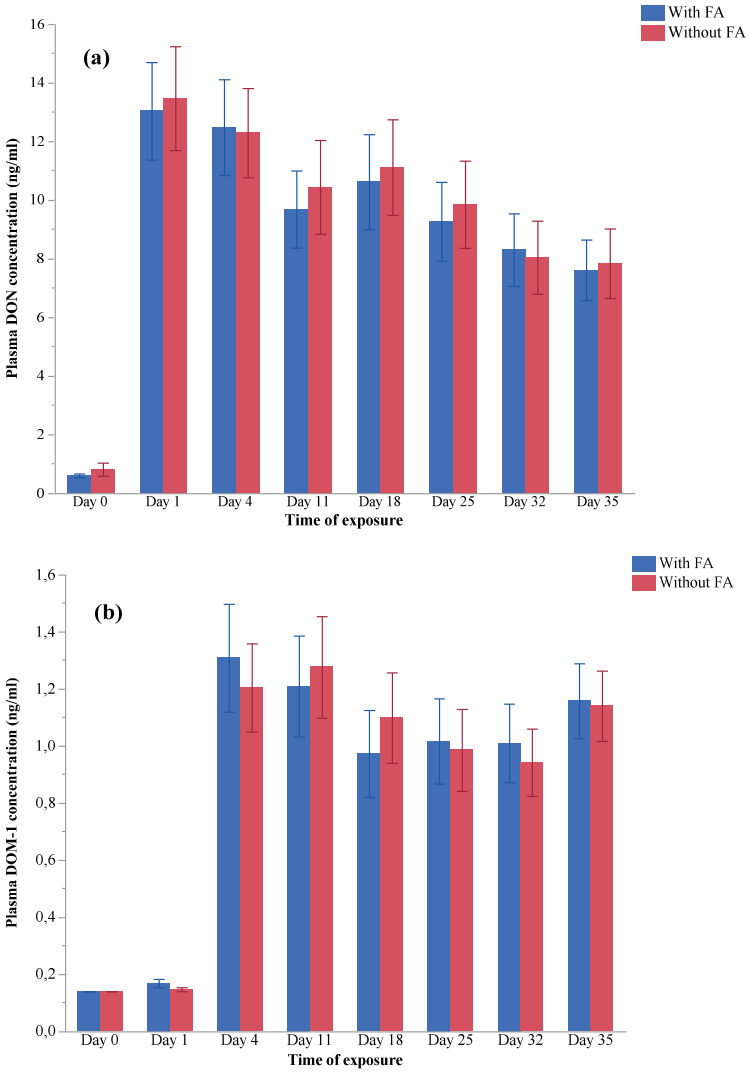


Figure 26. Effect of addition of DSM 11798 (FA) on plasma concentrations of DON (a) and DOM-1 (b). The values (ng/ml) of pigs over the course of experiment according to addition of DSM 11798 (n = 18 (only pigs fed contaminated diets are included) in each group, pooled data for the main effect" with or without FA"). Each error bar shows the standard error of the mean (SEM).

One possible – and in our opinion likely – explanation for our findings concerning the effect of DSM 11798 is related to the kind of feed. In our study, the experimental diets were pelleted, prepared by a standard pelleting process. We were aware that the heat involved in this process could impair the viability of bacteria. Therefore, the pelleting temperature was strictly controlled, and did not exceed 80°. DSM 11798 (in the form of BBSH 797) had been proven to survive common pelleting process with pelleting temperatures up to this limit (EFSA 2005; EFSA 2013). In addition, the survival of the DSM 11798 strain in our experimental diets were monitored after the pelleting process (Paper **I**, Table 1), and was assessed to be sufficient to ensure effective detoxification. However, the impact of the pelleting process and the embedding of the DSM 11798 microorganisms into a feed pellet in themselves may affect the microorganism's efficacy in pigs. Because the intestinal absorption of DON in pigs is fast, a delayed onset of the activity of the DSM 11798 in the pig gut may be sufficient to impair its efficacy against DON absorption and activity in pigs (Paper **I**)

If this explanation is correct, it would indicate that DSM 11798-based feed additives against DON-induced toxicity are less suited for use in pelleted feed. As far as we are aware, in the previous *in vivo* studies where DSM 11798-based products (BBSH 797) have shown effect against DON contamination, the products have been mixed directly into meal feed.

However, in our study, addition of DSM 11798 to DON-contaminated feed did significantly increase plasma concentration of DON-glucuronides (Paper **I**, Figures 2c and 2d). When calculating glucuronidation rates in the whole growing pig experiment, pigs fed contaminated diets with DSM 11798 had a significantly higher glucuronidation rate (68%, $p < 0.01$) compared to pigs fed contaminated diets without DSM 11798 (63%). One possible explanation for this finding is that the natural enteric or bacterial ß-glucuronidase activity might, hypothetically, be influenced by the presence of DSM 11798 in the gut, resulting in increased DON–glucuronide levels in the systemic circulation (Yang et al. 2017).

Conclusions

- Feeding growing pigs with naturally DON-contaminated diets resulted in a temporary effect on growth performance, which was significant in the first 7 days of exposure. The effects on growth performance parameters were reduced and levelled out towards the end of the experiment.
- During lactation, feed consumption of modern, high-yielding sows fed diets contaminated with moderate DON levels up to 1.7 mg DON/ kg feed was reduced, while sow BW changes were not significantly different from control sows.
- Production and reproduction performance of the sows fed DON-contaminated diets were not significantly affected.
- The loss of backfat during lactation in sows fed DON-contaminated diets was not significantly different from the control sows, although the average reductions in backfat were numerically higher in the DON-exposed groups.
- Skin temperature as an indicator for physiological effects was significantly lower in sows exposed to DON 12–36 h after farrowing, but this variable was not significantly affected at other assessment points. This may indicate that the lower skin temperature recorded 12–36 h after farrowing was accidental, but a higher sensitivity to effects of DON at this stage cannot be excluded.
- Feeding growing pigs with DON-contaminated diets led to reduced total serum protein, globulin and albumin, towards the end of the experiment. At the end of experiment, plasma DON concentrations and total serum protein concentrations were negatively correlated in DON-exposed growing pigs as well as in sows. Calculated for the whole experiment, there was a significant negative linear relationship between plasma DON concentrations and total serum protein of the growing pigs and sows fed contaminated diets.
- DON did not influence hematological parameters, neither in growing pigs nor in sows.
- DON and DON metabolites were detected in a dose-dependent pattern in the growing pig study in all assessment points, and in two of the three assessment points in the sow study; at 10 days after arrival and on day 21 in lactation.
- DON is transferred efficiently across the placenta from sows to their piglets in the last stage of gestation. The transfer of DON from the lactating sows to suckling piglets through milk during the lactation was less efficient. The results of this work therefore

suggest that piglets of sows fed DON-contaminated diets are at greater risk of DON exposure in late gestation and the first days after birth than during mid to late lactation.

- A somewhat higher rate of DON glucuronidation was found in sows compared with growing pigs, indicating a possible age-related effect on DON metabolism in swine.
- Sex-dependent differences in uptake, metabolism or effects of DON in pigs were not recorded in the present work.
- The addition of a feed additive containing the bacterial strain DSM 11798 (BBSH 797) in pelleted feed of growing pigs fed naturally DON-contaminated diets was found to be ineffective both in the biotransformation of DON to DOM-1, and in preventing DON-related toxicity.

Future perspectives

Based on the findings in this thesis, we may propose some possible directions for future research:

- The observed transient effect of DON on feed intake as a sickness behavior during the first week of exposure in the growing pig study may be associated with CNS effects of DON. Inspired by this, we have participated in *in vivo* research to explore neurobiological and behavioural impacts of chronic low-dose DON exposure in mice, as a model for pigs, together with another research group. The results of this study are still under processing.
- The sow study was conducted in a SPF piglet production unit. The sows' health was particularly good, since they were free from many common pathogens. Whether the SPF status may enhance sow resistance to DON-related toxicity is a natural issue for future research.
- Different patterns of DON-exposure was recorded at the first days of the piglet's life compared with other times throughout lactation. This did not seem to rely on changes in the transfer of DON from sow plasma to sow milk, but were probably more related to changes in DON uptake and metabolism in the piglets. Future studies should aim at closer investigation of metabolism and bioavailability of DON in new-born and suckling piglets.
- The inclusion of DSM 11798 in pelleted feed was ineffective in the degradation of DON in pigs *in vivo*. To further clarify the reasons for this finding, an investigation of the *in vivo* efficacy of this bacterial strain in an experimental design with both pelleted and meal diets would be appropriate.

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Effects and biotransformation of the mycotoxin deoxynivalenol in growing pigs fed naturally-contaminated grain pelleted with and without the addition of *Coriobacteriaceum* DSM 11798

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Abstract

Deoxynivalenol (DON) is one of the most prevalent *Fusarium* mycotoxins in grain and can cause economic losses in pig farming due to reduced feed consumption and lower weight gains. Biodetoxification of mycotoxins using bacterial strains has been a focus of research for many years. However, only a few *in vivo* studies have been conducted on the effectiveness of microbial detoxification of fusariotoxins. This study was therefore aimed at investigating the effect of a feed additive containing the bacterial strain *Coriobacteriaceum* DSM 11798 (the active ingredient in *Biomin*® BBSH 797) on growth performance and blood parameters, as well as uptake and metabolism of DON, in growing pigs. Forty-eight crossbred (Landrace-Yorkshire/Duroc-Duroc) weaning pigs were fed pelleted feed made from naturally-contaminated oats, with DON at four concentration levels: (1) control diet (DON < 0.2 mg kg^{-1}), (2) low-contaminated diet (DON = 0.92 mg kg^{-1}), (3) medium-contaminated diet (DON = 2.2 mg kg^{-1}), and (4) high-contaminated diet (DON = 5.0 mg kg^{-1}) and equivalent diets containing DSM 11798 as feed additive. During the first 7 days of

exposure, pigs in the highest-dose group showed a 20-28 % reduction in feed intake and a 24-34 % reduction in weight gain compared with pigs in the control and low-dose groups. These differences were levelled out by study completion. Towards the end of the experiment, dose-dependent reductions in serum albumin, globulin and total serum protein were noted in the groups fed DON-contaminated feed compared with the controls. The addition of DSM 11798 had no effect on the DON-related clinical effects or on the plasma concentrations of DON. The ineffectiveness of the feed additive in the present study could be a consequence of its use in pelleted feed, which might have hindered its rapid release, accessibility or de-toxification efficiency in the pig gastrointestinal tract.

Keywords: BBSH 797, deoxynivalenol (DON), microbial detoxification, blood parameters, biotransformation, feed additive, pigs

Introduction

Deoxynivalenol (DON), also known as vomitoxin, is produced by fungal pathogens belonging to the genus *Fusarium* and, in particular, *F. graminearum* and *F. culmorum*. DON can occur in oats, wheat, barley and corn and is the most commonly detected trichothecene mycotoxin in cereal grains in Northern countries (Vitenskapskomiteen for mattrygghet (VKM) 2013). Pigs are more sensitive to DON exposure than other farm animals, possibly because of differences in uptake (Bracarense et al. 2012). Acute exposure of pigs to high doses of DON can cause vomiting (Forsyth et al. 1977). Consumption of feed that is naturally contaminated with moderate levels of DON has been shown to induce dose-related feed refusal, unrest, decreased feed intake and decreased weight gain. Pigs fed with diets containing 1.7 and 3.5 mg DON kg^{-1} have shown partial feed refusal (Bergsjo et al. 1993), whereas 40 mg DON kg^{-1} has caused complete feed refusal (Forsyth et al. 1977). The reported Lowest Observed Adverse Effect Levels (LOAEL), which is based on reduced feed intake, has varied from 0.35 to 2 mg DON kg^{-1} in feed in different studies (Vitenskapskomiteen for mattrygghet (VKM) 2013). Toxic effects may impair swine health and welfare, production results, and lead to economic losses (Wu 2007).

DON and its fungal derivatives 3-*O*-acetyl-DON and 15-*O*-acetyl-DON, as well as the plant detoxification product DON-3-*O*-ß-D-glucoside, become bioaccessible in pigs after oral uptake either directly or after hydrolytic cleavage. Water-soluble DON is rapidly absorbed and reaches the systemic circulation in less than 15 min (Broekaert et al. 2017; Goyarts and Danicke 2006). The bioavailability of DON from ingested feed in pigs is high, with estimates between 50 and 90 %. DON is metabolised in pigs to the main conjugated products DON-3-*O*-ß-D-glucuronide and DON-15-*O*-ß-D-glucuronide, primarily by hepatic enzymes. Iso-DON-glucuronides and other metabolites are also detected in rodents, ruminants and pigs, but as minor metabolites (Schwartz-Zimmermann et al. 2017). The plasma half-life of the mycotoxin is in the range of 3 to 5 h. Excretion in pigs is relatively slow compared with that in other species, resulting in considerable exposure, which is regarded as one reason for the relatively high sensitivity of pigs to DON (Goyarts and Danicke 2006).

The risk to animal health from the exposure to DON has been assessed by the European Food Safety Authority (EFSA 2004). Considering the particular sensitivity of pigs, the recommended maximum acceptable level for DON, according to European Commission Recommendation 2006/576/EC, is set to 0.9 mg kg^{-1}. In Norway, the national feed safety authority recommends an even lower level of 0.5 mg DON kg^{-1} for pig feed (Mattilsynet 2015), causing feed manufacturers and farmers additional efforts to provide suitable feed grain sources. Thus, detoxifying measures allowing the utilisation of contaminated grain lots are of great interest.

Strategies to reduce exposure to mycotoxins include the supplementation of feed products with detoxifying additives, such as passive mycotoxin adsorbents, chemical supplements or active biotransforming agents containing bacteria, fungi or enzymes that can degrade mycotoxins into non-toxic metabolites (Jard et al. 2011). Adsorbing materials are less effective in binding trichothecene mycotoxins, such as DON (Awad et al. 2010). Therefore, biological detoxification methods targeting trichothecenes have been a research focus in recent years. Ruminal microbiota biotransform DON efficiently to products of less or no toxicity and are responsible for the relative tolerance of ruminants towards increased levels in feed (Seeling and Dänicke 2005). The safety and efficacy of the ruminal microbe *Coriobacteriaceae gen. nov. sp. nov.* DSM 11798, provided as the active ingredient of Biomin® BBSH 797 by Biomin (Herzogenburg, Austria), has been assessed by EFSA for use in pigs (European Food Safety Authority (EFSA) 2005; 2013) and has been approved by the European Commission (European Union 2013). Based on the available documentation, a minimum content of 1.7×10^8 colony-forming units (cfu) kg^{-1} feed (with 12 % moisture) was recommended to achieve sufficient detoxification (European Food Safety Authority (EFSA) 2013).

DSM 11798 detoxifies DON by the reduction of the 12,13-epoxy group to produce de-epoxy-DON (DOM-1). The de-epoxidation activity has been shown *in vitro* under anaerobic conditions and *ex vivo* in simulated swine gut (Fuchs et al. 2000; Fuchs et al. 2002; Schatzmayr et al. 2006). *In vivo* efficacy of DSM 11798 in weaned pigs fed with DON-spiked or naturally contaminated non-pelleted feed has been reported in short-term studies, showing significant clinical effect on performance (Cheng et al. 2006; Plank et al. 2009) and an effective decrease of DON serum concentrations (European Food Safety Authority (EFSA) 2013; Starkl et al. 2015), whereas the results were ambiguous in other performance studies with fattening pigs (European Food Safety Authority (EFSA) 2005). Another study, adding the clay-matrix stabilised bacterium (BBSH 797) as a ready-made product (Mycofix® Plus) to feed containing highly contaminated maize (3.5 - 8 mg DON kg^{-1} feed), in an experiment with fattening pigs, did not demonstrate significant performance improvement by the detoxifying supplement (Preißinger et al. 2016).

The aim of the present study was to elucidate the effects of chronic exposure to low, moderate and high levels of DON in pelleted feed produced from naturally contaminated oats with and without DSM 11798 addition by analysing feed uptake, growth performance, clinical parameters and plasma levels of DON and its metabolites in growing pigs.

Materials and methods

Animals and housing

In a 42-day experiment, 48 crossbred (Landrace-Yorkshire/Duroc-Duroc) five-week-old weanling pigs

of both sexes (24 castrated and 24 females) with a mean initial weight of 11.0 ± 1.5 kg were individually housed in floor pens with openings for social contact in environmentally controlled rooms. The experiment was run in two rounds with identical procedures including 24 pigs in each round of experiment. The first round was conducted in July and August 2014, and the second round, in September and October 2014. The temperature in the animal facility was at 25 °C during the first round, and 23 °C, during the second. At the beginning of each round, pigs were divided into 8 feeding groups of six animals, with equal numbers of females and castrated males within the groups. The pigs received routine anthelmintic treatment (Panacur vet. (fenbendazol), MSD Animal Health) and were allowed seven days acclimatisation to the environment and diet before the study was initiated. In this period, all pigs received the control diet. Throughout the experiment, both water and feed were provided *ad libitum* using automatic feeders (DOMINO-Feeder: Slop Feeder K-1 (08261) and drinking cups (DOMINO-Drinking cups: H2O (49014), Domino A/S, Tørring, Denmark). The automatic feeders were inspected daily, and the settings were adjusted individually to minimize feed loss. At study completion, the pigs were euthanized by captive bolt pistol. The animals were bled out, and the organs of interest dissected for further analysis. The Norwegian Animal Research Authority (Groenen et al.) approved the study and all experimental procedures (Approval no. 6707 – 2014).

Origin of the naturally contaminated oats

The oats used for the production of the experimental diets in the present study had been harvested in southern Sweden in 2013. Because of its high content of deoxynivalenol (DON), this specific oat batch was excluded from use in feed or food and was bought for use in the pig study.

Preparation of the experimental diets

After the 1-week (day -6 to day 0) acclimatisation, each group received pelleted feed containing different levels of naturally DON-contaminated oats (Table 1). The following DON levels were achieved in the feed: (1) control diet (DON < 0.2 mg kg^{-1}), (2) control diet supplemented with a feed additive containing the trichothecene-degrading bacteria DSM 11798, (3) low-contaminated diet (DON = 0.92 mg kg^{-1}), (4) low-contaminated diet (1.0 mg kg^{-1}) with DSM 11798, (5) medium-contaminated diet (DON = 2.2 mg kg^{-1}), (6) medium-contaminated diet (2.5 mg kg^{-1}) with DSM 11798, (7) high-contaminated diet (DON = 5.0 mg kg^{-1}) and (8) high-contaminated diet (5.7 mg kg^{-1}) with DSM 11798 (Table 1). The feed additive containing *Coriobacteriaceae Gen. nov. sp. nov.* DSM 11798 in a non-commercial form was supplied by Biomin®, Herzogenburg, Austria, as a dry grey-brown powder containing less than 35 % cell mass and 40 – 60 % coating agent. The different levels of DON were achieved by blending the naturally contaminated oats at three ratios with very low-contaminated oats harvested in southern Norway in 2013. The pelleted diets (Table 1) were formulated at Felleskjøpet Fôrutvikling (Trondheim, Norway) and produced at the Centre for Feed Technology at the Norwegian University of Life Sciences (Fôrtek, Ås, Norway) by a standard pelleting process, keeping the pelleting temperature close to 80 °C. The diets were produced immediately before the first round of the experiment and were stored dry at room temperature until the second round.

Analysis of number of active DSM 11798 bacteria in experimental diets

The numbers of cfu of DSM 11798-containing was measured at the Biomin Research Centre (Tulln, Austria) in the finished feed additive and in the different diets after pelleting using a validated enumeration method based on Koch's pour plate method: bacteria were extracted from the feed additive and the milled diets and serially diluted in a suitable medium under anaerobic conditions. Aliquots (1 ml) of three dilutions were transferred to Petri dishes and mixed with supplemented agar medium. After incubation under strictly anaerobic conditions at 37 °C in an incubator with 100 % CO_2 atmosphere, bacterial colonies were counted by transient light microscopy, and the number of DSM 11798 cfu g^{-1} in the different samples was calculated (Table 1). The test was performed in triplicate.

Reagents for chemical analyses

Acetonitrile, methanol and water (Fisher Scientific, Fair Lawn, NJ, USA) for liquid chromatography high-resolution mass spectrometry (LC–HRMS) analysis were of Optima™ LC–MS quality, while acetonitrile for sample preparation was from Romil (Cambridge, UK). Ammonium acetate and glacial acetic acid were of p.a. quality (Merck, Darmstadt, Germany). 4-Deoxynivalenol (DON), deepoxy-DON (DOM-1), 3-*O*-acetyl-DON (3-ac-DON) and DON-3-*O*-ß-D-glucoside (DON-3-Glc) were purchased from Romer Labs (Tulln, Austria), while DON-3-*O*-ß-D-glucuronide (DON-3-GlcA) and DON-15-*O*-ß-D-glucuronide (DON-15-GlcA) were available at the NVI, as described in a previous work (Uhlig et al. 2013).

Sample preparation of experimental diets for analysis of mycotoxins

Samples of 2.5 g from each of the experimental diets were milled with a Retsch ZM 100 mill (Retsch GmbH & Co. KG, Haan, Germany) and placed into 50-ml centrifuge tubes, and 10 ml of MeCN/H_2O/formic acid (80:19.9:0.1, v/v/v) was added. The mixture was vortexed for 30 s and extracted for 30 min using a horizontal shaker at 175 min^{-1}. After centrifugation at 4.000 × g for 10 min (4 °C), the supernatants were transferred into clean 50-ml tubes, and the remaining solid material was extracted with 10 ml of MeCN/H_2O/formic acid (20:79.9:0.1, v/v/v) by shaking for an additional 30 min. The two extracts were combined and kept at 4 °C before final centrifugation (4.000 × g, 10 min and 4 °C). Finally, a 0.5-ml aliquot of the combined supernatants was centrifuged for 1 min at 15.000 × g through 0.22-µm nylon filters (Costar Spin-X 0.22 Nylon filter; Corning, Inc., Corning, NY, USA) and analysed using liquid chromatography high-resolution mass spectrometry (LC-HRMS).

Analysis of mycotoxins in experimental diets

The samples of the experimental diets were analysed for DON, DON-3-Glc, 3-ac-DON and 15-ac-DON with a previously validated LC-HRMS method (Ivanova et al. 2017). The limits of detection (LOD) in a feed matrix were 14 µg kg^{-1} for DON, 26 µg kg^{-1} for DON-3-Glc, 5.9 µg kg^{-1} for 3-ac-DON and 52 µg kg^{-1} for 15-ac-DON. In addition, the eight experimental diets were analysed for a range of other mycotoxins using a semi-quantitative multi-toxin screening method at the Centre for Analytical Chemistry at IFA Tulln, Austria, using a semi-quantitative multi-toxin screening method (Malachova

et al. 2014).

Recording of feed intake and growth performance

Individual body weight (BW) was measured twice during the acclimatisation and thereafter twice weekly until the last day of the experiment using a digital platform scale (KRUUSE, PS250, Langeskov, Denmark). Feed consumption was also measured individually by weighing each of the automated feeding stations using the same platform scale every time new feed was added, and additionally three times a week throughout the study. Average Daily Feed Intake (ADFI), Average Daily Weight Gain (ADG) and Feed Conversion Ratio (FCR) were calculated based on the registration of feed consumption.

Clinical chemistry and haematology

All blood samples were collected from the jugular vein using 5-ml Vacutainer tubes. Blood samples for clinical evaluations were collected on day -3 (week 0) in the acclimatisation period and thereafter on d 4 (week 1), d 18 (week 3), and d 35 (week 5) during the period with DON exposure. Blood for haematology was collected in 5-ml tubes, and EDTA was used as an anticoagulant. The sampled blood was kept refrigerated and delivered to the Central Laboratory of the NMBU-Faculty of Veterinary Medicine (Oslo, Norway) for analysis on the same day. Blood for serum biochemistry was collected in 5-ml tubes with gel and clot activating factor. The tubes were kept at room temperature for 1–2 h. Serum was then separated by centrifugation at 1500 × g for 10 min at room temperature, stored frozen in 2-ml cryogenic vials (Nalgene, Nalge Company, Rochester, NY, USA), and delivered to the Central Laboratory.

Haematologic analyses (CBCs) were performed upon arrival in the laboratory using ADVIA® 2120 Haematology System and ADVIA® Multispecies software (Siemens Healthcare Diagnostics, Siemens AG, Germany) using the settings for swine. The clinical biochemical analyses were performed using ADVIA 1800® Clinical Chemistry System (Siemens Healthcare Diagnostics, Siemens AG, Germany), and serum protein electrophoresis was performed on a Sebia CapillarysTM 2 (Sebia, Norcross, GA, USA).

Sample preparation of plasma for analysis of mycotoxins

Blood samples for measuring plasma concentrations of DON, DOM-1, DON-3-GlcA and DON-15-GlcA were collected once on day -3 in the acclimatisation week and thereafter three times a day (08:00, 11:00 and 16:00) on days 1, 4, 11, 18, 25, 32 and 35 during the period with DON exposure. Blood for analysis of DON and its metabolites was collected in 3-ml lithium-heparin Vacutainer tubes. Plasma was separated by centrifugation at 1500 × g for 10 min at room temperature (approximately 20 °C) and stored frozen in 2-ml cryogenic vials (Nalgene, Rochester, NY, USA). Plasma samples (250 µL) were transferred into conical 15-ml plastic tubes (Corning Inc., Corning, NY, USA), mixed with 750 µL of acetonitrile, vortexed for 15 s, and sonicated (Branson 3200, Emerson, St. Louis, MO, USA) for 5 min. Proteins were precipitated by centrifugation at 2000 × g for 10 min at 4°C (Beckman Coulter, Brea, CA, USA), and supernatants were transferred to 10-ml conical glass tubes and evaporated to

dryness at 60°C using a gentle stream of pure nitrogen, quality 6.0. Dried samples were stored refrigerated, dissolved in 200 µL of water, vortexed for 15 s, sonicated for 5 min and transferred to HPLC vials prior to LC-HRMS analysis.

Analysis of mycotoxins in pig plasma

Plasma was analysed for DON, DOM-1, DON-3-GlcA and DON-15-GlcA. Samples were chromatographically separated at 30 °C using a UHPLC Dionex Ultimate 3000 system (Thermo Fisher Scientific, Waltham, MA, USA) with a 100 × 2.1 mm i.d. Acquity UPLC HSS T3 column (1.8 µm; Waters, Milford, MA, USA) and a 5 × 2.1 mm i.d. XSelect HSS T3 VanGuard pre-column (2.5 µm, 100 Å, Waters). The flow rate of the mobile phase was 0.5 ml min^{-1}, and the injection volume was 6 µL. Eluent A was water, and eluent B was 95% acetonitrile (both containing 5 mM ammonium acetate and 0.1% acetic acid). The column was eluted isocratically with 100 % A for 1 min and then, using a linear gradient, to 15 % B in 15 min. After flushing the column for 2.5 min with 100 % B, the mobile phase composition was returned to the initial conditions, and the column was eluted isocratically for 2.9 min. The LC-system was on-line coupled to a Q-Exactive™ Hybrid Quadrupole-Orbitrap high-resolution mass spectrometer (Thermo Fisher Scientific) equipped with a heated electrospray ion source (HESI-II). The HESI-II interface was operated at 300 °C in the negative ionisation mode, and the parameters were adjusted as follows: spray voltage, 4 kV; capillary temperature, 250 °C; sheath gas flow rate, 35 L min^{-1}; auxiliary gas flow rate, 10 L min^{-1}; and S-lens RF level, 55. The data were acquired in the selected ion monitoring (Boudergue et al.)/data-dependent MS2 (dd-MS2) mode targeting the $[M+acetate]^-$ ions for DON and DOM-1 (m/z 355.1387 and 339.1438, respectively) and the $[M-H]^-$ ions for the DON-glucuronides (m/z 471.1497) with a quadrupole isolation width of 2 *m/z* and a mass resolution of 70.000 full width half-maximum (FWHM) at *m/z* 200 for SIM. The presence of a target ion above a threshold intensity of 5×10^3 triggered a MS^2 scan for analyte verification (dd-MS2) using a normalised collision energy of 35%. The mass resolution during dd-MS2 was set to 17.500 FWHM. The automatic gain control (AGC) target was set to 5×10^5 ions including a maximum injection time (IT) of 250 ms during SIM, whereas for dd-MS2 the AGC target was 5×10^4, and the IT was 200 ms.

Matrix-matched, 1/x weighed calibration curves were plotted for DON, DOM-1, DON-3-GlcA and DON-15-GlcA using blank pig plasma. Xcalibur version 2.2 or 2.3 (Thermo Fisher Scientific) was used for data processing. Each round of analyses included at least one blank pig plasma sample, which had been fortified with 5.3 or 26 ng ml^{-1} of DON, DON-3-GlcA and DON-15-GlcA and 11 or 52 ng ml^{-1} DOM-1. The overall spike recoveries were (standard deviations in parentheses) DON 80 (11) %, DOM-1 74 (12) %, DON-3-GlcA 67 (17) % and DON-15-GlcA 65 (14) %. The limits of detection (LOD) were 0.1 ng ml^{-1} for DON, 0.2 ng ml^{-1} for DOM-1, 1.5 ng ml^{-1} for DON-3-GlcA and DON-15-GlcA.

Statistical analyses

Performance and haematological and biochemical data were analysed by repeated-measures analysis using a mixed model in JMP®, Version 10 (SAS Institute Inc., Cary, NC, USA). The level of significance was set to 0.05 in all models, and results with *p*-values between 0.05 and 0.1 were considered significant trends. If not otherwise specified, all results are expressed as the mean ± standard deviation (SD). The data were considered as a completely randomized block design with

eight treatments in six blocks. Each pig was considered as random effect and represented an experimental unit for the variables tested. The DON-levels and feed additive (DSM 11798) were considered independent variables. ADFI, ADG, FCR, haematological and biochemical parameters, and plasma concentrations of DON and its metabolites were defined as dependent variables. Performance and blood parameters were subjected to repeated-measure ANOVA according to a three-factorial design:

$Y_{ijkl} = \Pi + a_i + b_j + c_k + a.b_{(ij)} + a.c_{(ik)} + b.c_{(jk)} + a.b.c_{(ijk)} + e_{ijkl}$, *where* Y_{ijkl} is the *lth* observation related to the DON-levels *i*, feed additive (with or without DSM 11798) *j*, and time of exposure *k*; Π is the overall mean; a_i is the effect of DON-levels; b_j is the effect of feed additive (DSM 11798); c_k is the effect of time; $a.b_{(ij)}$ is the interaction between DON-levels and feed additive, $a.c_{(ik)}$ is the interaction between DON-levels and time; $b.c_{(jk)}$ is the interaction between feed additive and time; $a.b.c_{(ijk)}$ is the interaction between DON-levels, feed additive and time; and e_{ijkl} is the error term.

The normality of distribution of the different parameters was controlled by residual and predicted values plot, normal-percentile plots and Shapiro-Wilk test. If the *p*-value in the Shapiro-Wilk test was over 0.05, the data were considered normally distributed. The data that were not normally distributed were transformed or analysed by non-parametric models, such as Wilcoxon´s rank sum test. Levene's test was used to check the assumption of homogeneity of variances. If the *p*-value of Levene's test was over 0.05, variances were considered equal. If the output generated from the application of repeated-measure ANOVA was significantly different, the post hoc Tukey-Kramer HSD test was used for multiple comparisons and the identification of significant differences ($p < 0.05$).

Plasma concentrations of DON and its metabolites were evaluated by the non-parametric Wilcoxon Each Pair test ($p < 0.05$). In the statistical calculations, concentrations below the limit of detection (LOD) were represented by the LOD divided by the square root of 2.

Results

Effects of experimental diets on feed intake and growth performance.

The piglets accepted the oat-based feed (64 % oat; Table 1) easily, showed no signs of digestive problems, interacted socially with their neighbours, and became accustomed to being handled and weighed. The animals did not show obvious signs of toxicity, such as vomiting, disease or distress related to the experimental diets, at any time during the 42-day study.

Table 2 summarises the data on the effects of DON-contaminated diets with and without DSM 11798 on feed intake and growth performance during the experimental period. The data generated from the application of repeated-measure analysis of variance (ANOVA) on the growth performance parameters and related interactions between different effects are shown in Table 2b. The results from day 15 to 21 (week 3) and day 22 to 28 (week 4) of the exposure period (Table S2) were intermediate between results from week 2 and week 5 and are omitted from Table 2a to reduce the size of the table. The average daily feed intake (ADFI), average daily weight gain (ADG) and feed conversion ratio (FCR) were similar among all treatments during the pre-exposure period. The six-week old pigs ($n = 48$) had a mean ADFI of 337 ± 86 g day^{-1} and a mean ADG of 243 ± 85 g

day^{-1}, resulting in a mean FCR of 1.47 ± 0.37 and a total weight gain of 1.71 ± 0.59 kg in the acclimatisation week (week 0; day -6 to day 0) by consumption of the oat control diet.

When evaluated over the entire experimental period, neither ADFI nor ADG were significantly influenced by the exposure to DON or the presence of DSM 11798 in the experimental diets. However, on week 1 (day 1 to 7) of the exposure period, pigs in the high-DON group showed a significant ($p < 0.05$) 24% and 34% reduction in ADG compared with those fed with the control and low-DON groups, respectively. This trend was also observed, although the difference was not significant, in the medium-DON group (20 % ADG reduction in relation to the low-DON group). The addition of DSM 11798 did not show any effect on the ADFI, ADG or FCR results. On week 2 (day 8-14) of the DON exposure period, differences in ADG between the high-DON group and the control and low-DON groups were still noticeable, but the gap had decreased and was no longer significant. In subsequent weeks, the pigs appeared to adapt to the contaminated diets so that at the end of the feeding experiment (week 5), both ADFI and ADG were normalized and comparable between all DON-exposed and control groups. The mean total weight gain in the DON exposure period (days 1-35) in all diet groups was 24.7 ± 3.6 kg, the mean feed intake was 35.6 ± 6.1 kg, and the mean FCR was 1.57 ± 0.08 ($n = 48$).

Effects of experimental diets on haematological and biochemical parameters

Biochemical parameters

No differences in any of the measured biochemical parameters were observed between the two groups at the same dose level receiving feed with and without the addition of DSM 11798 throughout the experiment. Comparisons between DON dose levels are therefore based on $n = 12$ per group. Figure 1 shows the effects of experimental diets on some selected serum biochemical parameters of pigs in the experimental period. The studied serum biochemical parameters did not differ between the treatment groups on week 0 and week 1 (Figure 1). However, total serum protein concentrations were significantly ($p < 0.05$) lower in pigs in the high-DON group than in those in the control group in week 3 (Figure 1a). This difference was further increased in week 5, where pigs in the high-DON group had significantly lower total serum protein concentrations than pigs in the control ($p < 0.0001$) and low-DON groups ($p < 0.05$). In addition, pigs in the medium-DON group had significantly ($p < 0.05$) lower total serum protein concentrations than in pigs in the control group.

Serum albumin concentrations (Figure 1b) and total serum globulin concentrations (Figure 1c) showed a similar dependency. The pigs in the high-DON group had significantly ($p < 0.05$) lower serum albumin than pigs in the control group on week 5. Total serum globulin was significantly ($p < 0.05$) lower in the high-DON group than in the control group in week 3 and 5. These findings support the assumption that the observed effects were dose-dependent. The effects in the low-DON and medium-DON groups were also noticeably though not significantly different from those in the control group (Figure 1a, b and c).

The analysis of serum calcium concentrations showed that pigs in the high-DON group had significantly ($p < 0.05$) lower levels of calcium than pigs in the control group on week 5 (Figure 1d). Other serum biochemical parameters, such as aspartate aminotransferase, alkaline phosphatase, gamma-glutamyl transpeptidase, glutamate dehydrogenase, creatine kinase, C-reactive protein,

urine acid, creatinine, total bilirubin, cholesterol, glucose, inorganic phosphate, magnesium, sodium, potassium and iron, were not significantly affected by different DON levels in the feed.

Haematological parameters

Haematology parameters (CBS factors), including red and white blood cell counts, haematocrit, haemoglobin and platelets, did not change observably with dose scheme, time or addition of DSM 11798.

DON and its metabolites in pig blood

The plasma concentrations of DON and its main metabolites in pig, such as DOM-1, DON-3-GlcA and DON-15-GlcA, were determined in all pigs in all dose groups once in the acclimatisation period and at eight sampling days during the five weeks of DON exposure. On each sampling day, three samples were taken in the morning, around noon and in the afternoon, with the aim to mirror the feeding rhythm of the pigs. The plasma analysis showed, however, that the concentrations of DON and its metabolites were relatively constant throughout the day, likely due to the *ad libitum* access to feed. Therefore, the three measured values per animal on each sampling day were combined to obtain average values, with which a mean for each dose group ($n = 6$) was calculated.

Figure 2 presents the measured plasma concentrations of DON, DOM-1, DON-3-GlcA and DON-15-GlcA during the study. Whereas the average levels were low in the control pigs (0.6 ± 0.2 ng ml^{-1}), DON and DON-metabolites were detected in a dose-dependent pattern in the pigs fed with low-, medium- and high-DON contaminated diets.

The pairwise comparison between groups receiving the same dose, with or without the addition of DSM 11798, showed that the levels of DON (Figure 2a) and DOM-1 (Figure 2b) were very similar. In contrast, the glucuronide metabolites, DON-3-GlcA (Figure 2c) and DON-15-GlcA (Figure 2d), were elevated in samples from pigs in the low-DON, medium-DON and high-DON groups receiving DSM 11798-supplemented feed. The effect was significant for DON-3-GlcA on day 4 in high-DON and medium-DON, on day 11 in high-DON, medium-DON and low-DON, on day 18 in high-DON and medium-DON, on day 25 in medium-DON and low-DON, on day 32 in medium-DON and on day 35 in low-DON ($p < 0.05$). The effect was significant for DON-15-GlcA in medium-DON on days 4 and 11 ($p < 0.05$). No sex-dependent differences were found in the plasma levels; neither of DON, DOM-1 nor of the DON-glucuronides.

Considering the mean feed intake and pig weights including gain, the approximate average DON doses (µg kg^{-1} body weight day^{-1}) in the toxin-exposed groups were calculated for each week of the study period (Table 3). The estimated doses were almost constant throughout the study due to a practically parallel increase in body weight and feed intake. This resulted in nearly stable plasma concentrations in the pigs at each DON level during the experiment. Calculated for the whole exposure period, the mean DON plasma concentrations were 3.5 ± 1.6 ng ml^{-1}, 9.4 ± 3.9 ng ml^{-1} and 16.0 ± 6.0 ng ml^{-1} in pigs receiving low, medium and high-DON diets, respectively. The average DOM-1 plasma concentrations were found to be 0.4 ± 0.3 ng ml^{-1}, 0.9 ± 0.5 ng ml^{-1} and 1.6 ± 0.8 ng ml^{-1} in pigs fed low-, medium- and high-DON diets, respectively. The average DON-3-GlcA concentrations in plasma were 3.1 ± 1.8 ng ml^{-1}, 11.1 ± 5.7 ng ml^{-1} and 19.0 ± 9.0 ng ml^{-1}, and the average DON-15-GlcA

plasma concentrations were 3.0 ± 1.6 ng ml^{-1}, 8.1 ± 4.3 ng ml^{-1} and 15.0 ± 7.4 ng ml^{-1} in pigs fed with low-, medium- and high-DON contaminated diets, respectively.

Discussion

In this study, reductions in feed intake and reduced weight gain in the first week of the exposure period were the major adverse clinical effects of feeding animals with DON-contaminated diets. In this first week, feeding of high-contaminated diets led to 20-28 % reduction in feed intake and 24-34 % reduction in weight gain compared with the control and low-contaminated diet groups. However, these variables were normalised in the subsequent weeks. No significant differences in feed intake and daily weight gains were observed between groups in weeks 2, 3, 4 and 5 or over the entire 35-day exposure period.

An equivalent, transient effect during the first week of DON exposure has been reported previously in pigs fed diets naturally contaminated with DON (2.8 mg kg^{-1}) (Wache et al. 2009). Similar observations of feed aversion and particular effects on growth performance in the first weeks of feeding with DON-contaminated feed have also been reported by other authors (Bergsjo et al. 1993; Wache et al. 2009). Several mechanisms have been proposed to contribute to the reduction in feed intake induced by DON-contaminated feed. Some studies have shown that DON can cross the blood-brain barrier, leading to activation of central structures and affecting glial cell viability and function (Behrens et al. 2015; Razafimanjato et al. 2011). Other experiments have suggested a DON-induced reduction in plasma insulin-like growth factor acid-labile subunit (IGFALS) (Flannery et al. 2013) and an increased pro-inflammatory cytokine expression (Pestka and Amuzie 2008), effects that initiate anorexia and poor growth performance. In addition, it has been reported that acute ip-exposure of mice to DON (1-5 mg kg^{-1}) may elevate levels of the gut satiety hormones peptide YY (PYY) and cholecystokinin (CCK) and that this may be related to DON-induced feed refusal and growth suppression (Flannery et al. 2012).

Another possible explanation is that DON as a physiological or systemic stressor activates the hypothalamus-pituitary-adrenal (HPA) axis and can induce a corticosterone stress response, an effect that has been documented both in broiler chickens and in mice (Antonissen et al. 2016; Islam and Pestka 2003). A stress situation can in itself lead to reduced feed intake and poor growth performance in pigs (Campbell et al. 2013). The observed transience of the first-week effects of DON on feed intake could be in agreement with proposals in a review by Grissom and Bhatnagar (2009), suggesting possible habituation of HPA responses to repeated exposures to stressors.

Calculated over the entire 35-day exposure period, the effect of DON-contaminated feed on ADG was not significant. However, in previously reported studies with DON concentrations at similar levels, the effect over medium-length periods of exposure has also been variable. While Plank et al. (2009) showed a significant effect of 2 mg DON kg^{-1} feed in approximately 6 weeks of exposure and Bergsjø et al. (1993) showed significant effect of 3.5 mg DON kg^{-1} feed in 12 weeks of exposure. Bergsjø et al. (1992) in another study found statistically significant effect of 4 mg DON kg^{-1} on performance in the first 8 weeks of experiment. Øvernes et al. (1997) did not find significant effect of 4.7 mg kg^{-1} on performance during the first 8 weeks of exposure when the pigs were fed *ad libitum*.

The results of the present study also indicate that feeding pigs with DON-contaminated diets can contribute to significant changes in serum protein levels. In our experiment, total serum protein,

globulin and albumin were lower in pigs fed contaminated diets after 3 weeks of exposure and at the end of the feeding experiment (Figure 1). This finding is in agreement with other studies, which showed significant reductions in total serum protein and/or globulin in pigs fed diets contaminated with DON (3 to 4 mg kg^{-1}) (Bergsjo et al. 1993; Rotter et al. 1995). However, there are also studies that have not shown any significant effect of dietary exposure to DON on blood protein concentrations in pigs (Dänicke et al. 2004; Goyarts et al. 2006; Wu et al. 2015). It has been proposed that the reduction in serum protein levels observed in a proportion of feeding experiments may be a result of impaired protein synthesis in the liver related to reduced feed uptake when pigs are fed DON-contaminated diets (Rotter et al. 1995). In the present study, however, the effect on serum protein levels was evident in the last weeks of the experiment, when feed intake and weight gain were not significantly affected. This finding may indicate that the reduction in serum protein levels was an independent, direct effect of DON.

A significant inverse relationship with serum calcium and a trend ($p < 0.1$) of reduced serum phosphorous concentrations in pigs fed the high-DON feed (5.3 mg kg^{-1}) was another significant change in the last week of the present study. As these effects were only seen in week 5, and there was no tendency in the same direction in previous weeks, we cannot exclude that the result is accidental. However, if the finding reflects a real effect, it is in line with results from some other experiments (Bergsjo et al. 1993; Prelusky et al. 1994; Rotter et al. 1995). The possible mechanism behind the effect is unknown, but it might be related to changes in intestinal absorption related to DON-induced changes in intestinal morphology, permeability and transporter functions (Alizadeh et al. 2015; Maresca et al. 2002).

The *Coriobacteriaceae* strain DSM 11798, also known as BBSH 797, is a Gram positive, strictly anaerobic strain originally isolated from bovine rumen (European Food Safety Authority (EFSA) 2013). Several *in vitro* studies have confirmed that this bacterial strain can reduce DON to the less toxic metabolite DOM-1, also under simulated gut conditions including low pH values (Fuchs et al. 2000; Fuchs et al. 2002; Schatzmayr et al. 2006). Rather few *in vivo* studies in pigs have investigated the efficacy of the stabilised DSM 11798 strain (European Food Safety Authority (EFSA) 2005; 2013; Plank et al. 2009). Other studies made use of commercial, mixed feed additives, which contained a combination of DSM 11798 (as BBSH 797), patented specific enzymes and other components (Cheng et al. 2006; Grenier et al. 2013).

In this present study, DON-contaminated diets impaired performance parameters in the pigs to a moderate degree and addition of DSM 11798 to experimental diets had no influence on the growth performance. This finding is in agreement with some previous studies, where DSM 11798 did not show any effect on performance parameters (European Food Safety Authority (EFSA) 2005; Preißinger et al. 2016). However, our results are inconsistent with those of other previous studies, which have indicated a positive effect of DSM 11798/BBSH 797 on the performance of pigs fed DON-contaminated grain (European Food Safety Authority (EFSA) 2005; Plank et al. 2009).

In the current study, changes in serum chemical parameters in pigs fed DON-contaminated diets were not improved by feed supplementation with DSM 11798. This finding is inconsistent with another study, where minor effects of DON, such as a decreased albumin concentration at the end of the trial, were prevented by addition of DSM 11798 (Grenier et al. 2013).

The data from the plasma analyses of DON and its metabolites, especially that of DOM-1, confirmed that DSM 11798 was not able to reduce the epoxide group in DON in this *in vivo* study (Figure 2). The enzymatic reduction of DON to DOM-1 by DSM 11798 was expected to result in decreased DON plasma concentrations and increased DOM-1 plasma concentrations in pigs receiving

DON-contaminated diets supplemented with DSM 11798 compared to pigs fed contaminated diets without the detoxifying microorganism (Dänicke et al. 2004; Fuchs et al. 2000; Fuchs et al. 2002; Schatzmayr et al. 2006). However, the concentrations of both DON and DOM-1 (Figure 2) were not significantly different in the plasma of pigs fed contaminated diets supplemented with DSM 11798 compared with pigs fed diets without DSM 11798. On the other hand, the supplementation with DSM 11798 in the DON-contaminated feed resulted in significantly higher levels of DON–glucuronides (Figure 2c and Figure 2d). As no other studies we are aware of have included DON-glucuronides in investigations related to DSM 11798/BBSH 797, we cannot compare the result with previous research. At present, we have no explanation for the effect of DSM 11798 on the DON-glucuronide levels. However, presence of DSM 11798 in the gut might hypothetically influence enteric or bacterial ß-glucuronidase activity, and thereby change the enteric recycling of deoxynivalenol, resulting in increased DON-glucuronide levels in the systemic circulation (Yang et al. 2017).

Traditionally, DON-3-Glc and DON-15-Glc have been the main identified DON metabolites in non-ruminant mammals. Recent research has identified a range of additional metabolites in the urine of rodents and humans, including iso-DON-8-glucuronide, iso-DON-3-glucuronide, DOM-3-glucuronide and DON-3-sulfate (Schwartz-Zimmermann et al. 2017; Warth et al. 2016). In pigs, however, DON-3-Glc and DON-15-Glc still seem to be the dominating metabolites, and the iso-DON-glucuronides and DOM-glucuronides are found only in low concentrations (Schwartz-Zimmermann et al. 2017). In our experience, sulfate conjugation of DON is also of minor importance in pigs (unpublished results).

In this study, we found no differences in plasma concentrations of DON and its metabolites between male and female piglets. This is in contrast to the observations reported by Pestka et al. (2017) in mice, where they observed slower excretion and higher hepatic and renal concentrations of DON metabolites in male mice than in females.

The lack of activity of DSM 11798 to reduce the 12,13-epoxy group in DON and thereby detoxify the mycotoxin in the present study may have several explanations. One possible reason could be related to the feed production. In the present study, the experimental diets were prepared by a standard pelleting process. The heat involved in this process can impair the viability of bacteria. However, BBSH 797 has been proven to survive common pelleting process with pelleting temperatures up to 80 °C (European Food Safety Authority (EFSA) 2005; 2013), and the pelleting temperatures were held strictly at that level (Table 1). Furthermore, the survival of the DSM 11798 strain in our experimental diets was monitored after the pelleting process (Table 1) and was assessed to be sufficient to ensure effective detoxification.

It is possible that the impact of the pelleting process and the embedding of the DSM 11798 microorganism into a feed pellet themselves affect the microorganism's efficacy in pigs. Because the intestinal absorption of DON in pigs is fast, a delayed onset of the activity of the DSM 11798 in the pig gut may be sufficient to impair its efficacy against DON absorption and toxicity.

Another possibility for the lack of effect could be the stabilizing matrix used by Biomin in the preparation of the DSM 11798 product for our experiments, which was somewhat different from the commercial version. It should also be noted that the main feed ingredient in our study was DON-contaminated oats, while some of the other studies were based on contaminated maize. Further studies on the *in vivo* efficacy of DSM 11798 making use of an experimental design where both pelleted and meal diets are included are recommended.

In conclusion, in the present study, feeding pigs with pelleted, naturally DON-contaminated diets led to a transient effect on growth performance, which was significant in the first week of exposure. Some blood biochemical parameters were also influenced by the DON-contaminated diets. The inclusion of a feed additive containing the bacterial strain DSM 11798 in the pelleted feed was found to be ineffective both in the biotransformation of DON to less toxic components, such as DOM-1, and in preventing mycotoxin-related effects in the pigs fed the naturally DON-contaminated pelleted diets.

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Table titles

Table 1. Composition of the experimental diets.

Table 2. Effect of experimental diets on pig growth.

Table 2a. Growth performance results at the assessment stages in the study.

Table 2b. Probabilities derived from the fixed-effect tests (repeated measures).

Table 3. Daily DON doses in the pigs during the five-week exposure study.

Table footnotes

Table 1.

[a] Feed Additive (*Coriobacteriaceae* strain DSM 11798).

[b] FA dose in the finished pellets was determined with respect to an inert tracer substance in the DSM 11798 preparation.

Table 2a.

[a] *Coriobacteriaceae* strain DSM 11798.

[b] Mean ± standard deviation.

[c] ADG, average daily gain; [d] ADFI, average daily feed intake; [e] Feed conversion ratio (feed consumption/weight gain).

Means within a row with no common letter are significantly different ($p < 0.05$). The differences were evaluated by post-hoc Tukey test.

Results from weeks 3 and 4 (day 15-28) are omitted for space reasons. They are reported in the supplementary material (Table S2).

Table 2b.

[f] F-ratio and [g] *p*-values in the fixed-effect test table present the main effect and interactions.

Table 3.

[a] Standard deviation.

[b] Mean body weight in a week.

[c] Daily intake of DON in feed (mg day^{-1}): ADFI×DON conc. in feed.

[d] Daily DON dose (µg kg^{-1} body weight day^{-1}).

Figure captions

Figure 1. Effect of experimental diets on biochemistry parameters. Selected serum biochemical parameters of pigs over the course of the experiment according to DON levels (n = 12 in each group, pooled data for the main effect "DON levels", * $p < 0.05$). Each error bar shows the standard error of the mean.

Figure 2. Effect of experimental diets on plasma concentrations of DON (Figure 2a), DOM-1 (Figure 2b), DON-3-GlcA (Figure 2c) and DON-15-GlcA (Figure 2d). Plasma concentrations of DON and its metabolites (ng ml^{-1}) in pigs over the course of the experiment were dependent on DON levels and the addition of DSM 11798 (n = 6 in each group, pooled data for the main effect "DON levels and addition of DSM 11798"). The data points are the mean of plasma concentrations measured at three different time points per day. The first tick D0 (x-line) in each figure refers to the measurement on day 4 of acclimatisation. D1 baseline refers to the first measurement before the diet change at 08:00, and D1 refers to the average of plasma concentrations on two measurements after the diet changes at 11:00 and 16:00 on the first day of exposure. Error bars are the standard error of the mean.

Supplemental information.

Table titles

Table S1. Toxin contents in the experimental diets, as measured by multi-toxin LC-MS/MS.

Table S2. Growth performance results from days 15-28 in the feeding study (results omitted from Table 2 in the main text.)

Table footnotes

Table S1

[a] Feed Additive (*Coriobacteriaceae* strain DSM 11798).

Table S2

[a] *Coriobacteriaceae* strain DSM 11798.

[b] Mean ± standard deviation.

[c] ADG, average daily gain; [d] ADFI, average daily feed intake; [e] Feed conversion ratio (feed consumption/weight gain).

Figure captions

Figure S1. Average daily gain (g d^{-1}) of pigs over the course of the experiment in relation to DON levels [(n = 12 in each group), pooled data for the main effect "DON levels", $^{*}p < 0.05$]. Each error bar is constructed using one standard error of the mean.

Figure S2. Average daily feed intake (g d^{-1}) of pigs over the course of the experiment in relation to DON levels [(n = 12 in each group), pooled data for the main effect "DON levels"]. Each error bar is constructed using one standard error of the mean.

	Experimental Diets								
Ingredients	**Control**	**Control + FA[a]**	**Low-DON**	**Low-DON + FA[a]**	**Medium-DON**	**Medium-DON + FA[a]**	**High-DON**	**High-DON + FA[a]**	**Unit**
Oats, uncontaminated	64.0	64.0	55.0	55.0	36.0	36.0	8.0	8.0	%
Oats, DON-contaminated	_	_	9.0	9.0	28.0	28.0	56.0	56.0	%
Wheat, uncontaminated	9.6	9.5	9.6	9.5	9.5	9.5	9.5	9.5	%
Fish meal	11.4	11.4	11.4	11.4	11.4	11.4	11.4	11.4	%
Soya extracted	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	%
Rapeseed cake Mestilla	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	%
Soya oil (raw)	4.8	4.8	4.8	4.8	4.8	4.8	4.8	4.8	%
Calcium carbonate (limestone)	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	%
Monocalcium phosphate	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	‰
Salt (NaCl)	3.1	3.1	3.1	3.1	3.1	3.1	3.1	3.1	‰
Mikromin. Swine	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	‰
Selenpremix	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	‰
Normin ferrous fumarate	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	‰
Vitamin-A	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	‰
Vitamin-E V5	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	‰
Vitamin-ADKB	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	‰
Stay C 35%	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	‰
L-lysine	3.1	3.1	3.1	3.1	3.1	3.1	3.1	3.1	‰
DL-methionine	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2	‰
L-threonine	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8	‰
L-Valin	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	‰
L-Tryptophan	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	‰
Formic Acid 85%	8.8	8.8	8.8	8.8	8.8	8.8	8.8	8.8	‰
MAXAROME RP SWEET 1516	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	‰
PHYZYME XP 5000 TPT	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	‰
Deoxynivalenol	0.13	0.13	0.92	1.00	2.20	2.50	5.00	5.70	mg kg^{-1}
3-ac-DON	<0.01	<0.01	0.06	0.08	0.22	0.24	0.64	0.58	mg kg^{-1}
15-ac-DON	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	<0.05	mg kg^{-1}
DON-3-Glc	<0.03	<0.03	0.10	0.10	0.41	0.38	0.64	0.52	mg kg^{-1}
***Coriobacteriacea* strain DSM 11798 (FA)[b]**	_	0.10	_	0.10	_	0.10	_	0.10	%
Viable cell Count		3.1×10^8	_	2.5×10^8	_	1.9×10^8	_	1.2×10^8	cfu kg^{-1}

	–								
Pelleting temprature	81.8	79.5	79.7	79.9	80.3	80.5	79.9	80.2	℃

Table 1

	Diets					
	DON levels				Feed additive[a]	
	Control (n=12)[b]	Low-DON (n=12)[b]	Medium-DON (n=12)[b]	High-DON (n=12)[b]	FA (n=24)[b]	Non-FA (n=24)[b]
Pre-exposure (day -6 to day 0)						
Initial Body Weight (kg)	10.9 ± 1.2	10.9 ± 1.4	10.3 ± 1.8	11.9 ± 1.5	10.9 ± 1.5	11.1 ± 1.6
ADG (g day -1)[c]	251 ± 90	264 ± 80	213 ± 72	245 ± 96	233 ± 85	254 ± 85
ADFI (g day -1)[d]	338 ± 81	363 ± 82	303 ± 87	346 ± 91	323 ± 89	352 ± 82
FCR[e]	1.42 ± 0.25	1.43 ± 0.31	1.51 ± 0.51	1.52 ± 0.36	1.47 ± 0.32	1.48 ± 0.41
Final Body Weight (kg)	12.7 ± 1.6	12.8 ± 1.6	11.7 ± 2.1	13.6 ± 1.9	12.5 ± 1.9	12.8 ± 1.9
Exposure period (day 1-7)						
ADG (g day -1)	577 ± 141	**662 ± 117b**	532 ± 116	**437 ± 90a**	523 ± 144	581 ± 133
ADFI (g day -1)	640 ± 145	714 ± 127	571 ± 118	513 ± 84	574 ± 133	645 ± 139
FCR	1.13 ± 0.16	1.09 ± 0.17	1.08 ± 0.11	1.19 ± 0.13	1.12 ± 0.16	1.12 ± 0.13
Final Body Weight (kg)	16.7 ± 2.5	17.4 ± 2.1	15.5 ± 2.6	16.6 ± 2.3	16.2 ± 2.6	16.9 ± 2.2
Exposure period (day 8-14)						
ADG (g day -1)	650 ± 107	612 ± 83	582 ± 93	595 ± 120	595 ± 96	625 ± 107
ADFI (g day -1)	901 ± 221	922 ± 176	789 ± 165	801 ± 177	830 ± 187	876 ± 194
FCR	1.37 ± 0.15	1.50 ± 0.14	1.34 ± 0.11	1.35 ± 0.15	1.38 ± 0.09	1.39 ± 0.13
Final Body Weight (kg)	21.2 ± 3.1	21.7 ± 2.5	19.5 ± 3.2	20.8 ± 3.0	20.4 ± 3.2	21.3 ± 2.8
Exposure period (day 29-35)						
ADG (g day -1)	831 ± 141	883 ± 135	863 ± 138	818 ± 164	813 ± 151	885 ± 127
ADFI (g day -1)	1369 ± 267	1462 ± 263	1343 ± 278	1401 ± 264	1324 ± 287	1463 ± 223
FCR	1.65 ± 0.21	1.65 ± 0.12	1.55 ± 0.14	1.71 ± 0.16	1.62 ± 0.17	1.65 ± 0.17
Final Body Weight (kg)	37.6 ± 5.3	38.9 ± 3.8	35.6 ± 5.6	37.3 ± 4.7	36.4 ± 5.3	38.4 ± 4.2
Exposure period (day 1-35)						
ADG (g day -1)	714 ± 110	746 ± 89	686 ± 108	677 ± 99	681 ± 113	731 ± 85
ADFI (g day -1)	1121 ± 225	1195 ± 161	1055 ± 183	1083 ± 180	1064 ± 205	1162 ± 163
FCR	1.56 ± 0.11	1.60 ± 0.06	1.53 ± 0.07	1.59 ± 0.06	1.56 ± 0.08	1.58 ± 0.07

Table 2a

	Items and interactions						
	DON	FA	Time	DON × FA	DON × Time	FA × Time	DON × FA × Time
ADG (g day $^{-1}$)	F^{f} (3,40) =1.14, p^{g}=0.34	F (1,40) =2.84, p=0.09	**F (5,200) = 355.77, p <0.0001**	F (3,40) = 0.57, p= 0.63	**F (15,200) = 2.77, p= 0.0006**	F (5,200) = 0.65, p = 0.66	F (15,200) = 0.75, p = 0.72
ADFI (g day $^{-1}$)	F (3,40) =1.30, p=0.28	F (1,40) =3.32, p=0.07	**F (5,200)= 581.25, p <0.0001**	F (3,40) = 0.67, p= 0.57	F (15,200) = 1.17, p= 0.29	F (5,200) =1.82, p = 0.10	F (15,200) = 0.52, p = 0.92
FCR	F (3,40) =0.87, p=0.46	F (1,40) =0.97, p=0.32	**F (5,200)= 36.23, p <0.0001**	F (3,40) = 1.07, p= 0.37	F (15,200) = 0.86, p= 0.61	F (5,200) =0.24, p = 0.95	F (15,200) = 0.99, p = 0.45

Table 2b

Diets		Exposure time: Week 1 Mean ± SD [a] (*n*=6)	Week 2 Mean ± SD [a] (*n*=6)	Week 3 Mean ± SD [a] (*n*=6)	Week 4 Mean ± SD [a] (*n*=6)	Week 5 Mean ± SD [a] (*n*=6)
Control	Body Weight[b] (kg)	15.1 ± 1.9	19.8 ± 2.5	24.9 ± 3.1	30.5 ± 3.7	36.5 ± 4.2
	DON intake[c] (mg day^{-1})	0.10 ± 0.02	0.13 ± 0.03	0.14 ± 0.03	0.17 ± 0.03	0.19 ± 0.03
	DON dose[d]	6.3 ± 0.9	6.3 ± 0.8	5.6 ± 0.5	5.6 ± 0.3	5.2 ± 0.3
Control + FA	Body Weight[b] (kg)	14.2 ± 2.2	18.1 ± 3.0	22.6 ± 3.5	27.6 ± 4.1	32.9 ± 5.0
	DON intake[c] (mg day^{-1})	0.07 ±0.01	0.11 ± 0.03	0.12 ± 0.02	0.14 ± 0.03	0.16 ± 0.04
	DON dose[d]	5.0 ± 0.2	5.9 ± 0.6	5.4 ± 0.6	5.1 ± 0.7	5.0 ± 0.6
Low-DON	Body Weight[b] (kg)	14.8 ± 1.7	19.2 ± 2.2	23.9 ± 2.3	29.3 ± 2.3	35.2 ± 2.8
	DON intake[c] (mg day^{-1})	0.66 ± 0.10	0.84 ± 0.19	0.99 ± 0.04	1.17 ± 0.10	1.34 ± 0.17
	DON dose[d]	44.7 ± 4.5	43.5 ± 6.9	41.4 ± 4.3	40.0 ± 2.5	38.0 ± 2.3
Low-DON + FA	Body Weight[b] (kg)	15.1 ± 1.6	19.9 ± 2.6	24.8± 3.1	30.4 ± 3.6	36.4 ± 4.3
	DON intake[c] (mg day^{-1})	0.72 ± 0.16	0.93 ± 0.16	1.09 ± 0.16	1.30 ± 0.26	1.47 ± 0.35
	DON dose[d]	46.7 ± 6.4	47.2 ± 8.1	44.1 ± 5.0	42.7 ± 6.4	40.2 ± 7.3
Medium-DON	Body Weight[b] (kg)	14.5 ± 2.6	18.6 ± 3.2	23.2 ± 3.5	28.5 ± 4.2	34.4 ± 5.4
	DON intake[c] (mg day^{-1})	1.38 ± 0.26	1.80 ± 0.37	2.24 ± 0.23	2.74 ± 0.50	3.16 ± 0.68
	DON dose[d]	96.4 ± 22.0	96.4 ± 7.0	97.3 ± 10.0	96.1± 9.0	91.1 ± 9.1
Medium-DON + FA	Body Weight[b] (kg)	12.7 ± 1.8	16.5 ± 2.5	20.8 ± 3.0	25.7 ±3.9	29.4± 3.2
	DON intake[c] (mg day^{-1})	1.30 ± 0.28	1.91 ± 0.44	2.26 ±0.42	2.70 ± 0.54	3.14 ± 0.62
	DON dose[d]	103 ± 21	115 ± 11	108 ± 11	105 ± 8	107 ± 18
High-DON	Body Weight[b] (kg)	15.1 ± 2.1	18.8 ± 2.5	23.6 ± 3.1	29.1 ± 3.5	35.1 ± 3.8
	DON intake[c] (mg day^{-1})	2.59 ± 0.53	4.06 ± 0.74	5.07 ±0.83	6.71 ± 0.89	7.60 ±1.03
	DON dose[d]	172 ± 32	217 ± 31	215 ± 21	231 ± 23	217 ±22
High-DON + FA	Body Weight[b] (kg)	15.0 ± 2.4	18.6 ± 3.0	23.1 ± 3.6	28.3 ± 4.1	33.7± 5.1
	DON intake[c] (mg day^{-1})	2.90 ± 0.39	4.51 ± 1.23	5.42 ± 0.75	6.64 ± 1.58	7.32 ±1.63
	DON dose[d]	195 ± 26	238 ± 28	235 ± 16	233 ± 29	216 ± 16

Table 3

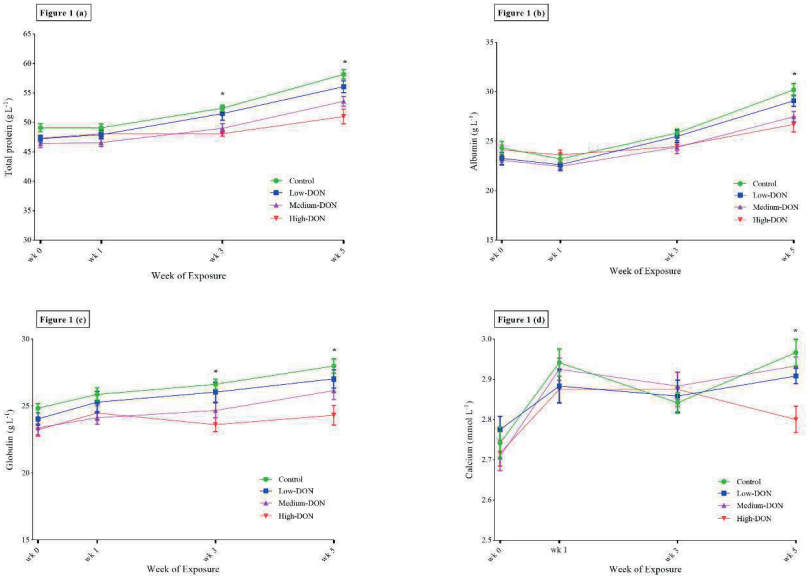
Figure 1 (a)
Total protein (g L-1)
Week of Exposure
Control
Low-DON
Medium-DON
High-DON
Figure 1 (b)
Albumin (g L-1)
Week of Exposure
Figure 1 (c)
Globulin (g L-1)
Week of Exposure
Figure 1 (d)
Calcium (mmol L-1)
Week of Exposure

Figure 1

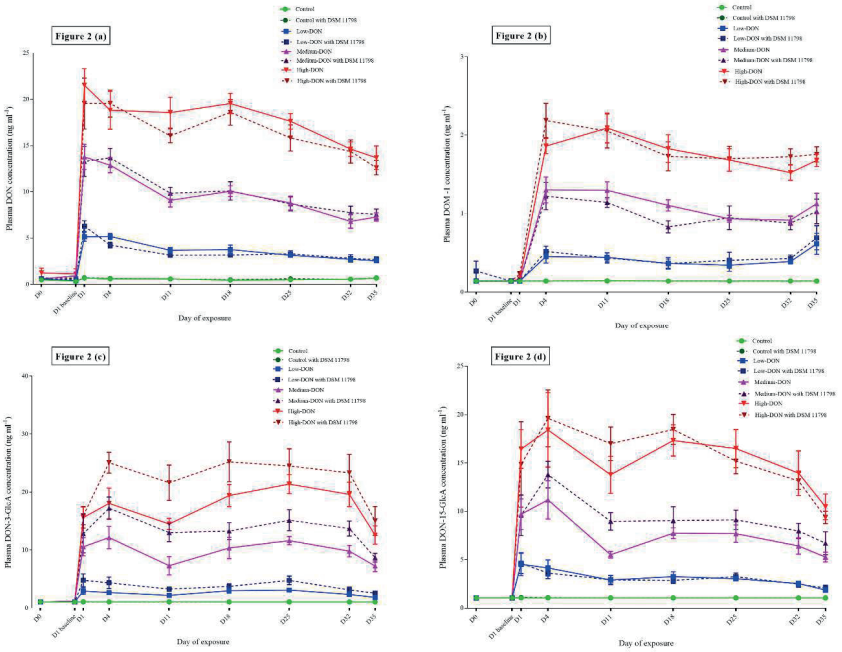
Figure 2 (a)
Plasma DON concentration (ng ml-1)
Day of exposure
Figure 2 (b)
Plasma DOM -1 concentration (ng ml-1)
Day of exposure
Figure 2 (c)
Plasma DON-3-GlcA concentration (ng ml-1)
Day of exposure
Figure 2 (d)
Plasma DON-15-GlcA concentration (ng ml-1)
Day of exposure
Control
Control with DSM 11798
Low-DON
Low-DON with DSM 11798
Medium-DON
Medium-DON with DSM 11798
High-DON
High-DON with DSM 11798

Figure 2

	Experimental Diets							
Mycotoxin (μg kg^{-1})	Control	Control + FA[a]	Low-DON	Low-DON + FA[a]	Medium-DON	Medium-DON + FA[a]	High-DON	High-DON + FA[a]
15-Hydroxyculmorinculmorin	20	< LOD	298	85	319	266	488	589
3-Nitropropionic acid	6.0	4.8	6.3	11.4	8.7	5.0	2.3	5.2
Alternariol	< LOD	< LOD	0.90	< LOD	< LOD	3.63	< LOD	2.70
Alternariolmethylether	0.45	0.33	0.41	0.37	< LOD	3.03	0.29	< LOD
Antibiotic Y	2207	874	1145	1318	1547	713	271	339
Apicidin	< LOD	0.9	4.8	13.6	16.0	18.3	28.2	31.2
Asperglaucide	61.5	54.0	56.4	54.5	55.5	54.3	55.2	56.5
Aurofusarin	59	67	46	63	62	53	257	190
Beauvericin	0.9	1.1	4.8	4.7	14.4	16.1	25.7	29.9
Brevianamid F	99	120	152	80	74	78	81	78
Butenolid	15.5	27.8	22.3	67.6	36.1	32.4	71.2	42.3
Chanoclavin	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	0.15	< LOD
Chrysogin	62	64	77	73	90	91	106	96
Culmorin	59	95	466	550	1426	1354	2346	2637
cyclo(L-Pro-L-Tyr)	286	322	397	278	253	268	285	242
Cytochalasin B	10.2	7.5	< LOD	< LOD	12.6	7.8	19.0	< LOD
Deoxynivalenol	136	211	828	895	2220	2284	4124	4097
DON-3-glucoside	16	12	65	74	196	204	423	391
Emodin	3.7	4.5	3.9	2.5	3.8	4.5	3.3	6.6
Enniatin A	2.2	2.4	1.8	1.9	1.3	2.0	1.1	1.3
Enniatin A1	6.3	6.7	6.9	7.2	5.5	9.6	7.2	8.3
Enniatin B	64	66	78	62	40	50	23	25
Enniatin B1	42	41	47	43	29	38	24	24
Enniatin B2	2.6	2.6	2.9	2.5	1.5	1.7	1.1	1.3
Enniatin B3	0.02	0.02	0.02	0.02	0.01	0.01	< LOD	0.01
Epiequisetin	0.50	0.18	0.22	0.15	0.09	0.27	0.25	0.14
Equisetin	1.12	0.30	0.40	0.36	0.30	0.37	0.06	0.21
Ergocornine	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	1.18	< LOD
Ergocorninin	0.21	< LOD	< LOD	0.30	0.29	0.31	0.90	0.56
Ergocristine	< LOD	37.03	< LOD	< LOD	< LOD	2.08	1.99	< LOD
Ergocristinine	< LOD	6.30	< LOD	< LOD	0.21	0.30	0.51	0.27
Ergocryptine	2.60	4.46	3.54	3.71	3.48	3.26	7.89	5.71
Ergocryptinine	< LOD	< LOD	< LOD	< LOD	1.08	< LOD	2.37	1.75
Ergometrinin	3.04	< LOD	7.41	3.82	< LOD	< LOD	< LOD	< LOD
Ergometrinine	0.32	0.30	0.41	0.25	0.31	0.20	0.25	0.36
Ergosin	< LOD	15.6	3.8	< LOD	1.0	1.0	1.6	3.0
Ergosinion	0.15	3.17	1.01	0.15	0.22	0.23	0.56	0.53
Ergotamine	< LOD	< LOD	42.82	< LOD	< LOD	< LOD	< LOD	< LOD
Ergotaminine	< LOD	< LOD	3.19	< LOD	< LOD	< LOD	< LOD	< LOD
HT-2 toxin	< LOD	< LOD	< LOD	< LOD	< LOD	6.04	< LOD	< LOD
Infectopyron	91	100	94	80	95	100	94	104

Lotaustralin	6.8	12.9	6.4	< LOD	5.6	5.1	2.9	3.3
Macrosporin	< LOD	0.42	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD
Moniliformin	8.4	12.2	9.0	9.1	5.9	6.4	3.6	3.5
Nivalenol	< LOD	< LOD	30	31	83	120	252	216
Rugulusovin	5.78	< LOD	< LOD	< LOD	< LOD	7.95	< LOD	6.86
Secalonic acid D	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	5.82	< LOD
T2-toxin	4.18	1.65	< LOD	< LOD	6.69	< LOD	< LOD	< LOD
Tentoxin	3.13	3.25	3.54	3.17	2.52	2.21	3.01	1.97
Zearalenone	0.7	1.3	2.6	4.5	9.9	11.5	23.8	21.0
Zearalenone-sulfate	< LOD	< LOD	< LOD	< LOD	< LOD	< LOD	2.40	< LOD

Table S1

	Diets					
	DON levels				Feed additive[a]	
	Control (n=12)[b]	Low-DON (n=12)[b]	Medium-DON (n=12)[b]	High-DON (n=12)[b]	FA (n=24)[b]	Non-FA (n=24)[b]
Exposure period (day 15-21)						
ADG (g day -1)[c]	720 ± 90	760 ± 110	704 ± 97	731 ± 69	706 ± 99	751 ± 85
ADFI (g day -1)[d]	1010 ± 204	1079 ± 109	959 ± 141	982 ± 146	974 ± 166	1041 ± 140
FCR[e]	1.39 ± 0.11	1.43 ± 0.11	1.37 ± 0.12	1.34 ± 0.11	1.38 ± 0.10	1.39 ± 0.13
Final Body Weight (kg)	27.1 ± 2.8	26.0 ± 3.3	26.2 ± 3.7	24.4 ± 3.6	26.5 ± 3.1	25.4 ± 3.7
Exposure period (day 22-28)						
ADG (g day -1)	792 ± 123	811 ± 127	750 ± 183	803 ± 149	767 ± 161	811 ± 127
ADFI (g day -1)	1203 ± 245	1287 ± 193	1162 ± 223	1252 ± 239	1164 ± 251	1289 ± 178
FCR	1.52 ± 0.17	1.59 ± 0.17	1.58 ± 0.23	1.56 ± 0.11	1.52 ± 0.17	1.60 ± 0.17
Final Body Weight (kg)	32.8 ± 3.2	31.7 ± 4.1	31.7 ± 4.3	29.6 ± 4.5	32.1 ± 3.6	30.8 ± 4.6

Table S2

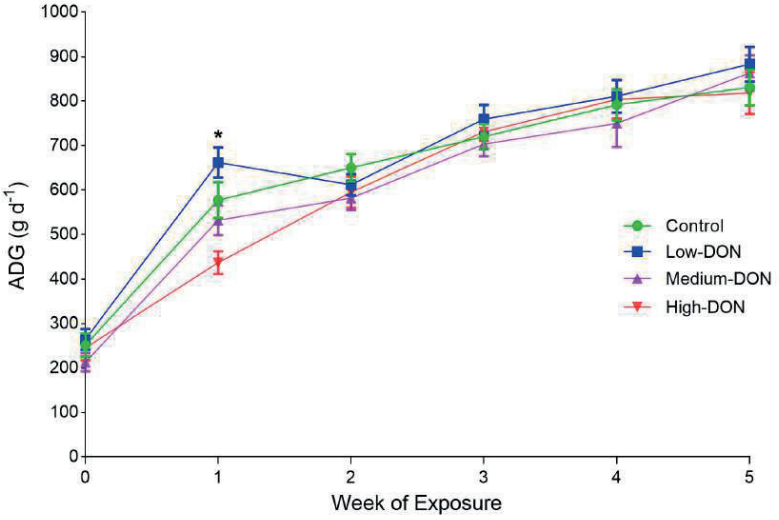

Figure S1

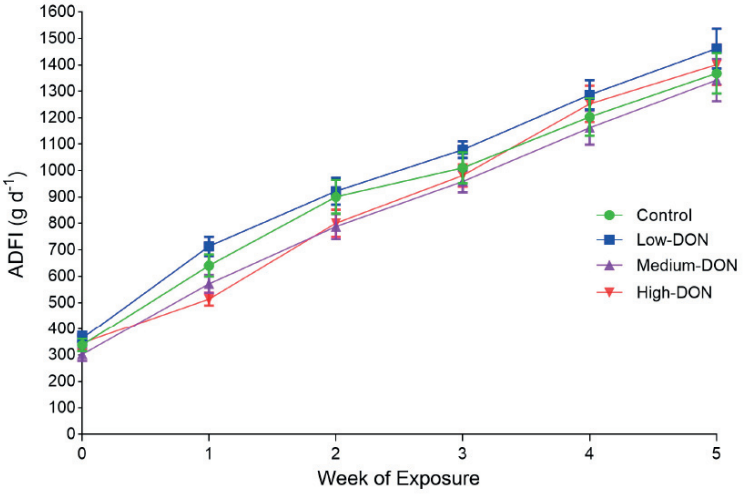

Figure S2

Paper II

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Porcine Health Management

RESEARCH Open Access

Effects of feeding naturally contaminated deoxynivalenol diets to sows during late gestation and lactation in a high-yield specific pathogen-free herd

Amin Sayyari*, Tore Framstad, Anette Kristine Krogenæs and Tore Sivertsen

Abstract

Background: The most prevalent *Fusarium* mycotoxin in grains is deoxynivalenol (DON). Contamination of swine feed with DON can result in reduced consumption and poor growth performance. Gestating and lactating sows need sufficient feed intake for fetus development during late gestation and milk production and body maintenance during lactation. Therefore, there is considerable concern in modern piglet production about the effects of DON contamination in sow feed. Most previous studies in sows have been done under experimental conditions, with DON levels ≥2.8 mg/kg feed. The aim of the current field trial was to investigate the effects of feeding grains that are naturally contaminated with more realistic levels of DON on sows during late gestation and lactation.

Methods: In a commercial, high-yield specific pathogen-free piglet production unit, 45 Norwegian Landrace × Yorkshire sows were fed three diets from 93 ± 1 days of gestation until weaning of the piglets, and average daily feed intake (ADFI), body weight (BW), production and reproduction performance, as well as sow blood parameters were recorded. Diets were made from naturally contaminated oats, with three concentration levels: 1) control (DON < 0.2 mg/kg), 2) DON level 1 (1.4 mg DON/kg), and 3) DON level 2 (1.7 mg DON/kg).

Results: Sows that were fed DON level 1 and 2 diets showed a 4–10% reduction in feed consumption during lactation, compared with sows in the control group. However, the DON-contaminated diets did not significantly affect sow BW or backfat thickness. Similarly, there were neither effects on production or reproduction performance, nor on blood parameters in the sows. The effects on skin temperature were variable.

Conclusion: Naturally contaminated diets with realistic, moderately increased DON levels, fed during late gestation and lactation in a modern high-yield piglet production farm, had limited effects on sow health and production.

Keywords: Deoxynivalenol, *Fusarium*, Sow, Production, Reproduction, Performance

Background

For many years, deoxynivalenol (DON) has been known as one of the most important trichothecene mycotoxins that commonly contaminates grain. Swine are particularly sensitive to DON exposure because of delayed excretion and poor detoxification [1]. Dose-related feed refusal, reduction in feed intake, and reduced weight gain of growing pigs have been associated with DON-contaminated diets [2, 3]. However, only a limited number of studies have researched the effects of feeding diets that are naturally contaminated with DON to sows during late gestation and lactation. Some of these studies did not show a significant effect of DON levels from 2.8 to 6.2 mg/kg feed; moreover, these levels did not affect feed intake, body weight (BW) change, or litter performance [4, 5]. Chavez [6], on the other hand, reported significantly higher BW loss in the sows that were fed diets contaminated with 3.3 mg DON/kg, while feed intake and litter performance were not affected. Diaz-Llano and Smith [7] and Diaz-Llano et al. [8] also reported significantly lower feed

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intake and higher BW loss during lactation when fed diets that contained 5.5 mg DON/kg [7] or 3.6 mg DON/kg [8], compared to the control groups. However, litter performance was not significantly affected [7]. Jakovac-Strajn et al. [9] reported lower feed intake and litter weight gain and longer duration of farrowing in sows that received diets containing 5 mg DON/kg, compared to control sows, while sow BW was not affected. Feeding lactating sows with diets containing up to 3 mg DON/kg decreased feed intake, decreased BW, and increased backfat loss, although litter performance and sow reproductive performance were not affected [10].

Most of these studies were conducted under controlled, experimental conditions, and with levels of DON contamination ≥2.8 mg/kg. However, in Norway, some farmers and veterinarians have reported suspected cases of negative DON-related effects on sow health and litter performance after intake of feed with more moderate DON levels [11]. In the last decade, average litter size and lactation performance of sows in Norway have increased substantially [12]. It is, therefore, a relevant question whether high-yielding sows in modern pig production may be more sensitive to moderate levels of DON than results from previous studies should indicate. Accordingly, we investigated the effects of diets that were naturally contaminated with realistic, moderately increased levels of DON on sows in late gestation and during lactation. We studied the effects on sow feed intake, BW development, litter gain, and reproduction performance in a field trial, conducted under practical conditions in a modern, high-yield piglet production unit. In addition, we studied the effect of different levels of DON contamination on hematology and biochemistry parameters, skin temperature, litter size, number of stillborn piglets, and production results in the subsequent litter.

Methods

Animals, housing, and management

The trial was done in a commercial specific pathogen-free (SPF) high-yield piglet production unit in southeastern Norway. Table 1 shows the key performance indicators of the farm in 2015. Norwegian SPF herds are free from specific swine diseases and parasites such as *Sarcoptes scabiei*, *Brachyspira hyodysenteriae*, *Brachyspira pilosicoli*, *Actinobacillus pleuropneumonia*, *Mycoplasma hyopneumoniae*, toxin-producing *Pasteurella multocida*, *Lawsonia intracellularis*, *Salmonella* sp. and *Ascaris suum*.

This trial was performed in a farrowing group of 47 Norwegian Landrace × Yorkshire sows for 53 days from December 2015 to February 2016. All sows were kept individually and without fixation in standard farrowing pens (7.0 m^2) with a piglet creep area (1.3 m^2), from approximately 3 weeks before expected farrowing until weaning. Each pen had a solid concrete floor, except for a slatted draining floor at one end of the pen (2.3 m^2). The sows were distributed based on parity numbers, to ensure even distribution of parity within groups.

Table 1 Key performance indicators of the piglet farm in 2015

Item	Value
Total pigs weaned	8898
Farrowing rate, %	83.6
Pigs born alive / Litter	14.1
Stillborn pigs / Litter	1.5
Pigs weaned / Litter	12.3
Average age at weaning, *days*	33
Average individual weaning weight, *kg*	11.6
Pre-weaning mortality, %	13.1
Average gestation length, *days*	115
Pigs weaned / sow /year	27.2
Litter / sow / year	2.22
Weaning to 1st service interval, *days*	5.3
Non-productive days / Litter	15

The sows were further divided into three feeding groups: (1) control (n = 16), (2) DON level 1 (n = 15), and (3) DON level 2 (n = 16). They were fed twice daily during gestation until 1 week postpartum, when feeding frequency was increased to three times daily, and four times daily from 2 weeks postpartum to weaning. The sows were offered a restricted amount of feed during late gestation, with a maximal allowance of 4 kg/day to each sow in this period. After farrowing, the amount of feed offered to each sow was gradually increased and the feeding automates adjusted to meet the requirements for a modified ad libitum feeding strategy. The amount of feed offered to each sow by the automated feeding system was adjusted continuously; in such a way that a small amount of feed was left over in the trough before the next feeding, as a confirmation that the sows had access to feed ad libitum. After weaning, the sows were removed from the farrowing unit and fed uncontaminated diets. All sows were offered 0.2 kg of hay daily. Both sows and piglets had access to water ad libitum. Farrowing occurred naturally, although under constant staff surveillance. Commercial husbandry procedures were performed within 48 h after birth, including teeth grinding and a 200-mg oral iron supplement (1.5 mL plus iron pasta, Felleskjøpet, Norway). Thereafter, a creep feed mixed with iron-fortified peat was offered to the piglets on the concrete floor of the piglet creep area until 4 days before weaning. Between 5 and 10 days after farrowing, a local veterinarian performed surgical castration using local anesthesia and analgesia. Necessary cross-fostering of piglets was performed within or across treatments within 48 h after farrowing and recorded. All litters were weaned on the same day, and average lactation length was 33.3 days (range 29–38 days).

Two sows (one from the control group and one from the DON level 2 group) were removed from the trial. The omitted sow from the control group died during the lactation period due to a penetrating gastric ulcer, and the other one was excluded from the study because the sow did not eat the experimental diet at all, and consequently received other feed. The final study therefore included 45 sows; 15 in each feeding group.

Origin of the naturally contaminated oats

The DON-contaminated oats that were used to produce the experimental diets in the present study were harvested in southern Sweden in 2013 and provided by Lantmännen, Sweden. The experimental diets in this study contained oats from the same DON-contaminated batch that was used in our recent study on growing pigs [13]. This batch of oats was analyzed for a wide range of mycotoxins, using a semi-quantitative multi-toxin screening method, at the Centre for Analytical Chemistry at IFA Tulln, Austria (Additional file 1: Table S1) [14].

Preparation of the experimental diets

From arrival in the farrowing unit, the sows were fed pelleted feed with different levels of naturally DON-contaminated oats (Table 2). The experimental diets were produced by a standard pelleting process by Felleskjøpet Agri, Lillestrøm, Norway (Production site: Trondheim, Norway), and were based on the formula of a commercial lactation diet (FORMAT, Felleskjøpet Fôrutvikling, Trondheim, Norway). The three diets were formulated to provide the following treatments: (1) control-diet (DON < 0.2 mg/kg), (2) DON-contaminated diet level 1 (DON = 1.4 mg/kg), and (3) DON-contaminated diet level 2 (DON = 1.7 mg/kg). We initially planned to run the trial with DON levels of approximately 0.5 mg/kg and 1 mg/kg, in the two DON-contaminated diets. The different dose levels were sought by blending the naturally contaminated oats with very low-contaminated oats, which were harvested in Norway in 2014. However, due to an unexplained factor in the feed production at the factory, the final contaminated diets contained higher and more similar levels of DON than originally planned.

Analysis of mycotoxins in experimental diets

The experimental diets were analyzed at the Norwegian Veterinary Institute for DON, DON-3-Glc, 3-ac-DON, and 15-ac-DON with a previously validated liquid chromatography-high resolution mass spectrometry method [15]. The limits of detection (LOD) in the feed matrix were 14 μg/kg for DON, 26 μg/kg for DON-3-Glc, 5.9 μg/kg for 3-ac-DON, and 52 μg/kg for 15-ac-DON. Increased DON concentrations were the dominant mycotoxin contamination recorded in the experimental feeds in the current study, and the other commonly occurring mycotoxins in Norwegian cereal grain were below the LOD or detected at insignificant levels. The hay provided to the sows was not analyzed for mycotoxin content. However, all the hay was of good quality, with no sign of fungal contamination.

Clinical examination and skin temperature

The animals were monitored for clinical diseases and disorders during the experiment. We recorded all diseases and treatments in both sows and piglets. Skin temperatures from the sow's ear were recorded upon arrival in the farrowing unit, 14 days after arrival, within 10–36 h after farrowing, on day 7 and 21 during the lactation period and at weaning, using a thermal imaging camera (FLIR i7, FLIR System, Inc., Wilsonville, OR, USA).

Feed intake and growth performance

In each farrowing pen, a plastic dispenser showed the amount of feed intake at each feeding. In this study, daily feed consumption refers to feed disappearance from the dispenser and was not adjusted for feed spillage or consumption by piglets.

Upon arrival at the farrowing unit, all sows were weighed individually, using a digital scale (EC2000, TEO teknikk AS, Nærbø, Norway) with a dimension of 1 × 2 m and an accuracy of 500 g. They were weighed again after 14 days, in late gestation period. Within 12–36 h after parturition, the sows and litters were weighed. Litter weight was measured using a bucket placed on a digital walk-on scale (Slim-Line small animal weight scale, Eickemeyer Medizintechnik für Tierärzte, KG, Tuttlingen, Germany) with a dimension of 90 × 55 cm and an accuracy of 100 g. Sows and litters were also weighed on days 7 and 21 of lactation and at weaning.

Backfat measurement and body condition score

Backfat measurements were done by a trained technician from Norsvin (Norwegian Pig Breeding Association). Due to practical limitation, backfat measurement was performed only twice: postpartum (1–7 days postpartum, as all sows did not farrow on the same day) and at weaning. Backfat was measured using RKU 10, a veterinary B mode ultrasound scanner (KAI XIN, Jiangsu, China), over the second-to-last rib of the sows. The measurements are presented in *mm*.

Body condition scores (BCS) were performed on all sows, using the scale developed by Animalia and Norsvin [16]. All sows were given a BCS ranging from 1 to 5, with half-point gradations: 1 is emaciated, 2 is thin, 3 is good condition, 4 is overweight, and 5 is obese. BCS was given upon arrival in the farrowing unit, after 14 days, 12–36 h after parturition, on days 7 and 21 during lactation, and at weaning.

Table 2 Experimental diet composition

	Experimental Diets			
Ingredients (as-fed basis)	Control	DON- Level 1	DON- Level 2	Unit
Barley, uncontaminated	18.8	18.8	18.7	%
Wheat, uncontaminated	25	25	25	%
Oats, uncontaminated	20	13.6	5.5	%
Oats, contaminated	0	6.4	14.5	%
Soybean meal	12	12	12	%
Rapeseed cake Mestilla	5	5	5	%
Corn gluten meal	1.04	1.04	1.04	%
Corn grits	2	2	2	%
Wheat bran meal	2	2	2	%
Molasses	2	2	2	%
Cossettes	3	3	3	%
Fish meal	1	1	1	%
Fish protein concentrate (by Scanbio)	3	3	3	%
Animal fat	3.05	3.05	3.05	%
Calcium carbonate (Limestone)	1.26	1.26	1.26	%
Monocalcium phosphate	0.6	0.6	0.6	%
Sodium bicarbonate	0.03	0.03	0.03	%
Salt	0.43	0.43	0.43	%
Sel-plex 1000	0.015	0.015	0.015	%
Selen premix	0.04	0.04	0.04	%
Mikromin. Swine	0.16	0.16	0.16	%
Vit.premix	0.06	0.06	0.06	%
Vitam-ADKB	0.07	0.07	0.07	%
L-lysin	0.26	0.26	0.26	%
DL-methionin	0.04	0.04	0.04	%
L-treonin	0.10	0.10	0.10	%
L-valin	0.01	0.01	0.01	%
Tryptophan	0.01	0.01	0.01	%
Betain	0.01	0.01	0.01	%
Digestrarom 1310	0.02	0.02	0.02	%
Ronozyme hiphos (0-50 g/t)	0.01	0.01	0.01	%
Vitamin E 50%	0.03	0.03	0.03	%
Biotin 10%	0.002	0.002	0.002	%
Calculated composition (as-fed basis)				
Protein	16.4	16.4	16.4	%
Fat SOX	5.58	5.59	5.59	%
Fat HCL	6.2	6.2	6.21	%
Water	12.3	12.3	12.3	%
Starch	34.8	34.8	34.8	%
Lysine	0.99	0.99	0.99	%
Methionine	0.3	0.3	0.3	%
Methionine + cysteine	0.62	0.62	0.62	%
Threonine	0.69	0.69	0.69	%

Table 2 Experimental diet composition *(Continued)*

	Experimental Diets			
Tryptophan	0.2	0.2	0.2	%
Ca	0.84	0.84	0.84	%
P	0.53	0.53	0.53	%
Na	0.23	0.23	0.23	%
NE	10.2	10.2	10.2	(MJ/kg)
Deoxynivalenol	< 0.2	1.4	1.7	(mg/kg)
3-ac-DON	< 0.01	0.14	0.17	(mg/kg)
15-ac-DON	< 0.05	≤ 0.05	< 0.05	(mg/kg)
DON-3-Glc	< 0.03	0.32	0.36	(mg/kg)

Litter and reproductive performance

On the day of parturition, we recorded gestation length and duration of farrowing (time between the first and the last piglet born). The number of total born, born alive, and stillborn piglets were recorded for each sow. Within 12–36 h after parturition, each litter of live piglets was weighed. Stillborn piglets in each litter were registered. Litters were also weighed and litter size was recorded on days 7 and 21 during the lactation period and at weaning. After weaning, we recorded the fate of all sows used in the trial, and the number of sows kept for further production that returned to heat after 5 days. Non-pregnant sows were culled and urogenital organs were examined for type of fertility disturbances. Finally, we recorded the number of conceptions at first service, and the number of live piglets born to the sows in the subsequent parity.

Clinical chemistry and hematology

Blood samples from the sows were taken from the milk vein (*v. subcutanea abdominis*) [16], using 9-mL heparin tubes for chemistry and 5-mL ethylenediaminetetraacetic acid (EDTA) tubes for hematology. Blood samples were taken upon arrival to the farrowing unit, 10 days after arrival, within 12–36 h after parturition, and at weaning. The EDTA tubes were kept refrigerated and delivered to the Central Laboratory at the Norwegian University of Life Sciences (NMBU), Faculty of Veterinary Medicine (Oslo, Norway), on the same day or in the next morning. Samples for plasma chemistry and biochemistry were prepared by centrifugation at 1500×g for 10 min at room temperature (approximately 20 °C), stored in 2-mL cryogenic vials (Nalgene, Nalge Company, Rochester, NY, USA), and delivered to the Central Laboratory.

For hematological analyses, we used ADVIA® 2120 Hematology System ADVIA® Multispecies software (Siemens Healthcare Diagnostics, Siemens AG, Erlangen, Germany), with the settings for swine species. The clinical biochemical analysis was performed using ADVIA 1800® Clinical Chemistry System (Siemens Healthcare Diagnostics, Siemens AG, Germany), and serum protein electrophoresis was performed on Sebia CapillarysTM 2 (Sebia, Norcross, GA, USA). The biochemical analyses included aspartate aminotransferase (AST), alkaline phosphatase (ALP), gamma-glutamyl transpeptidase (GGT), glutamate dehydrogenase (GLDH), creatine kinase (CK), C-reactive protein (CRP), total serum protein, total serum globulins, urea, creatinine, total bilirubin, cholesterol, glucose, inorganic phosphate, and calcium.

Blood samples from the sows and selected piglets were also collected for analysis of DON uptake, metabolism and vertical transmission. These results will be published in a separate paper.

Statistical analysis

After the trial, the analyses of DON concentrations in plasma indicated a mistake in the feeding of one of the sows in the DON level 2 group. The plasma concentrations of DON and its metabolites in this sow were below the LOD at 5 of 8 measurements. This sow was therefore excluded from all statistical analyses. Thus, the final dataset included 44 sows (15 in the control, 15 in the DON level 1, and 14 in the DON level 2), where 11 were first parity sows (4 in the control, 4 in the DON level 1, and 3 in the DON level 2), 12 were second parity sows (4 in each treatment), and 21 sows ranged from parity 3–6 (7 in each treatment).

All statistical analysis in this study was performed in JMP®, Version 10 (SAS Institute Inc., Cary, NC, USA). The level of significance was set to 0.05 in all models, and results with p-values between 0.05 and 0.1 were considered trends. If not otherwise specified, all results are expressed as means ± standard deviation (SD). The data were considered as a completely randomized block design with three treatments in 15 blocks. Each sow and its litter were considered as a random effect and represented an experimental unit for tested variables. The experimental diets were the independent variables. Growth performance parameters, skin temperature, and hematological and biochemical parameters were defined as dependent variables. The

normality of distribution was controlled by residual and predicted values plot, normal-percentile plots, and Shapiro-Wilk test. If the *p*-value in the Shapiro-Wilk test was over 0.05, data were considered normally distributed. Data that were not normally distributed were transformed or analyzed by non-parametric models, such as Wilcoxon rank-sum and Kruskal-Wallis tests. The same tests were performed when the data failed the assumptions of the analysis of variance (ANOVA). If the data generated from the application of ANOVA were significantly different, the post hoc, Tukey-Kramer honest significant difference (HSD) test was used for multiple comparisons and distinction of significant differences ($p < 0.05$). The Steel-Dwass test was used for multiple comparisons of the data generated from the application of Kruskal-Wallis test ($p < 0.05$).

Statistical differences between the treatment groups (control diet, DON level 1, and DON level 2 diets) for changes in sow weight, feed consumption, weight loss, litter weight, skin temperature, hematological and biochemical data, total backfat, and total litter gain were analyzed using a mixed effects model, with treatment, parity, and their interaction as fixed effects and individuals nested to treatments as a random effect. When appropriate, number of piglets born alive, number of weaned piglets, and lactation length were included in the models as covariates. Covariates and interactions that were not statistically significant were removed from the models by backward elimination.

Log-linear regression with normal distribution was used for data such as total number born, number born alive, and number of liveborn piglets in subsequent parity, with a Poisson distribution for the number of weaned piglets and negative binomial distribution for gestation length and duration of farrowing.

Results

We conducted a post-mortem investigation of the sow in the control group that died during lactation, and encountered penetrating gastric ulcers. There were no DON-related health issues, such as vomiting, diarrhea, or other pathological conditions, in any of the other sows during the trial.

Effects of experimental diets on feed consumption

During late gestation, ADFI of the sows were 3.76 ± 0.21, 3.36 ± 0.30 and 3.66 ± 0.33 *kg* in the control, DON level 1 and DON level 2 groups, respectively (Table 3). During lactation, sows fed the DON level 2 diet had significantly lower ADFI (6.12 ± 0.41 *kg*) ($p < 0.01$; Table 3) than the control group (6.76 ± 0.56 *kg*; Table 2). In the same period, sows receiving the DON level 2 diet had a non-significant lower ADFI than sows in the DON level 1 group (6.52 ± 1.20 *kg*) ($p = 0.33$) while there was no significant difference between the DON level 1 and the control groups (Table 3). The interaction between parity and treatment (DON) had a statistically significant effect on feed consumption during lactation, $F(10, 25) = 5.90$, $p = 0.0002$.

Effects of experimental diets on sow and litter performance and on subsequent reproduction

No statistically significant differences were found in sow weights at the start of the trial, 14 days after arrival, within 12–36 h after farrowing, on day 7, day 21, or at weaning (Table 3). The sows in the two DON groups had higher average weight losses during lactation compared to sows in the control group (Table 3). However, due to large individual variation, the group differences were not statistically significant. Similarly, the interaction between parity and treatment (DON) did not have a statistically significant effect on sow weight, $F(10, 26) = 1.35$, $p = 0.26$.

There were also no statistically significant differences between the treatment groups in backfat depth measured at 1–7 days postpartum and at the time of weaning. ($p > 0.05$). However, sows in the DON level 2 (5.5 ± 4.3 *mm*) and DON level 1 (4.3 ± 4.6 *mm*) groups had a higher average backfat loss at the end of the trial than the control group (2.5 ± 3.8 *mm*). Again, due to large individual variation, the group differences were not statistically significant (Table 3). Likewise, there were no statistically significant differences in BCS. The interaction between parity and treatment (DON) did not have a statistically significant effect on total backfat loss, $F(10, 26) = 1.07$, $p = 0.41$.

No statistically significant differences were found in gestation length or duration of farrowing, although, the mean duration of farrowing was 69 min longer in the DON level 2 group than the control group. The total number born, born alive, and weaned piglets did not differ between the groups (Table 4).

Litter weight at birth (12–36 h after parturition), day 7 and day 21 after parturition, and at weaning did not differ significantly between the treatment groups ($p > 0.05$). On average, the sows that received the DON level 2 diet had lower litter gain, but the difference was not statistically significant (Table 5).

The day after removal from the farrowing unit, one sow in the DON level 1 group died; also from a penetrating gastric ulcer. Four sows were sorted out for slaughter; due to age (2 sows), damaged teats (1) and lameness (1). Of the remaining sows, one in the control group did not return to heat in 5 days, and was sorted out on that basis. All the other sows returned to heat at 5 days post-weaning. The number of conceptions at first service was similar in all treatment groups, and there were no significant differences in the average number of liveborn piglets per sow in the subsequent litter ($p > 0.05$; Table 4). The results from pathological examination of urogenital organs of non-pregnant sows

Table 3 Effect of DON on sow growth performance and skin temperature

Item	Diets						Comparison *p*-values		
	1: Control (*n*=15)		2: DON-Level 1 (*n*=15)		3: DON- Level 2 (*n*=14)		1 vs. 2	1 vs. 3	2 vs. 3
	Mean	SD	Mean	SD	Mean	SD			
Average Daily feed intake (ADFI), *kg/day*									
During late gestation	3.76	0.21	3.36	0.30	3.66	0.33	–	–	–
During lactation [a]	6.76	0.56	6.52	1.20	6.12	0.41	0.83	0.01	0.33
Sow weight, *kg*									
Arrival farrowing unit	260	37	265	30	262	24	0.89	0.97	0.96
Day 14 after arrival [b]	296	38	301	33	303	25	0.93	0.95	0.99
12–36 h after farrowing	268	37	280	38	277	26	0.45	0.84	0.83
Day 7 after farrowing	260	36	273	38	265	26	0.44	0.99	0.54
Day 21 after farrowing	254	39	262	39	257	33	0.30	0.90	0.17
Weaning	251	42	258	40	255	34	0.83	0.99	0.81
Weight gain in late gestation [c]	36.5	5.8	36.7	7.2	40.6	7.6	0.99	0.29	0.29
Weight loss wk. 1–2 after farrowing	7.8	3.4	7.2	6.3	11.6	9.2	0.98	0.26	0.19
Weight loss wk. 2–3 after farrowing	6.3	12.0	10.8	6.4	8.57	12.6	0.47	0.91	0.73
Weight loss in lactation	17.2	17.8	22.1	14.9	22.3	18.9	0.72	0.71	0.99
Backfat rib, *mm*									
1–7 days after farrowing	14.6	5.9	16.9	5.9	18.3	5.3	0.33	0.19	0.79
weaning	12.1	5.0	12.7	3.6	13.5	6.7	0.53	0.95	0.99
Total backfat loss rib	2.5	3.8	4.3	4.6	5.5	4.3	0.51	0.16	0.71
Skin temperature, ℃									
Arrival farrowing unit	32.2	2.0	31.8	1.7	31.8	2.2	0.51	0.94	0.94
Day 14 after arrival	34.0	0.8	33.9	1.2	33.9	0.8	0.97	0.65	0.97
12–36 h after farrowing	35.2	1.2	35.0	1.3	33.8	1.5	0.67	0.02	0.24
Day 7 after farrowing	34.2	1.5	34.5	1.0	34.1	1.0	0.93	0.75	0.46
Day 21 after farrowing	35.3	1.1	35.3	1.2	35.5	0.8	0.99	0.97	0.98
Weaning	35.9	1.0	35.9	0.6	35.1	0.8	0.94	0.06	0.03

[a]One sow in control was excluded from the final analysis of this variable
[b]Two sows in control were excluded from the final analysis of this variable
[c]Two sows in control and one sow in DON-level 2 were excluded from the final analysis of this variable

revealed endometritis due to infection with *Escherichia coli* or *Staphylococcus hyicus* in 3 sows from the control, 2 sows from DON level 1 and 2 sows from DON level 2.

Regardless of treatment and parity, a negative correlation was observed between the sows' total feed consumption and weight loss during lactation ($R = -0.39$, $p < 0.01$). Several interactions were positively correlated (Table 6): total feed consumption during lactation and litter weaning weight ($R = 0.28$, $p = 0.06$); sows' total weight loss during lactation and litter weaning weight ($R = 0.44$, $p < 0.01$); and sows' total weight loss during lactation and backfat loss ($R = 0.47$, $p < 0.01$).

Effects of experimental diets on skin temperature

There were no significant differences in skin temperature between sows fed experimental diets and the control, throughout most of the experiment. However, sows in the DON level 2 group had a significantly lower skin temperature (33.8 ± 1.5 °C; $p < 0.05$) than sows in the control group (35.2 ± 1.2 °C) immediately after farrowing. Sows in the DON level 2 group also had a significantly lower skin temperature (35.1 ± 0.8 °C; $p < 0.05$) than sows in the DON level 1 group (35.9 ± 0.6 °C) at weaning. Additionally, they had a tendency ($p = 0.06$) to have a lower temperature than the control group (35.9 ± 1.0 °C) at weaning (Table 3).

Effect of experimental diets on hematological and biochemical parameters

Hematological parameters, including red and white blood cell counts, hematocrit, hemoglobin, and platelets did not change with DON level or time.

Table 4 Effect of DON on sow reproductive performance

Item	Diets						*p*-value
	Control		DON- Level 1		DON- Level 2		
	Mean	SD	Mean	SD	Mean	SD	
Reproduction							
Gestation length, *days*	114.53	1.13	115.13	1.06	115.07	1.68	0.99
Duration of farrowing, *minutes* [a]	448	190	454	138	517	208	0.54
Time interval between birth of each piglet, *minutes* [a]	33	25	34	22	36	18	0.74
Total born, *n*	16.9	5.1	15.9	4.0	15.4	4.7	0.67
Born alive, *n*	15.3	4.5	14.7	3.3	13.6	4.2	0.56
Weaned, *n*	11.6	2.1	12.9	0.9	11.0	2.1	0.34
Sows returning to heat at 5 days after weaning, *n* [b]	13	–	14	–	11	–	–
Conceptions at first subsequent service, *n* [b]	10	–	12	–	9	–	–
Born alive subsequent litter, *n*	16.9	3.0	15.3	4.2	14.7	2.6	0.33

[a]One sow in DON-level 2 was excluded from the final analysis of this variable
[b]Total number of sows returning to heat and total number of conceptions

No differences were observed between the groups, throughout the experiment, in any of the measured biochemical parameters.

Discussion

Feed consumption

In the present study, the sows fed contaminated diets had a lower feed consumption rate during lactation than the control group did. This finding is in line with those of Diaz-Llano and Smith [7], Diaz-Llano et al. [8], Jakovac-Strajn et al. [9], and Herkelman et al. [10], who found reduced feed intake in sows fed diets with higher DON levels (3 to 5.5 mg/kg). Diaz-Llano et al. [8] observed a reduction in feed intake only during lactation. Our results contrast those of Chavez [6], who reported that feed intake of gilts was not affected by diets containing up to 3.3 mg/DON kg. In that study, the feeding was restricted in late gestation (2.3 kg/day) and ad libitum during lactation. The results of the present study also diverge from those of Friend et al. [4], Diaz-Llano and Smith [17], and Gutzwiller [5], who reported that restricted feeding of gilts, with diets containing 2.8 to 6.2 mg DON/kg, did not reduce feed consumption during gestation or lactation. Given that the feeding was restricted (up to 4 kg/day) in late gestation in the present study, the differences in ADFI between sow groups in this period should be treated with considerable caution. Thus, we have not analyzed these differences statistically. The ADFI in late gestation was strongly influenced by the feeding method, as the sows were offered a standard amount of feed, according to the routines of the farmer. Therefore, the differences recorded in feed intake in this period do probably not reflect the effect of DON content in the feed.

When comparing results from different feeding experiments, one should consider that the recorded effects of DON-contaminated feed in pigs may be influenced by different feeding strategies [18]. Some studies reported in sows have used restricted feeding in both gestation and lactation, while the other studies have used a combination of restricted feeding during late gestation and ad libitum feeding during lactation. In the present study, we have used a restricted feeding during late gestation and a modified ad libitum feeding strategy, using a semi-automatic feeder during lactation.

Table 5 Effect of DON on sow productive performance

Item	Diets						Comparison *P*-values		
	1: Control		2: DON- Level 1		3: DON- Level 2		1 vs. 2	1 vs. 3	2 vs. 3
	Mean	SD	Mean	SD	Mean	SD			
Production									
Litter weight at birth, *kg*	21.1	3.6	20.5	2.7	21.6	4.5	0.37	0.74	0.14
Litter weight day 7, *kg*	34.7	7.0	35.3	3.3	32.9	8.2	0.94	0.58	0.52
Litter weight day 21, *kg*	83.3	14.7	88.1	10.7	81.4	16.9	0.85	0.99	0.81
Litter weaning weight, *kg*	138	26	142	12	128	27	0.24	0.93	0.41

Table 6 Correlations between feed consumption, weight loss, litter weaning weight, and backfat loss during lactation

Variable	1	2	3	4
1. Feed consumption, *kg*	1.00			
2. Weight loss sow, *kg*	−0.39 **	1.00		
3. Litter weaning weight, *kg*	0.28	0.44**	1.00	
4. Total backfat loss sow, *mm*	−0.15	0.47**	0.16	1.00
Mean	229.1	20.5	136.2	4.1
SD	32.5	17.0	22.8	4.3
Range	(138.0-278.1)	((−13.0)-49.0)	(82.7-175.8)	((−5.0)-16)

**$p < 0.01$

Furthermore, one cannot exclude the possibility that the feed consumption results in the current study were partially affected by feed wastage, since feed consumption was defined as feed disappearance without adjusting for feed spillage or the amount of feed that may have been eaten by piglets. However, the feeders were inspected several times a day and significant feed spillage was not observed.

Sow and litter performance

There were no significant differences in average sow BW between the treatment groups throughout the experiment. On average, the sows that received the contaminated diets had a higher weight loss during lactation than the control sows, but the difference was not statistically significant. The lack of significant effect of DON on sow BW changes in the current study concurs with Friends et al. [4], Jakovac-Strajn et al. [9], and Gutzwiller [5]. They found that feeding gestating and lactating sows with diets containing higher DON levels (2.8 to 6.2 mg/kg) had no significant effect on BW changes. However, our findings differ from Chavez [6], who found a significant reduction in BW gain in sows fed a diet containing 3.3 mg/DON kg during gestation, and significantly greater BW losses in sows fed diets containing 1.3 and 2.4 mg DON/kg than controls during lactation. Diaz-Llano and Smith [7, 17] also reported that sows fed diets containing 5.5 mg DON/kg showed a reduction in BW gain during the last 3 weeks of gestation and greater BW losses during lactation. In another experiment by Diaz-Llano et al. [8], feeding diets containing 3.6 mg DON/kg to sows during gestation and lactation resulted in significantly greater BW losses compared to controls during lactation, while BW gain was not affected during late gestation. Our findings differ also from Herkelman et al. [10], who reported a significant reduction in sow weaning weight and significant higher BW losses in sows fed diets containing 3 mg DON/kg during lactation.

Some previous studies have shown that a high concentration of DON (6.2 mg/kg) exhibited no effect on BW [4], while a lower level of DON (3 to 3.6 mg/kg) reduced BW in the lactating sows [8, 10]. This variability in results may be due to the presence of other mycotoxins in Diaz-Llano et al. [8]. However, in Friend et al. [4] and in the current study, increased DON concentrations were the dominant mycotoxin contamination recorded in the experimental feeds.

In the current study, the backfat thickness of the sows during lactation was measured as an additional indicator of feed efficiency and body condition [19, 20]. We are only aware of one previous study that measured the effect of DON on sow backfat [10]. Our results showed that diets with 1.4 and 1.7 mg DON/kg did not have a statistically significant effect on backfat thickness, although the average losses in backfat thickness in the sows fed contaminated feeds were somewhat higher than in the controls. These results are in accordance with our findings on BW changes in sows. Our findings are in contrast with the results from Herkelman et al. [10], who found significant higher backfat losses in sows fed diets containing 3 mg DON/kg during lactation.

The dietary treatments in the present study did not have significant effect on litter performance. This finding indicates that milk production of the sows was not affected by the treatment diets. This concurs with similar previous studies [4–7, 10, 17]. However, Jakovac-Strajn et al. [9] showed that litter weight gain in the group receiving 5 mg DON/kg was significantly lower than the control. In the same experiment, farrowing was significantly longer in sows receiving 5 mg DON/kg than in the control sows.

The positive correlation between total weight loss during lactation and litter weaning weight, regardless of treatment and parity, was also reported by Thingnes et al. [21]. In the case of feeding sows with DON-contaminated diets, the positive correlation between these two parameters may have contributed to a reduction in the statistical significance of the DON-related effects. In lactation, there is a priority to produce milk over maintenance of sow body tissues [22], but there is considerable individual variation. The reduction in ADFI that was induced by DON-contaminated feed in this study may have led to a reduction in BW in some of the sows, but affected milk production and litter performance more strongly in others. Taken together, this may have reduced the effect of the DON-contaminated feed on both parameters, statistically.

A disturbance of the normal weaning-to-service interval has a considerable negative impact on results in modern piglet production units, because of the use of group farrowing. In the current study, DON-contaminated diets had no effect on the subsequent number of sows that returned to heat at 5 days post-weaning. This finding is in line with the limited, non-significant effect of DON-contaminated diets on BW loss during lactation. Gutzwiller [5] also found no effect of a diet containing 2.8 mg DON/kg on the weaning-to-service interval. However, Diaz-Llano and Smith [7] reported that sows fed contaminated diets with 5.5 mg DON/kg had a trend (p = 0.09) toward a longer weaning-to-service interval than the controls, in line with the higher BW losses observed in that experiment.

Skin temperature

We recorded sow skin temperature as an indicator of the clinical and physiological effects on the animals. Soerensen and Pedersen [23] reviewed using skin temperature for monitoring pig health. In the present study, the sows that received the DON level 2 diet had significantly lower skin temperature than the control group immediately after farrowing. Additionally, their temperature was significantly lower than the DON level 1 group at weaning. However, for all the other skin temperature recordings, no differences between the feeding groups were observed. We cannot exclude the possibility that the significant differences that we found immediately after farrowing and at weaning were accidental. However, sows might have been more sensitive to the effects of DON on skin temperature around farrowing than during the lactation [24]. In some previous studies, feeding weaning pigs with diets containing 3 mg DON/kg resulted in lower skin temperature than in the control pigs [25, 26].

Hematological and biochemical parameters

There are few reports concerning the hematological and biochemical effects of DON-contaminated diets on gestating and lactating sows. However, DON-related changes in blood parameters have been studied more thoroughly in growing pigs [2, 13]. Our study did not detect any effects of DON on the evaluated hematological and biochemical parameters. Our results concurred with Diaz-Llano and Smith [17], who fed gilts in late gestation with 5.5 mg DON/kg. In contrast, serum urea concentration was reduced in sows that were fed diets with 3.6 and 5.5 mg DON/kg during late gestation and lactation [7, 8].

Conclusion

In summary, we observed reduced ADFI during lactation in sows that were fed naturally contaminated diets (up to 1.7 mg DON/kg). However, this level of contamination did not have statistically significant effects on sow BW changes during gestation and lactation. Likewise, there were neither effects on production or reproduction performance, nor on blood parameters of the sows.

The highest level of DON contamination in the feed that was used in our study was lower than in most of the previous studies. Nevertheless, it was about twice that of the recommended maximum acceptable level for DON (0.9 mg/kg), according to European Commission Recommendation 2006/576/EC, and more than three times higher than the maximum level (0.5 mg DON/kg) recommended by the Norwegian Food Safety Authority. One of our objectives was to investigate whether sows in a modern, commercial, high-yield piglet production unit might be more sensitive to the effects of DON-contaminated feed than previously documented under other experimental conditions. Our results do not give any indication in that direction. On the contrary, the effects of naturally contaminated feed with 1.4 and 1.7 mg DON/kg were not stronger than the effects previously observed in fattening pigs that were fed similar DON levels [2]. Whether moderate DON levels may have other, more subtle effects on sow health and performance in the long run, is a natural question for further research.

The current study was conducted in a SPF piglet production unit. The sows' health was particularly good, since they were free from many common pathogens. The SPF status of our study herd might have enhanced sow resistance to DON-related toxicity. This issue may also require future study.

Additional file

Additional file 1: Table S1. Toxin contents in the oats used for the production of the experimental diets, as measured by multi-toxin LC-MS/MS by the Centre for Analytical Chemistry at IFA Tulln, Austria. (DOCX 34 kb)

Abbreviations

ADFI: Average daily feed intake; ALP: Alkaline phosphatase; ANOVA: Analysis of variance; AST: Aspartate aminotransferase; BCS: Body condition scores; BW: Body weight; CK: Creatine kinase; CRP: C-reactive protein; DON: Deoxynivalenol; EDTA: Ethylenediaminetetraacetic acid; GGT: Gamma-glutamyl transpeptidase; GLDH: Glutamate dehydrogenase; HSD: Honest significant difference; NMBU: Norwegian University of Life Sciences; SD: Standard deviation; SPF: Specific pathogen-free

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Availability of data and materials
The datasets generated and/or analyzed during the current study are not publicly available due to protection of privacy and details of production of the farmer, but are available from the corresponding author on reasonable request.

Authors' contributions
AS, TS, TF and AKK designed the study. AS, TS and TF visited the farm and performed data collections. AS, TF and AKK participated in post-mortem reproductive investigation. AS did the analysis of data and statistics. AS wrote the paper. All authors reviewed, read and approved the final manuscript.

Ethics approval
The study was conducted in accordance with Norwegian regulations for animal testing (FOR-2015-06-18-761), which comply with EU Directive 2010/63/EU. A detailed report was evaluated and confirmed approvable by the Norwegian Animal Research Authority after completion. The paper is written in accordance with the ARRIVE guidelines for reports of animal experiments.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Paper III

Transfer of Deoxynivalenol (DON) through Placenta, Colostrum and Milk from Sows to Their Offspring during Late Gestation and Lactation

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Abstract: Deoxynivalenol (DON) contamination of feed may result in reduced growth, feed refusal, immunosuppression, and health problems in swine. Piglets can be exposed to DON via placenta before birth and via milk during lactation. The extent of early-life exposure of piglets to DON is, however, not fully known. This study was therefore aimed at investigating the DON uptake in sows fed with naturally contaminated diets, DON transfer across placenta during late gestation and transfer of DON to piglets via colostrum and milk. Forty-five crossbred sows were fed from day 93 ± 1 of gestation until weaning of the piglets with feed made from naturally contaminated oats, with DON at three concentration levels: (1) control (DON < 0.2 mg/kg); (2) DON level 1 (1.4 mg DON/kg); and (3) DON level 2 (1.7 mg DON/kg). The transfer of DON to the piglets was evaluated in 15 of the sows, with repeated sampling of blood and milk from the sows and blood samples from five piglets of each litter. The piglet/sow plasma DON ratio and milk/plasma (M/P) DON ratio in sows were calculated to estimate the degree of transfer. Piglet/sow plasma ratios were 2.14 at birth, 2.30 within 12–36 h after parturition, 0.08 on day 7, 0.16 on day 21 and 0.20 at weaning. M/P ratios were 0.92, 1.11, 0.94, 1.21 and 0.90 at the same time points. The results indicate that DON is efficiently transferred across placenta and into milk. However, the low piglet/sow plasma ratios at mid-lactation to weaning indicate that the piglets were most strongly exposed to DON in early life, despite the high M/P ratios and efficient secretion of DON in milk over the entire lactation.

Keywords: deoxynivalenol; placental transfer; lactational transfer; piglets; sows

Key Contribution: New-born and suckling piglets of sows fed with diets naturally contaminated with DON are exposed to this mycotoxin through the placenta in the late gestation, colostrum in the first day of life and transitory and mature milk during lactation. Our data show that the DON exposure of the piglets is highest in early life.

1. Introduction

Mycotoxins are secondary metabolites of fungi, which can cause a variety of adverse effects on animals and humans [1,2]. One of the most common mycotoxins produced by *Fusarium* fungi is the trichothecene deoxynivalenol (DON) [1]. DON is produced mainly by *F. graminearum* and *F. culmorum,* and is the most commonly detected trichothecene worldwide [3]. This mycotoxin can contaminate wheat, oats, maize, and barley, and is known to cause significant economic losses in

farm animal production, particularly in pigs [4]. The following rank order of sensitivity, pigs > mice > rats > poultry ≈ ruminants, shows that susceptibility to DON differs between animal species [2,5]. This is in part related to differences in metabolism, absorption, distribution, and elimination of DON among different animal species [2]. Other factors that can be involved in differences in the severity of DON-related responses and susceptibility are age and sex [6,7]. Young pigs fed contaminated diets with DON had a greater reduction in weight gain compared with older animals [7]. This may be associated with a reduced capacity of DON metabolism and detoxification in the liver and gastrointestinal tract in younger animals [7]. A meta-analytical study of mycotoxins in pigs fed DON-contaminated diets showed that male pigs were more sensitive than females when growth performance was evaluated [7]. DON-related adverse effects vary from acute symptoms such as emesis, diarrhea and abdominal pain to subacute and chronic effects, including reduced feed intake and body weight (BW) [2]. Immunotoxic, neurotoxic and reprotoxic effects of DON are also of particular concern [3]. Evidence for direct effects on the foetus, including embryo-foetal toxicity, foetal malformation and developmental disorders following maternal DON exposure, indicates a substantial risk for vertical DON transfer [8,9]. Understanding DON exposure during prenatal development and in early life has received much attention in the past decade, and has emphasized the need for knowledge about vertical transmission of DON. The placental transfer of DON has been observed both in vitro using cell lines and in ex vivo studies [10,11]. However, only a few in vivo studies have investigated the transfer of DON across the placenta in pigs. Following feeding pregnant sows at mid-gestation (day 35–70) with diets containing 4.42 mg DON/kg feed, DON passed the placental barrier and was detected in fetal physiological samples, including urine, bile, serum, liver and kidney [12]. In another experiment, the exposure of pregnant sows in the last third of gestation (day 75–110) with diets containing 9.57 mg DON/kg feed resulted in noticeable DON concentrations in urine, bile and serum of the developing fetus, and serum concentrations of DON in piglets and sows were nearly similar [13]. It has been suggested that specificities in the placental structures in different species as well as the time of exposure during gestation may influence the extent of transplacental DON transfer [13].

Previous studies on DON transfer from dams to progeny have mainly focused on the placental transfer of DON. We are, however, not aware of any previous study on DON transfer from sow plasma into sow milk and the resulting lactational transfer to new-born piglets. In contrast, there are some reports on the transfer of feed-borne mycotoxins and their metabolites into the milk of dairy cattle [14,15]. Feeding dairy cows with contaminated diets containing 8.21 mg DON/kg resulted in very low levels of DON in the milk due to a rapid biotransformation of DON to de-epoxy DON (DOM-1), a less toxic metabolite, in the bovine rumen [15]. The microbial breakdown of trichothecenes in the rumen leads to significant lower DON uptake in ruminants than in pigs and other monogastric species. Consequently, higher DON plasma levels in pigs may contribute to a higher transfer rate into sow milk as compared with cow milk [2].

Against this background, it was the aim of the present experiment to study DON uptake in sows fed naturally contaminated feed during late gestation and lactation, and the transfer of DON from sows to foeti/new-born piglets and suckling piglets. Additionally, the transfer rates of DON from sow plasma to the sow milk were determined.

2. Results

2.1. DON-Uptake in Sows

2.1.1. DON and DON Derivatives in Sow Feed

DON was detected at concentrations of 1.4 and 1.7 mg/kg in the DON-level 1 and DON-levels 2 diets, respectively. The levels of 3-ac-DON were 0.14 and 0.17 mg/kg in the DON-level 1 and DON-levels 2 diets, respectively. DON-3-Glc was detected at concentrations of 0.32 and 0.36 mg/kg in the DON-level 1 and DON-level 2 diets, respectively. The levels of 15-ac-DON were below the LOD (0.05

mg/kg) in both DON-contaminated groups. The concentrations of DON, DON-acetates and DON-3-Glc were all below the LOD (0.2 mg/kg for DON, 0.01 mg/kg for 3-ac-DON, 0.05 mg/kg for 15-ac-DON and 0.03 mg/kg for DON-3-Glc) in the control feed.

Considering the mean feed intake and pig weights including gain during the study, the approximate average DON doses (μg/kg BW/day) in the exposed groups were calculated for the gestation and lactation periods (Table 1). The estimated doses were not constant throughout the study, due to the changes in BW and feed intake during gestation and lactation. One should particularly be aware that the feed consumption of sows is considerably higher during lactation compared to late gestation [16].

Table 1. Daily DON doses in the sows during late gestation and lactation.

Diets		Late Gestation	Lactation
		Mean ± SD [a]	Mean ± SD
Control (*n* = 15)	Body Weight [b]	290 ± 40	251 ± 42
	ADFI [c]	3.8 ± 0.2	6.6 ± 0.7
	DON intake [d]	<0.8	<1.3
	DON dose [e]	<2.6	<5.4
DON level 1 (*n* = 15)	Body Weight	301 ± 34	258 ± 40
	ADFI	3.4 ± 0.3	6.5 ± 1.2
	DON intake	4.7 ± 0.4	9.1 ± 1.7
	DON dose	15.7 ± 1.5	35.4 ± 3.3
DON level 2 (*n* = 14)	Body Weight	303 ± 25	255 ± 34
	ADFI	3.7 ± 0.3	6.1 ± 0.4
	DON intake	6.2 ± 0.5	10.3 ± 0.9
	DON dose	20.6 ± 1.9	40.9 ± 5.0

[a] Standard deviation; [b] Mean body weight (kg); [c] Average daily feed intake (kg/d); [d] Daily intake of DON in feed (mg/d): ADFI × DON conc. in feed; [e] Daily DON dose (μg/kg BW/d).

2.1.2. DON and DON Metabolites in Sow Plasma

The measured plasma concentrations of DON, DON-3-GlcA and DON-15-GlcA of 44 sows sampled at different time points during the study are presented in Figure 1. In the samples taken at arrival in the farrowing unit, DON concentrations were below 0.1 ng/mL, in all sows. During the experimental period, there was a gradual increase in the mean DON plasma levels, in both groups fed with contaminated diets. The same was true for the levels of both DON-glucuronides in the DON level 1 group, while in the DON level 2 group, the mean DON-glucuronide levels decreased noticeably from day 21 in lactation until weaning (Figure 1). The plasma concentrations of DOM-1 were below the LOD in all sows at all sampling points.

The mean plasma concentrations of DON and DON glucuronides in the combined DON level 1 and 2 groups are shown in Table S1. The sow glucuronidation rates were calculated as the sum of plasma DON-3-GlcA + DON-15-GlcA concentrations divided by total DON (sum of plasma DON + DON-3-GlcA + DON-15-GlcA concentrations). The mean glucuronidation rates were 69%, 75% and 67%, respectively, at 10 days after arrival, on day 21 in the lactation, and at weaning.

The transfer rate of DON from the diet to sow plasma was calculated as plasma/diet ratio (plasma DON concentration divided by the DON concentration in feed). The resulting median plasma/diet ratios of the DON level 1 and DON level 2 groups were, respectively, 0.003 and 0.003 at 10 days after arrival, 0.004 and 0.004 on day 21 in the lactation period, and 0.004 and 0.003 at weaning.

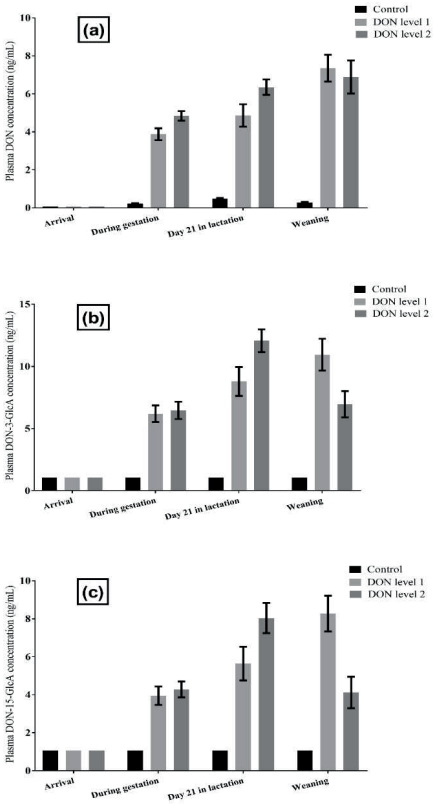

Figure 1. Effect of the experimental diets on plasma concentrations of DON (**a**), DON-3-GlcA (**b**) and DON-15-GlcA (**c**) in sows (n = 44) over the course of the exposure study. Error bars indicate the standard error of the mean (SEM).

2.2. DON Transfer from Sows to Piglets

2.2.1. DON Plasma Concentrations in Sows in the Transfer Study

DON was detected in almost all plasma samples from the sows included in the transfer study and fed with DON-contaminated diets (Table 2a and Figure S1a). In the control group, plasma DON concentrations higher than the LOD were measured at several time points in some sows (Table 2a). The highest DON plasma concentrations were found in the sows fed DON level 1 diet at weaning (Table 2a). During the lactation period, the mean DON concentrations in the two exposure groups were increasing toward weaning and concentrations at weaning were ~5 times higher than concentrations at farrowing.

DON-3-GlcA and DON-15-GlcA were detected in almost all plasma samples from the sows fed with DON-contaminated diets (Figure S1b, c). In the control group, plasma concentrations of DON glucuronides were below the LOD at all time points. The plasma concentrations of DON glucuronides were increasing from farrowing to weaning. The highest concentrations of DON glucuronides were found in sows fed DON level 1 diets at weaning. DOM-1 was not detected in any sow plasma sample.

2.2.2. Piglet Survival and Growth Performance

In total 75 new-born piglets were included, born by the 15 sows included in the transfer study (4 from the control group, 6 from DON level 1 group and 5 from DON level 2 group). Nine piglets died during the experiment: 3 piglets died within 24 ± 8 h after parturition, 4 died between day 1 and day 7 after parturition, 1 died between day 7 and day 21, and 1 piglet died shortly before weaning. The data on the effects of DON-contaminated diets on piglet weight during the experimental period are shown in Table 3. There was a statistically significant effect of time on piglet weight, F (3, 191) = 1366, $p < 0.0001$. However, the interaction between time and treatments (DON) did not have a statistically significant effect on this variable F (6, 191) = 1.4, $p = 0.22$. No sex-dependent differences were found in the piglet BW.

Table 3. Effect of diet on piglet growth performance.

		Diets		
Items	***n***	**Control** [a]	**DON Level 1** [a]	**DON Level 2** [a]
Piglet body Weight (kg)				
48 ± 12 h after parturition	71	2.2 ± 0.4	1.8 ± 0.3	1.8 ± 0.4
Day 7	68	3.1 ± 0.8	2.7 ± 0.5	2.9 ± 0.8
Day 21	67	7.4 ± 1.8	7.1 ± 1.2	7.2 ± 1.6
Weaning	66	11.9 ± 2.4	10.7 ± 1.6	11.0 ± 2.1
Average weaning age (d)		35	33	33
Total average daily gain (g/d)		297 ± 67	290 ± 48	292 ± 59
		(n = 18)	(n = 27)	(n = 21)

[a] Mean ± SD.

2.2.3. DON and DON Metabolites in Piglet Plasma

DON was detected in all plasma samples from piglets born and nursed by sows fed with DON-contaminated diets (Table 2a and Figure S2). In the control group, plasma DON concentrations close to the LOD were measured at several time points in some piglets. The highest DON plasma concentrations were found in the DON level 1 and 2 groups at birth (Table 2a). During the lactation period, the mean DON concentrations in the two exposure groups decreased by 52% from birth to 12–36 h after parturition, by 89% until day 7 and by 74% until day 21. At weaning, the DON plasma concentrations were again slightly higher and were 43% lower as compared to new-borns.

The plasma concentrations of the metabolites DON-3-GlcA and DON-15-GlcA were below the LOD at most sampling points. At weaning, DON glucuronides were detected in the plasma of a few piglets at low levels. DOM-1 was not detected in any piglet plasma sample.

Table 2. (**a**) Plasma concentrations of DON and ratios between matched sows (n = 15) [a] and piglets (n = 75) [a] plasma over the course of experiment; (**b**) Piglet/sow plasma ratios for the combined results of the DON level 1 and DON level 2 groups [b].

(a)

Sampling point	10 days after arrival			At farrowing			Day 1			Day 7			Day 21			Weaning		
Experimental Diets	**Con**	**level 1**	**level 2**	**Con**	**level 1**	**level 2**	**Con**	**level 1**	**level 2**	**Con**	**level 1**	**level 2**	**Con**	**level 1**	**level 2**	**Con**	**level 1**	**level 2**
Sow plasma (ng/mL)																		
Mean ± SD	0.22 ± 0.04	3.71 ± 1.81	4.90 ± 0.57	0.10 ± 0.04	1.79 ± 1.12	1.13 ± 0.90	<LOD	2.65 ± 0.70	0.81 ± 0.76	0.24 ± 0.05	4.22 ± 1.14	3.67 ± 0.37	0.46 ± 0.05	4.31 ± 2.65	5.70 ± 0.69	0.30 ± 0.11	9.06 ± 1.57	7.15 ± 1.99
Median	0.22	3.98	4.79	0.09	1.77	0.96	<LOD	2.88	0.53	0.23	3.96	3.56	0.46	4.52	6.02	0.27	9.42	7.27
Range	0.17–0.25	0.48–5.52	4.36–5.66	<LOD–0.16	0.68–3.71	0.23–2.38	<LOD	1.67–3.50	<LOD–1.69	0.18–0.30	3.03–6.06	3.20–4.16	0.41–0.53	0.52–7.82	4.80–6.44	0.20–0.45	7.2–11.4	5.0–10.1
Piglet plasma (ng/mL)																		
Mean ± SD	–	–	–	0.47 ± 0.28	2.97 ± 0.85	2.35 ± 0.65	0.14 ± 0.12	0.94 ± 1.11	1.62 ± 0.20	<LOD	0.26 ± 0.13	0.32 ± 0.18	0.12 ± 0.11	0.80 ± 0.43	0.58 ± 0.16	0.11 ± 0.09	1.69 ± 1.39	1.20 ± 1.41
Median	–	–	–	0.41	2.69	2.26	<LOD	0.73	1.61	<LOD	0.25	0.32	<LOD	0.71	0.51	<LOD	1.05	0.61
Range	–	–	–	<LOD–1.61	1.74–4.74	1.34–3.43	<LOD–0.37	0.49–6.66	1.20–2.08	<LOD	<LOD–0.57	<LOD–0.79	<LOD–0.38	0.45–2.70	0.38–0.94	<LOD–0.41	0.32–5.27	0.20–6.33
Piglet/sow plasma ratio [c]																		
Mean ± SD	–	–	–	–	2.05 ± 0.87	3.64 ± 3.25	–	0.30 ± 0.08	6.78 ± 8.41	–	0.06 ± 0.03	0.09 ± 0.03	–	0.20 ± 0.09	0.11 ± 0.04	–	0.21 ± 0.17	0.20 ± 0.19

(b)

Sampling point	10 days after arrival	At farrowing	Day 1	Day 7	Day 21	Weaning
Sow plasma (ng/mL)						
Mean ± SD	4.25 ± 1.47	1.53 ± 1.04	1.73 ± 1.19	3.97 ± 0.88	4.94 ± 2.06	8.19 ± 1.96
Median	4.61	1.24	1.68	3.73	5.56	7.80
Range	0.48–5.66	0.23–3.71	<LOD–3.50	3.03–6.06	0.52–7.82	5.0–11.4
Piglet plasma (ng/mL)						
Mean ± SD	–	2.69 ± 0.81	1.25 ± 0.89	0.29 ± 0.16	0.70 ± 0.35	1.48 ± 1.41
Median	–	2.55	1.11	0.30	0.64	0.86
Range	–	1.34–4.74	0.49–6.66	<LOD–0.79	0.38–2.70	0.20–6.33
Piglet/sow plasma ratio [c]						
Mean ± SD	–	2.14 ± 0.80	2.30 ± 2.81	0.08 ± 0.03	0.16 ± 0.08	0.20 ± 0.17

[a] n = 4 control sows, n = 6 sows fed with DON level 1, n = 5 sows fed with DON level 2; n = 75 live-born piglets; day1: n = 72; day 7: n = 68; day 21: n = 67; weaning: n = 66. [b] Combined plasma concentrations in sows in the DON level 1 and DON level 2 groups (n = 11) and the matched piglets. The number of the piglets in each sampling point; n = 55 live-born piglets; day1: n = 53; day 7: n = 49; day 21: n = 49; weaning: n = 48. [c] Missing values and outliers are treated.

2.2.4. Association between Plasma DON Concentrations in Sows and Piglets

Plasma DON concentrations of the selected sows and their piglets in the transfer study are summarized in Table 2a and in the supplementary information (Figure S1a; for the sows). Figure 2 shows plasma DON concentrations in the selected sows and their offspring combined for both DON exposure levels. The relation between plasma samples of the sows and the piglets were expressed as the ratio between the concentrations of DON in piglet and sow plasma samples, and designated "piglet/sow plasma ratios" (Table 2a). The piglet/sow plasma ratio refers to the mean of plasma DON concentrations in each litter of selected piglets, divided by that of their mothers. The ratios combined for both DON exposure levels were 2.14 at birth and 2.30 within 12–36 h after parturition (Table 2b). From then on, there was a significant reduction in the transfer ratios, with values of 0.08, 0.16 and 0.20 on day 7, day 21 and at weaning, respectively (Table 2b).

The transfer of DON from the sows into their offspring is also illustrated by direct correlation between both DON plasma concentrations. DON plasma concentrations in piglets at day 1 postpartum and towards weaning may reflect several sources of intake, see discussion. Therefore, correlations were determined for the respective plasma concentrations at birth as an indicator for placental transfer and on day 7 during lactation as an indicator for lactational transfer. The plasma DON concentrations showed strong and significant sow-piglet correlation at birth ($r = 0.88$, $p > 0.001$). However, there was no association between plasma DON concentrations in sows and piglets on day 7.

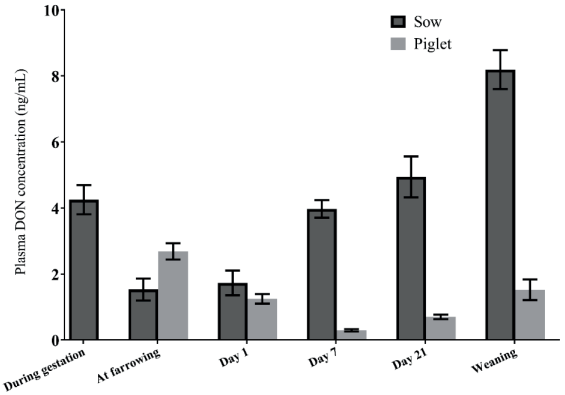

Figure 2. Plasma concentrations of DON in sows and piglets (combined DON level 1 and 2 groups) at birth, in the lactation period, and at weaning. The data represent means of $n = 11$ sows, and piglets (at farrowing: $n = 55$; day 1: $n = 53$; day 7: $n = 49$; day 21: $n = 49$; weaning: $n = 48$). Day 1 refers to sampling within 12–36 h after parturition. Error bars indicate standard error of the mean (SEM).

2.2.5. DON and DON Metabolites in Sow Milk

The repeated collection of milk samples allowed us to study the concentrations of DON over the course of lactation (Table 4). We used immunoaffinity columns for the solid-phase extraction of milk samples, which also retained DOM-1 and DON-15-GlcA, but not DON-3-GlcA. DON and DON-15-GlcA were detected in most (100% and 63%, respectively) of all the milk samples collected from the sows in the transfer study, while DOM-1 was detected in 11% of the milk samples. The DON concentrations in the milk of DON-exposed sows increased consistently with about 55–60% from farrowing until weaning for both DON level groups (Table 4). DON-15-GlcA milk concentrations showed a comparable, but not so uniform increase. The results show considerable inter-individual variation.

Table 4. DON, DOM-1 and DON-15-GlcA concentrations in milk of sows included in the transfer study [a].

Sampling point	At Farrowing			Day 1			Day 7			Day 21			Weaning		
Diets	**Con**	**Level 1**	**Level 2**	**Con**	**Level 1**	**Level 2**	**Con**	**Level 1**	**Level 2**	**Con**	**Level 1**	**Level 2**	**Con**	**Level 1**	**Level 2**
DON (ng/mL)															
Mean ± SD	0.42 ± 0.22	1.35 ± 0.52	1.20 ± 0.39	0.29 ± 0.09	2.14 ± 0.30	1.43 ± 1.49	0.48 ± 0.05	3.72 ± 1.98	4.14 ± 1.19	0.50 ± 0.00	5.67 ± 1.72	5.75 ± 1.52	0.73 ± 0.17	7.49 ± 1.99	7.03 ± 2.79
Range	0.25–0.75	0.82–2.29	0.81–1.65	0.23–0.43	1.68–2.44	0.46–4.06	0.43–0.53	1.33–5.97	3.08–6.20	0.50–0.50	2.97–7.09	4.61–8.29	0.57–0.97	5.4–10.5	4.1–10.4
DOM-1 (ng/mL)															
Mean ± SD	<LOD	<LOD	0.15 ± 0.02	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	<LOD	0.18 ± 0.05	<LOD	0.17 ± 0.04	0.22 ± 0.06
Range	–	–	<LOD–0.19	–	–	–	–	–	–	–	–	<LOD–0.24	–	<LOD–0.23	<LOD–0.30
DON-15-GlcA (ng/mL)															
Mean ± SD	<LOD	3.18 ± 1.42	4.39 ± 0.77	<LOD	2.42 ± 1.57	2.17 ± 0.86	<LOD	1.89 ± 1.12	3.52 ± 3.95	<LOD	2.62 ± 1.04	7.00 ± 4.55	0.82 ± 0.27	6.37 ± 4.65	10.8 ± 1.9
Range	–	1.86–5.02	3.49–5.14	–	0.66–4.37	1.20–3.08	–	<LOD–3.51	1.20–10.5	–	1.75–4.51	2.3–13.2	0.57–1.06	2.6–13.1	8.6–13.6

[a] $n = 4$ control sows, $n = 6$ sows fed with DON level 1, $n = 5$ sows fed with DON level 2.

2.2.6. DON Transfer from Sow Plasma to Milk (Lactational Transfer)

Lactational transfer was evaluated by calculating the sow milk/plasma (M/P) ratio at different time points during the lactation period, in accordance to Fleishaker [17]: M/P ratio = DON concentration in sow milk divided by DON plasma concentration in the sow.

M/P ratios were calculated for both DON level groups combined and at all sampling points. They varied from 0.41 to 1.7, indicating high inter-individual variability (Figure 3). The differences in M/P ratios between samples collected at birth (0.92 ± 0.28), within 12–36 h after parturition (1.11 ± 0.41), on day 7 (0.94 ± 0.26) and 21 (1.21 ± 0.36) in lactation and at weaning (0.90 ± 0.24) were not statistically significant ($p = 0.24$).

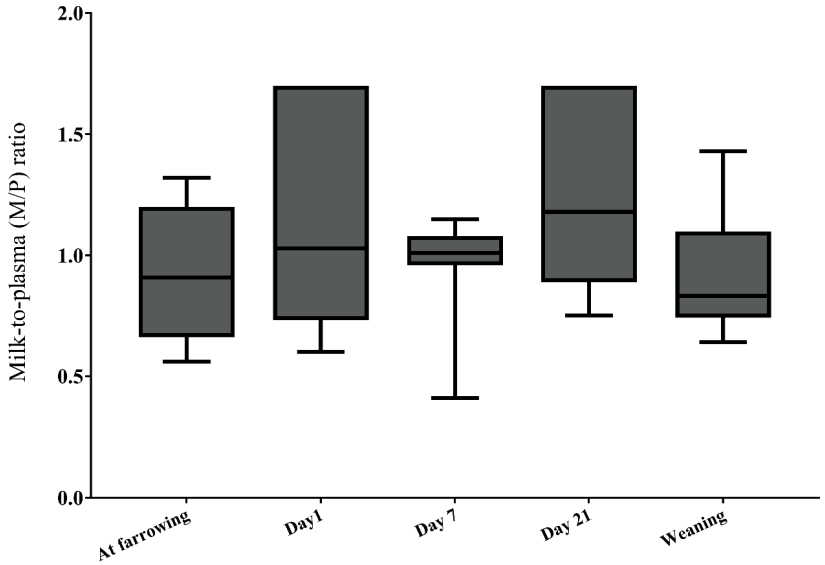

Figure 3. Milk/plasma (M/P) ratios at different stages in the lactation period for both DON level groups combined. Day 1 refers to the assessment within 12–36 h after parturition.

The transfer of DON from sow plasma to sow milk was also evaluated by correlation analyses using the quantitative data for each sow (Figure S3a). DON concentrations in sow plasma and milk were correlated throughout the lactation period, at birth ($r = 0.87$, $p < 0.001$), within 12–36 h after parturition ($r = 0.84$, $p < 0.01$), on day 7 ($r = 0.74$, $p < 0.01$), on day 21 ($r = 0.63$, $p < 0.05$) and at weaning ($r = 0.59$, $p = 0.08$). Additionally, we looked for a possible correlation between DON concentrations in sow milk and in the plasma of the respective piglets (mean value) (Figure S3b). There was not a strong correlation between DON in milk and DON in piglet plasma, neither on day 7 ($r = 0.25$, $p = 0.46$ nor at weaning ($r = 0.42$, $p = 0.20$), and DON in milk and DON in piglet plasma was not associated within 12–36 h after parturition and on day 21.

3. Discussion

Studies on the placental and lactational transport of DON are important in order to acquire knowledge of the foetal and early life exposure. In the present in vivo study, we demonstrated that DON and the metabolites DON-3-GlcA and DON-15-GlcA were present in the plasma of sows which had been exposed to naturally DON-contaminated diets in the last stage of gestation and during lactation. Furthermore, we proved transfer of DON from exposed sows to their new-born piglets

across the placenta and subsequently through milk. The data showed considerable variation among individuals and at the different sampling points in the lactation period, underlining that uptake and vertical transfer of contaminants is a complex process. Additionally, the individual differences in sow feeding patterns may have affected DON concentrations in plasma and milk. The milk composition may also vary depending on demands made by the litter, the time of day, and the nutritional status of the sow.

3.1. DON-Uptake in Sows

DON, DON-3-GlcA and DON-15-GlcA were detected in dose-dependent plasma concentrations in sows in the lactation period, but not at weaning, where plasma concentrations for DON and DON metabolites were lower for DON level 2 than for DON level 1. The incongruence for DON at weaning might result from increased variation between sows at this time point (DON < LOD for one sow). This explanation is, however, not applicable for DON-3-GlcA and DON-15-GlcA, for which the concentration differences between both dose levels were significant. The differences might also result from observed changes in the feed intake and BW during the study, which led to higher DON doses during lactation compared to late gestation. Placenta and uterus grow extensively in late gestation and contribute, together with the unborn piglets, to the higher BW that in combination with decreased appetite decreased the actual DON dose [18]. In contrast, the sow energy and nutrients requirements during lactation far exceed requirements in any other phase of the production cycle, and the higher feed intake, together with a lower BW after farrowing, increased the DON doses in this phase [19].

In the present study, the transfer of DON into plasma of sows (plasma/diet ratio) increased from arrival to weaning, where the ratio reached up to 0.008 in DON level 1 group. The increasing DON uptake in sows in the lactation period was reflected in the observed rise in the plasma/diet ratio. Accordingly, the median plasma/diet ratio for both DON level groups during the whole study was 0.004, which is higher than that observed for sows fed a *Fusarium*-contaminated diet containing 4.42 mg DON/kg for 35 days in mid-gestation (serum/diet ratio 0.002) [12] and that reported in sows (0.001) after consuming a *Fusarium* toxin-contaminated diet of 9.57 mg DON/kg for 35 days in the last third of gestation [13]. In fattening pigs exposed to a *Fusarium* toxin-contaminated diet containing 6.68 mg DON/kg for 12 weeks the mean serum/diet ratio was 0.002 [20]. Taken together, the pigs' age, race and the different stages in production cycle could all contribute to the observed differences in DON uptake. Comparing the uptake of DON in the current study with results from our recent study in growing pigs [21] leads to some interesting results. Calculated over the whole experiment, the DON plasma/diet ratios in the low DON (0.9 mg/kg feed) and medium DON (2.2 mg/kg) groups in the growing pig study were both close to 0.004, which is quite similar to the average value in the present study. This would indicate that there is not a strong effect of age on DON uptake in pigs. However, if average plasma DON concentrations in the two studies are compared to average DON dose in µg/kg BW/day, the ratio between plasma concentrations and DON dose is about twice as high in the sows than in the growing pigs. Adult sows and young, growing pigs may not be directly comparable, but these results still indicate that the DON uptake in sows in late gestation and lactation is not less efficient than in younger pigs.

3.2. Plasma Profiles of DON and DON–Glucuronides in Different Age Groups in Pigs

The present study allowed the comparison of plasma profiles of DON and DON–glucuronides in pigs of different age after DON exposure via the diet. Using the data for sows and their offspring, as well as data from a previous study on growing pigs [21] we found that DON-3-GlcA is the main metabolite in pigs of all ages. Plasma concentrations of this metabolite were higher than those for DON and DON-15-GlcA after DON exposure with feed. In new-born piglets, measuring of DON and its metabolites in blood plasma immediately postpartum and before they received colostrum showed the extent of the placental transfer in the last stage of gestation. At this stage DON–glucuronides were not detected in piglet plasma, indicating impaired or reduced placental transfer of these hydrophilic

metabolites compared to DON. In suckling piglets, measurable levels of DON-3-GlcA and DON-15-GlcA were only found at weaning, although the metabolite concentrations in sow milk were in the same range as those for DON. However, after oral uptake in the piglets, the DON-glucuronides are probably not absorbed in the gut and excreted [22], or enzymatically split so that free DON is taken up. Furthermore, another possible explanation for this finding is that metabolic DON conjugation to DON-glucuronides appears to be not fully developed in newborn piglets.

The mean glucuronidation rate for sows in both dose groups was 71% (Table S1) as compared to 63% in growing pigs [16], which appears to be in line with the observation that younger animals have a lower capacity for DON metabolism [7]. It should be noted that the lower glucuronidation rate in the growing pig study might in theory be explained by the higher DON exposure (up to 5 mg DON/kg) compared to the present study (up to 1.7 mg DON/kg). DON is known to have hepatotoxic effects at higher levels [23], which might affect the glucuronidation capacity. However, an increase in serum hepatic enzymes, as an indication of hepatic damage, was not detected in the growing pig study [21]. We are not aware of any previous studies that have evaluated age-related glucuronidation rates in DON exposed pigs. However, one previous study suggested that young mice are more susceptible than adult mice to DON-related effects, due to higher plasma DON concentrations in weanling mice compared to adults given identical doses of DON [24]. At 120 min after DON administration, DON plasma concentrations in young mice were close to the levels in adults, suggesting that age-related differences might be more pronounced for DON uptake than for clearance [24].

3.3. Growth Performance of Piglets of DON-Exposed Sows

Adverse effects of feeding gestating and lactating sows with DON contaminated diets on their piglets might relate indirectly to DON-induced reduction in feed intake of the exposed sows [16], and directly to placental or lactational transfer of this toxin to piglets. Therefore, one should take both these aspects into account in evaluation of the results from the present study. Average BW and daily weight gain of piglets in DON level 1 and DON level 2 was not significantly different from the control piglets. To the best of our knowledge, there are not any previous studies that have evaluated individual BW development of piglets of sows exposed to DON during lactation. However, comparing effects on litter weight gain, the results of the current study concur with similar previous studies [25-27], but are in contrast with a report by Jakovac-Strajn et al. [28], which showed that litter weight gain in sows receiving 5 mg DON/kg was significantly lower than the control. DON had no sex-related effects on the piglet BW in the current study. This finding is in agreement with some previous studies, where no sex differences were observed in piglets of the sows fed a *Fusarium* toxin-contaminated diet containing 9.52 mg DON/kg in the last third of gestation [13] and in the growing piglets receiving contaminated diets with up to 5 mg DON/kg for 42 days [21]. In contrast, male pigs have been shown to be more sensitive to DON-related toxicity than females in a previous meta-analytic study [11], in feeding experiments on fattening pigs [29], and in a study of foeti of sows in mid-gestation [12].

3.4. DON Transfer from Sows to Unborn Piglets

Placental transfer of DON has been investigated in vitro using human cell lines and ex vivo using human placenta [10]. The results from the in vitro study suggested that DON transferred slowly from the maternal side to the fetal side, and the ex vivo study showed that 21% of the initially incubated DON was transferred to the fetus [10]. In pigs, placental DON transfer was studied in vivo in pregnant sows in mid-gestation [12] and during late gestation [13]. The high DON levels in piglet plasma at birth in the present study indicated an efficient transfer of DON across the placenta in the last stage of pregnancy. There was a statistically significant association ($r = 0.88$, $p < 0.001$) in plasma DON concentrations between the sows and their new-born, comparable to the linear association observed in the reference study ($r^2 = 0.69$) [13]. The mean and median piglet/sow plasma ratio at birth (mean:

2.14, n = 72; median: 1.80) was considerably higher than the median piglet/sow serum ratio (0.75, n = 91) reported for DON-exposed sows in the last third of gestation [13]. A transfer ratio close to unity would mean that DON absorbed by sows is present in the circulation of the foeti at almost the same concentration as in the sows [13]. The differences in transfer ratios might result from the significantly reduced feed intake during farrowing in the present study, whereas in the previous experiment [13] the sows had been euthanized in late gestation and before start of the stressful farrowing period. Since the plasma half-life of DON in pigs is relatively short (diet t½ 2–6 h) [5], plasma concentrations in the sows may have decreased considerably during the farrowing period. The piglets selected for the transfer study were consistently the first-born, and plasma was sampled immediately at birth, while the samples from the corresponding sows were often collected after finished farrowing of the whole litter. As an estimate for plasma DON concentrations in sows at the start of farrowing, we used the DON plasma concentrations in sows at 10 days after arrival/during gestation and calculated an expected plasma DON concentration just before farrowing, taking the reduction in feed allowance from approximately 2 days prior to expected farrowing into consideration. Using this estimate, piglet/sow plasma ratio was near unity, which was close to that of the referenced study.

3.5. DON Transfer from Sows to Suckling Piglets

The DON concentrations measured in colostrum and milk and the calculated M/P ratios indicated an efficient transfer of DON from sow plasma into milk, and showed no significant differences of transfer efficiency at different stages of lactation since the M/P ratios were almost the same throughout the lactation period. The observed higher DON plasma concentrations in one-day-old piglets as compared to later in lactation may indicate a better uptake of DON in the gut of piglets in the colostrum period than at a later stage. Furthermore, some DON present in piglet plasma 12–36 h after birth could be a remnant of placental transfer, which would indicate a slower DON metabolism in new-borns than in older animals. The gradual increase of DON in piglet plasma towards weaning may reflect that some piglets started to eat sow feed during late lactation. Due to practical limitations, it was not possible to prevent this in the present study.

To our knowledge, the plasma-to-milk transfer of mycotoxins in different stages of lactation (i.e., colostrum versus mature milk) had previously been investigated only in an ochratoxin A (OTA) exposure study in humans, where the OTA in colostrum was 2.5 times higher than in transient and mature milk [30]. These findings can, however, not be compared due to species differences and chemical differences between OTA and DON. Colostrum has a higher protein content than mature milk, and OTA is known for its high affinity to proteins [31]. We are not aware of any studies that have investigated the binding of DON to milk proteins in pigs.

A limitation of our study was that neither the blood samples from sows and piglets nor the milk samples were collected at fixed time points during the day, because of necessary adjustments to the course of farrowings and other practical limitations. However, the results from a previous study on growing pigs [21], where plasma DON concentrations were recorded at three times a day (08:00, 11:00 and 16:00) during 35 days of exposure showed that the concentrations of DON and its metabolites were relatively constant throughout the day, likely due to the *ad libitum* access to feed. Therefore, we assumed that also the DON plasma concentrations of the sows were relatively stable throughout the day in the present study, except during farrowing and the first 12–36 h after parturition, when the sows had a temporarily reduced feed intake [16].

4. Conclusions

DON is transported efficiently across the placenta from sows to their piglets during the last stage of gestation. The transfer of DON from the lactating sows to suckling piglets via colostrum and milk was less efficient, particularly in mid-lactation. The differences in DON exposure of piglets at different times during lactation were apparently related to changes in DON uptake and metabolism in the piglets and not to changes in the transfer of DON from sow plasma to sow milk. The study results

suggest that piglets of sows receiving DON-contaminated diets are at greater risk of DON exposure during late gestation and the first days after birth than during mid to late lactation.

5. Materials and Methods

5.1. Animals, Housing, and Management

This study was performed on 45 Norwegian Landrace × Yorkshire sows, used in a field trial during late gestation and lactation, using diets that were naturally contaminated with deoxynivalenol (DON). It was conducted in a high-yielding specific pathogen free (SPF) unit from December 2015 to February 2016 [16]. All sows were kept individually and without fixation in standard farrowing pens (7.0 m^2) with a piglet creep area (1.3 m^2) from approximately 3 weeks prior to expected farrowing until weaning. Each pen had a solid concrete floor except for a slatted draining floor at one end of the pen (2.3 m^2).

From the onset, the study included 47 sows. However, two sows (one from the control group and one in the group receiving feed with the highest DON level) were removed from the trial. The sow from the control group died during the lactation period due to a penetrating gastric ulcer, while the sow in the highest dose group was excluded due to complete refusal of the contaminated feed. The final study therefore included 45 sows, divided into three groups (Figure 4): (1) control (DON < 0.2 mg/kg) (n = 15); (2) DON level 1 (1.4 mg DON/kg) (n = 15); and (3) DON level 2 (1.7 mg DON/kg) (n = 15). The sows were fed twice daily during gestation until 1 week postpartum when the feeding frequency was increased to 3 times daily, and 4 times daily from 2 weeks postpartum to weaning. They were offered a restricted amount of feed during late gestation, with a maximal allowance of 4 kg/day for each sow in this period. From approximately 2 days before expected farrowing, the daily feed allowance was reduced and adjusted to a daily feed allowance of between 2 and 3 kg per sow. After farrowing, the amount of feed offered to each sow was gradually increased, and the feeding automates were adjusted to meet the requirements for a modified *ad libitum* feeding strategy. The amount of feed offered to each sow by the automated feeding system was adjusted continuously; in such a way that a small amount of feed was left over in the trough before the next feeding, as a confirmation that the sows had access to feed *ad libitum*. All sows were offered 0.2 kg hay daily. Both sows and piglets had access to water *ad libitum*. Farrowing was allowed to occur naturally, although under constant surveillance by the staff. Commercial husbandry procedures were performed within 48 h after birth, including teeth grinding and a 270 mg oral iron supplementation (1.5 mL plus iron paste, Felleskjøpet, Lillestrøm, Norway), and thereafter a creep feed mixed with iron fortified peat was offered to piglets on the concrete floor in the piglet creep area until 4 days before weaning. Between five and ten days after farrowing, surgical castration was performed by a veterinarian, using local anesthesia (Lidocaine (Lidokel-Adrenalin vet.), Kela Laboratoria NV, Hoogstraten, Belgium) and analgesia (NSAIDs (Metacam vet.), Boehringer Ingelheim Vetmedica GmbH, Ingelheim am Rhein, Germany). All litters were weaned on the same day, and average lactation length was 33.3 days (range 29–38 days). Further details are given in Sayyari et al. [16]. All 45 sows in the field trial were included in the DON-uptake study. However, one sow in the DON level 2 group and its piglets were later excluded from all calculations, because the plasma results of DON indicated a mistake in the feeding procedure for this sow; see comments under statistical analysis (Figure 4).

In the farrowing period, four sows from the control group, six sows from the DON level 1 group and six sows from DON level 2 group (one later excluded: see above) were selected for the transfer studies. The selection was done randomly, but sows that started farrowing during the night were excluded. The farrowing of the selected sows was monitored continuously in order to take plasma samples from the new-born piglets before they received colostrum from the sows. The first five live-born and healthy piglets from each of the sows were tagged with plastic ear tags, COMBI 2000® (OS ID®, Os, Norway), and gender was recorded. Each piglet were weighed within 48 ± 12 h after parturition, on day 7 and 21 in the lactation period and at weaning, using a digital scale with 10-g

accuracy according to the manufacturer (Premium 8006 GR-ST, EKS international SAS, Wittisheim, France).

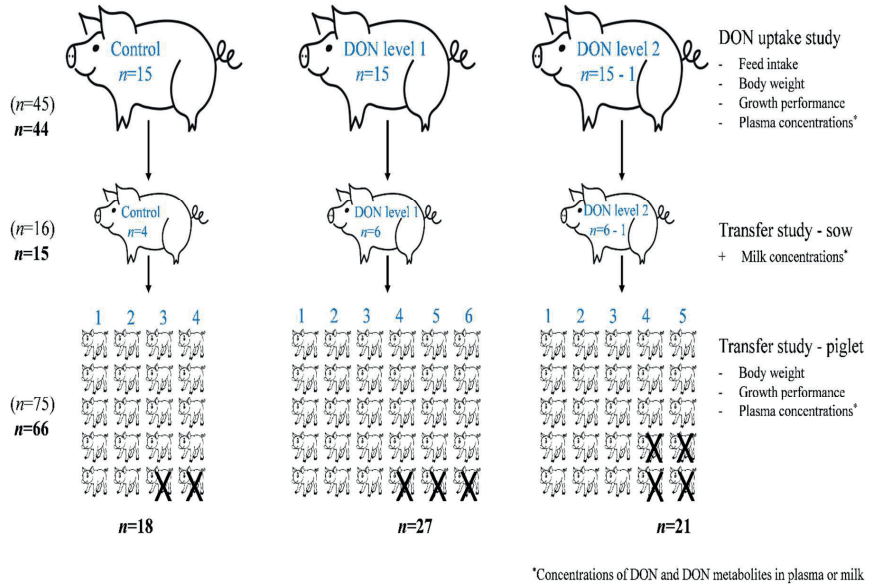

Figure 4. Flow chart of study design and animal groups in the DON uptake and transfer study.

This study was conducted according to the Norwegian regulations for animal testing (FOR-2015-06-18-761), which comply with EU Directive 2010/63/EU. A detailed description of the experiment, in accordance with the ARRIVE guidelines for reports of animal experiments, was evaluated and confirmed approvable by the Norwegian Animal Research Authority after completion.

5.2. Origin and Preparation of the Naturally Contaminated Experimental Diets

The DON-contaminated oats used for the production of the experimental diets for this study had been harvested in southern Sweden in 2013 and provided by Lantmännen, Sweden, and was identical to that used in our recent study on growing pigs [21]. This batch of oats was analyzed for a wide range of mycotoxins, using a semi-quantitative multi-toxin screening method, at the Centre for Analytical Chemistry at IFA Tulln, Austria (Table S2) [32].

From arrival in the farrowing unit, the sows were fed pelleted feed with different levels of naturally DON-contaminated oats. Detailed information on the composition of the experimental diets is given by Sayyari et al. [16]. The experimental diets were produced with a standard pelleting process by Felleskjøpet Agri, Lillestrøm, Norway (Production site: Trondheim, Norway), and were based on the formula of a commercial lactation diet (FORMAT, Felleskjøpet Fôrutvikling, Trondheim, Norway). The three diets were formulated to provide the following treatments: (1) control-diet (DON < 0.2 mg/kg), (2) DON-contaminated diet level 1 (DON = 1.4 mg/kg) and (3) DON-contaminated diet level 2 (DON = 1.7 mg/kg). We initially planned to run the trial with DON levels of approximately 0.5 mg/kg and 1 mg/kg in the two DON-contaminated diets. The different dose levels were obtained by blending the naturally contaminated oats with low-contaminated oats, which were harvested in Norway in 2014. However, due to an unexplained factor in the feed production at the factory, the final contaminated diets contained higher and more similar levels of DON than originally planned.

5.3. Measuring of Feed Intake and Growth Performance of the Sows

In each farrowing pen, a plastic dispenser showed the amount of feed consumption at each feeding, so that the daily feed intake could easily be registered. In this study, daily feed consumption refers to feed disappearance from the dispenser and was not adjusted for feed spillage or consumption of sow feed by piglets.

Upon arrival at the farrowing unit, all sows were weighed individually, using a digital scale (EC2000 by TEO teknikk AS, Nærbø, Norway) with the dimensions 1 × 2 m and an accuracy of 500 g. They were weighed again after 10 days in late gestation period, within 12–36 h after parturition, on day 7 and day 21 of lactation and at weaning.

5.4. Reagents for Chemical Analyses

Acetonitrile, methanol and water (Fisher Scientific, Fair Lawn, NJ, USA) for liquid chromatography high-resolution mass spectrometry (LC–HRMS) analysis were of Optima™ LC–MS quality, while acetonitrile for sample preparation (gradient quality) was from Romil (Cambridge, UK). Ammonium acetate and glacial acetic acid were of p.a. quality (Merck, Darmstadt, Germany). 4-Deoxynivalenol (DON), deepoxy-DON (DOM-1), 3-*O*-acetyl-DON (3-ac-DON), 15-*O*-acetyl-DON (15-ac-DON) and DON-3-*O*-ß-D-glucoside (DON-3-Glc) were purchased from Romer Labs (Tulln, Austria), while DON-3-*O*-ß-D-glucuronide (DON-3-GlcA) and DON-15-*O*-ß-D-glucuronide (DON-15-GlcA) were available from work performed in a previous study [33].

5.5. Analysis of Mycotoxins in Experimental Diets

Samples of 2.5 g from each of the experimental diets were milled with a Retsch ZM 100 mill (Retsch GmbH & Co. KG, Haan, Germany) and placed into 50-mL centrifuge tubes, and 10 mL acetonitrile/H_2O/formic acid (80:19.9:0.1, *v*/*v*/*v*) was added. The mixture was vortexed for 30 s and extracted for 30 min using a horizontal shaker at 175 min^{-1}. After centrifugation at 4000× *g* for 10 min (4 °C), the supernatants were transferred into new 50-mL tubes, and the remaining solid material was extracted with 10 mL of acetonitrile/H_2O/formic acid (20:79.9:0.1, *v*/*v*/*v*) by shaking for an additional 30 min. The two extracts were combined and kept at 4 °C before final centrifugation (4000× *g*, 10 min, 4 °C). Finally, 0.5-mL aliquots of the combined supernatants were centrifuged for 1 min at 15,000× *g* through 0.22-µm nylon filters (Costar Spin-X, 0.22 µm; Corning, Inc., Corning, NY, USA). The prepared samples were analyzed for DON, DON-3-Glc, 3-ac-DON, and 15-ac-DON with a previously validated LC-HRMS method [34]. The limits of detection (LOD) in feed matrix were 14 µg/kg for DON, 26 µg/kg for DON-3-Glc, 5.9 µg/kg for 3-ac-DON and 52 µg/kg for 15-ac-DON. The prepared experimental diets contained DON as the main contaminant, while other mycotoxins that commonly occur in Norwegian cereal grain were below the LOD or detected at low levels (Table S2). The hay provided to the sows was not analyzed for mycotoxin content. However, all hay was of good quality with no signs of fungal contamination.

5.6. Blood and Milk Sampling for Analysis of Mycotoxins

5.6.1. DON-Uptake Study

Blood samples from all sows were taken from the milk vein (*v. subcutanea abdominis*) without any form of immobilization while they were lying down or standing [35], using 9-mL Lithium-Heparin tubes. The samples were collected upon arrival in the farrowing unit, after 10 days, on day 21 in lactation, and at weaning. Plasma was prepared by centrifugation at 1500× *g* for 10 min at room temperature, stored frozen in 2-mL cryogenic vials (Nalgene, Nalge Company, Rochester, NY, USA), and delivered to the Norwegian Veterinary Institute for analysis.

5.6.2. Transfer Study

Blood samples of the sows included in the transfer study were taken from the milk vein (*v. subcutanea abdominis*), using 9-mL heparin tubes at farrowing, within 12–36 h after parturition (day 1), on day 7 and 21 in the lactation period and at weaning. Blood samples from the respective piglets were collected from the jugular vein (*v. jugularis*), using 2.7-mL S-Monovette® Lithium-Heparin tubes (Sarstedt AG & Co. KG, Nümbrecht, Germany) immediately after birth (before the new-borns received colostrum), within 12–36 h after parturition, on day 7 and 21 in the lactation period and at weaning.

Colostrum was collected shortly after the onset of parturition. Transient milk was sampled on day 1 after parturition and mature milk on days 7 and 21 in the lactation period and at weaning. In this study, 2 to 5 mL milk was sampled from the first available teat into plastic vials by manual expression and without oxytocin administration, after the milk ejection was initiated by the suckling behaviour of sows and piglets. The collected samples were stored frozen (−20 °C) in 5-mL cryogenic vials (Greiner Bio-One International GmbH, Monroe, NC, USA) until analyzed.

Neither the blood samples from sows and piglets nor the milk samples were collected at fixed times during the day. Sample collections were always started in the morning, but due to necessary adjustments to the course of farrowings and other practical limitations, sampling was sometimes not finished until late in the evening.

5.7. Sample Preparation of Plasma for Analysis of Mycotoxins

Plasma samples (250 µL) were transferred into conical 15-mL plastic tubes (Corning Inc., Corning, NY, USA), mixed with 750 µL acetonitrile, vortexed for 15 s, and sonicated (Branson 3200, Emerson, St. Louis, MO, USA) for 5 min. Proteins were precipitated by centrifugation at 2000× *g* for 10 min at 4 °C (Beckman Coulter, Brea, CA, USA), and supernatants were transferred to 10-mL conical glass tubes and evaporated to dryness at 60 °C using a gentle stream of nitrogen. Dried samples were stored refrigerated, re-dissolved in 200 µL water, vortexed for 15 s, sonicated for 5 min and transferred to HPLC vials for LC–HRMS analysis. Each round of analyses included at least one blank pig plasma sample, which had been fortified with 5.3 or 26 ng/mL of DON, DON-3-GlcA and DON-15-GlcA and 11 or 52 ng/mL DOM-1.

5.8. Sample Preparation of Sow Milk for Analysis of DON, DOM-1 and DON-15-GlcA

Milk samples were allowed to thaw at room temperature, transferred to 1.5-mL Eppendorf tubes, and then centrifuged at 20,000× *g* for 20 min. Positive control samples containing either 27.5 ng/mL of DON, 27.6 ng/mL of DOM-1, and 16.5 ng/mL of DON-15-GlcA, or 2.75 ng/mL of DON, 2.76 ng/mL of DOM-1, and 1.65 ng/mL of DON-15-GlcA were prepared by spiking blank colostrum or milk samples with 20 µL of the appropriate standard solution in water. Aliquots of the supernatants (400 µL) were transferred to immunoaffinity columns (ImmunoClean CF for DON, aokin AG, Berlin, Germany), which had been rinsed with 3 mL of PBS. The immunoaffinity columns are designed for the clean-up of DON, but were previously shown to retain also DOM-1 and DON-15-GlcA, but not DON-3-GlcA [36]. Following application of the supernatants, they were washed with 5 mL PBS and dried under vacuum for approximately 30 s. The columns were then allowed to soak with 1.5 mL methanol for 2 min and eluted. The eluates were evaporated to dryness at 60 °C using a gentle stream of nitrogen. Residues were dissolved in 200 µL water, vortexed and then sonicated for 5 min. The solutions were filtered through 0.22 µm Nylon membranes (Spin-X, Corning Inc., New York, NY, USA) and transferred to chromatography vials.

5.9. Quantitative Analysis of Mycotoxins in Plasma and Milk

The plasma samples were analyzed for DON, DOM-1, DON-3-GlcA and DON-15-GlcA. Samples were chromatographed at 30 °C using a Vanquish UHPLC (Thermo Fisher Scientific, Waltham, MA,

USA) and a 100 × 2.1 mm i.d. Acquity UPLC HSS T3 column (1.8 µm; Waters, Milford, MA, USA) including a 5 × 2.1 mm i.d. XSelect HSS T3 VanGuard pre-column (2.5 µm, 100 Å, Waters). The flow rate of the mobile phase was 0.5 mL/min, and the injection volume was 6 µL. Eluent A was water, and eluent B was 95% acetonitrile (both containing 5 mM ammonium acetate and 0.1% acetic acid). The column was eluted isocratically with 100% A for 1 min, and then the mobile phase composition was changed to 15% B using a linear gradient over 15 min. After flushing the column for 2.5 min with 100% B, the mobile phase composition was returned to the initial conditions, and the column was re-equilibrated for 2.9 min. The UHPLC-system was coupled to a Q-Exactive™ Hybrid Quadrupole-Orbitrap high-resolution mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a heated electrospray ion source (HESI-II). The HESI-II interface was operated at 300 °C in the negative ionisation mode, and the parameters were adjusted as follows: spray voltage, 4 kV; capillary temperature, 250 °C; sheath gas flow rate, 35 units; auxiliary gas flow rate, 10 units; and S-lens RF level, 55. The data were acquired in the selected ion monitoring (SIM)/data-dependent MS^2 (dd-MS2) mode targeting the $[M + acetate]^-$ ions for DON and DOM-1 (m/z 355.1387 and 339.1438, respectively) and the $[M - H]^-$ ions for the DON-glucuronides (m/z 471.1497) with a quadrupole isolation width of 2 m/z and a mass resolution of 70.000 full width half-maximum (FWHM) at m/z 200 for SIM. The presence of a target ion above a threshold intensity of 5×10^3 triggered a MS^2 scan for analyte verification (dd-MS2) using a normalised collision energy of 35%. The mass resolution during dd-MS2 was set to 17.500 FWHM. The automatic gain control (AGC) target was set to 5×10^5 ions including a maximum injection time (IT) of 250 ms during SIM, whereas for dd-MS2 the AGC target was 5×10^4, and the IT was 200 ms.

Matrix-matched, 1/x weighed calibration curves were plotted for DON, DOM-1, DON-3-GlcA and DON-15-GlcA using plasma of untreated pigs (blank). Xcalibur version 2.2 or 2.3 (Thermo Fisher Scientific) was used for data processing. The overall spike recoveries were (standard deviations in parentheses) DON 80 (11) %, DOM-1 74 (12) %, DON-3-GlcA 67 (17) % and DON-15-GlcA 65 (14) %. The LODs were 0.1 ng/mL for DON, 0.2 ng/mL for DOM-1 and 1.5 ng/mL for DON-3-GlcA and DON-15-GlcA.

Instrument calibration for quantification of DON, DOM-1 and DON-15-GlcA in sow milk was carried out using 1/x weighed calibration curves and water as solvent for standards. The overall spike recoveries were (standard deviations in parentheses) DON 54 (16) %, DOM-1 59 (8.5) % and DON-15-GlcA 119 (55) % in colostrum, and DON 55 (18) %, DOM-1 58 (8.7) % and DON-15-GlcA 89 (26) % in milk. The LODs were estimated to 0.2 ng/mL for DON and DOM-1, and to 0.7 ng/mL for DON-15-GlcA.

5.10. Statistical Analysis

The analyses of DON concentrations in sow plasma indicated that a mistake in the feeding of one sow in the DON level 2 group had occurred. The plasma concentrations of DON and its metabolites in this sow were below the LOD at 5 of 8 sampling points. This sow and its piglets was therefore excluded from all calculations and statistical analysis. Thus, the final dataset included 44 sows in the DON-uptake study (15 controls, 15 at DON level 1, and 14 at DON level 2); and 15 sows (4 controls, 6 at DON level 1, and 5 at DON level 2) in the transfer study.

Piglet growth performance and plasma concentration data were analyzed by a mixed model in JMP®, Version 10 (SAS Institute Inc., Cary, NC, USA). The level of significance was set to 0.05 in all models, and results with p-values between 0.05 and 0.1 were considered significant trends. If not otherwise specified, all results are expressed as the mean ± standard deviation (SD). The normality of distribution of the different parameters was controlled by residual and predicted values plot, normal-percentile plots and Shapiro–Wilk test. If the p-value in the Shapiro–Wilk test was greater than 0.05, the data were considered normally distributed. Data that were not normally distributed were analyzed by non-parametric Wilcoxon's rank sum test. Levene's test was used to check the assumption of homogeneity of variances. If the p-value of Levene's test was greater than 0.05, variances were considered equal. If the output generated from the application of one-way Analysis of

Variance (ANOVA) indicated significant differences, the post–hoc Tukey–Kramer Honestly Significant Difference (HSD) test was used for multiple comparisons and the identification of significant differences ($p < 0.05$). Interactions that were not statistically significant were removed from the models by backward elimination.

DON concentrations measured in plasma samples from the sows fed experimental diets and in selected piglets (pooled per sow) were compared by the non-parametric Wilcoxon Each Pair test ($p < 0.05$) as these data were not normally distributed.

Correlations between DON concentrations in the plasma of sows and their respective piglets were analyzed by a non-parametric Spearman's rank-order correlation test for non-normally distributed data and by Pearson correlation test for normally distributed data (r_s for Spearman's rank-order and r for Pearson correlation test: $p < 0.05$). In order to analyze the correlation between plasma DON concentrations in sows and piglets, we have merged the data from both groups fed DON-contaminated feed (DON level 1 and DON level 2) into a single data set for piglets and sows. Wilcoxon's rank sum test ($p < 0.05$) was used for statistical comparisons of M/P ratios. In the statistical calculations, concentrations below the LOD were represented by the LOD divided by the square root of 2 [37].

During the data evaluations and statistical analyses, missing values and outliers were encountered. Therefore, imputation analysis for missing values and winsorization was used whenever appropriate [38]. Results from the control groups were not included in the calculation and presentation of ratios and correlations, because the low values in the control groups may give false impressions of these variables.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1, Figure S1: Effect of experimental diets on plasma concentrations of DON (a), DON-3-GlcA (b) and DON-15-GlcA (c) in sows included in the transfer study, Figure S2: Effect of experimental diets on plasma DON concentrations in piglets over the course of study, Figure S3: The relationship between the DON concentrations in milk samples and plasma concentrations of DON in sows (a) and piglets (b) regardless of treatments (for DON level 1 and DON levels 2 groups) over the course of study, Table S1: Sow plasma concentrations of DON and DON–glucuronides, and glucuronidation rates determined over the course of the experiment, Table S2: Toxin contents in the oats used for the production of the experimental diets, as measured with multi-toxin LC–MS/MS at the Centre for Analytical Chemistry at IFA Tulln, Austria.

Author Contributions: A.S., T.S., T.F., S.U. and C.K.F. designed the study. A.S., T.S. and T.F. visited the farm and performed the collection of data, blood and milk samples. A.S. and S.U. conducted the preparation of samples and mycotoxin analysis. A.S. did the analysis of data and statistics. A.S. wrote the paper. All authors reviewed, read and approved the final manuscript.

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

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Supplementary information

Figure S1

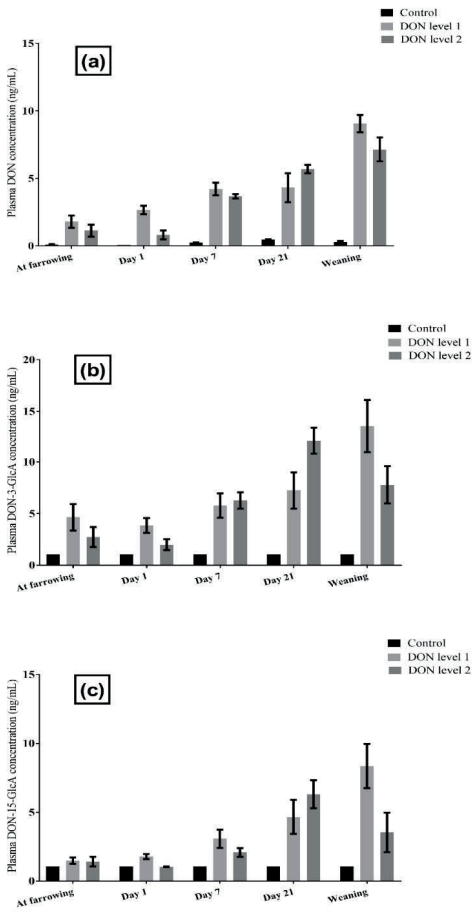

Figure S1. Effect of experimental diets on plasma concentrations of DON (a), DON-3-GlcA (b) and DON-15-GlcA (c) in sows included in the transfer study. Day 1 refers to 12–36 h after parturition. Error bars are the standard error of the mean (SEM).

Figure S2

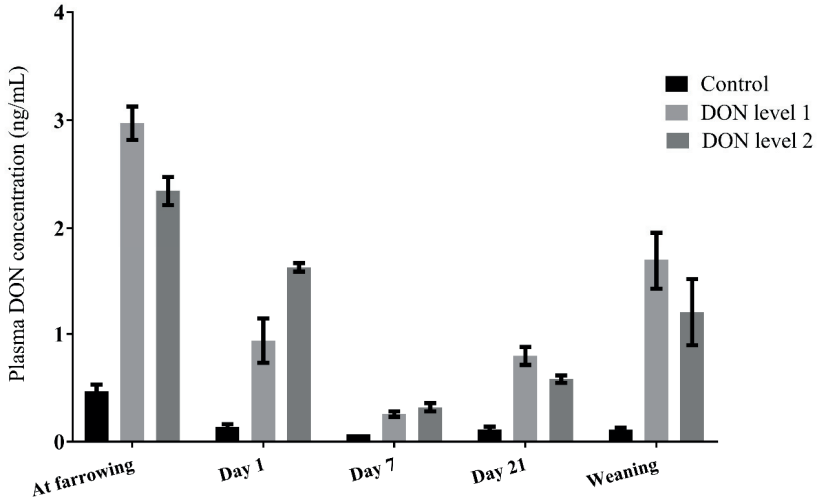

Figure S2. Effect of experimental diets on plasma DON concentrations in piglets over the course of study. Day 1 refers to the assessment within 12–36 h after parturition. Error bars are the standard error of the mean (SEM).

Figure S3

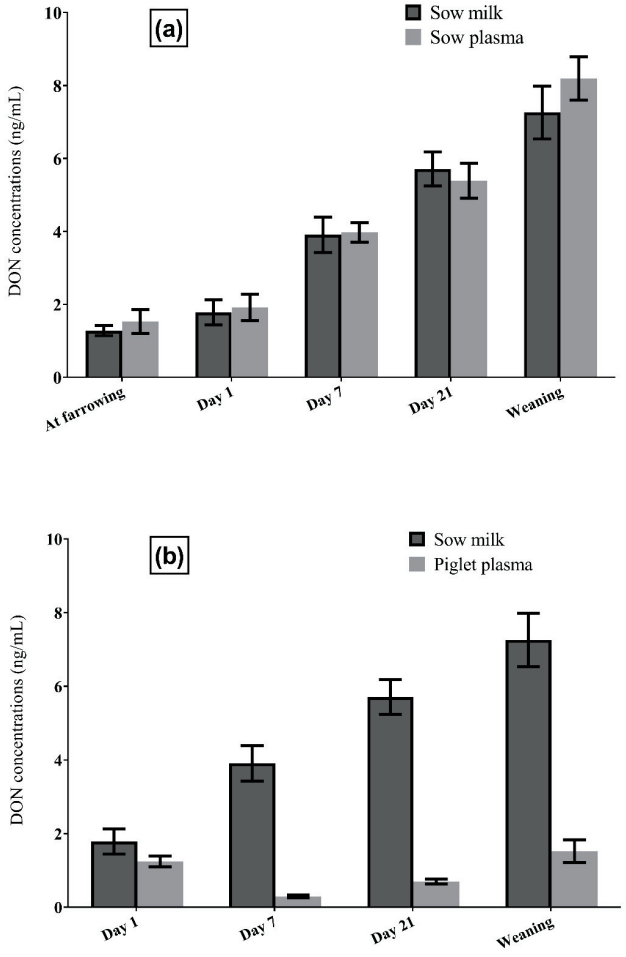

Figure S3. The relationship between the DON concentrations in milk samples and plasma concentrations of DON in sows (a) and piglets (b) regardless of treatments (for DON level 1 and DON levels 2 groups) over the course of study. Each column is the average of DON levels in milk and plasma DON concentrations of all individuals in sow or piglet groups. Day 1 refers to within 12–36 h after parturition. Error bars are the standard error of the mean (SEM).

Table S1. Sow plasma concentrations [a] of DON and DON–glucuronides, and glucuronidation rates determined over the course of the experiment.

Sampling point	DON (*ng/mL*)	DON-3-GlcA (*ng/mL*)	DON-15-GlcA (*ng/mL*)	Σ total DON (*ng/mL*)	Glucuronidation rate (%)
Arrival	< LOD	< LOD	< LOD	< LOD	–
10 days after arrival	4.34 ± 1.18	6.31 ± 2.55	4.11 ± 1.71	14.77 ± 4.83	69
Day 21 in lactation	5.58 ± 2.06	10.37 ± 4.28	6.80 ± 3.38	22.75 ± 9.06	75
Weaning	7.13 ± 2.96	9.02 ± 4.87	6.27 ± 3.97	22.42 ± 9.57	67
Average [b]	5.68 ± 1.22	8.57 ± 2.18	5.72 ± 2.02	19.98 ± 4.33	71

[a] The concentrations presents the average of sow plasma concentrations in two contaminated groups, DON level 1 and DON level 2.

[b] The values are calculated based on the average of plasma concentrations at the three sampling points for the combined DON level 1 and 2 groups sows in the DON uptake study, excluding the value at arrival.

Table S2. Toxin contents in the oats used for the production of the experimental diets, as measured with multi-toxin LC–MS/MS at the Centre for Analytical Chemistry at IFA Tulln, Austria.

	Oats [a]	
Toxins (µg/kg)	**Sample 1**	**Sample 2**
15-Hydroxyculmorin	499	500
15-Hydroxyculmoron	182	193
5-Hydroxyculmorin	387	441
Alternariol	15.3	2.12
Alternariolmethylether	1.58	0.64
Andrastin A	< LOD	< LOD
Apicidin	54.5	49.5
Ascochlorin	6.37	0.98
Asperfuran	< LOD	< LOD
Asperglaucide	5.38	3.91
Asperphenamate	12.08	9.51
Aspterric acid	< LOD	< LOD
Aurofusarin	1771	1310
Beauvericin	13.3	14.4
Brevianamid F	10.4	9.15
Butenolid	19.6	21.9
Chanoclavin	0.24	0.27
Chrysogin	134	144
Citreorosein	5.42	5.83
Culmorin	2139	1996
Curvularin	14.4	7.52
cyclo(L-Pro-L-Tyr)	79.2	63.9
cyclo(L-Pro-L-Val)	95.5	75.9
Deoxynivalenol	3788	3975
Diplodiatoxin	< LOD	< LOD
DON-3-glucoside	843	869
Elymoclavine	< LOD	< LOD
Emodin	4.86	5.70
Enniatin A	0.39	0.36
Enniatin A1	2.83	3.09
Enniatin B	14.9	14.7
Enniatin B1	13.0	13.2
Enniatin B2	0.69	0.81
Enniatin B3	0.00	0.00
Epiequisetin	0.85	0.91
Equisetin	1.88	1.16
Ergometrine	< LOD	10.44
Ergometrinine	0.35	0.82
Fallacinol	< LOD	< LOD

Fellutanine A	15.5	11.9
Fonsecin	< LOD	< LOD
Fumonisin B1	< LOD	< LOD
Fumonisin B2	< LOD	< LOD
Fumonisin B3	< LOD	< LOD
Fusaric acid	< LOD	< LOD
Fusarinolic acid	< LOQ [b]	< LOQ
HT-2 toxin	9.82	< LOD
Infectopyron	175	189
Iso-Rhodoptilometrin	< LOD	0.30
Kojic acid	< LOD	< LOD
Macrosporin	< LOD	< LOD
Moniliformin	9.46	28.3
Mycophenolic acid	< LOD	< LOD
N-Benzoyl-Phenylalanine	5.07	3.48
Neoechinulin A	22.6	33.1
Nivalenol	402	399
Ochratoxin A	< LOD	< LOD
Pestalotin	< LOD	< LOD
Physcion	< LOD	< LOD
Questiomycin A	< LOD	< LOD
Quinolactacin A	0.14	0.10
Rugulusovin	5.00	3.58
Siccanol	< LOQ	< LOQ
T-2 toxin	10.1	< LOD
Tentoxin	1.24	2.08
Tenuazonic acid	82.6	40.2
Tryptophol	111	85.3
Xanthotoxin	< LOD	< LOD
Zearalenone	38.1	58.5

[a] The samples 1 and 2 are the replicates of the same batch of oats.

[b] Limit of Quantitation (LOQ)

Paper IV

Prediction of deoxynivalenol toxicokinetics in humans by in vitro-to-in vivo extrapolation and allometric scaling of in vivo animal data

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Abstract

Deoxynivalenol (DON) is the most prevalent mycotoxin in cereals worldwide. It can cause adverse health effects in humans and animals, and maximum levels in food and feed have been implemented by food authorities based on risk assessments derived from estimated intake levels. The lack of human toxicokinetic data such as absorption, distribution, and elimination characteristics hinders the direct calculation of DON plasma levels and exposure. In the present study, we have, therefore, used in vitro-to-in vivo extrapolation of depletion constants in hepatic microsomes from different species and allometric scaling of reported in vivo animal parameters to predict the plasma clearance [0.24 L/(h × kg)] and volume of distribution (1.24 L/kg) for DON in humans. In addition, we have performed a toxicokinetic study with oral and intravenous administration of DON in pigs to establish benchmark parameters for the in vitro extrapolation approach. The determined human toxicokinetic parameters were then used to calculate the bioavailability (50–90%), maximum concentration, and total exposure in plasma, and urinary concentrations under consideration of typical DON levels in grain-based food products. The results were compared to data from biomonitoring studies in human populations.

Keywords

Allometric scaling
Deoxynivalenol (DON)
Human exposure
IVIVE
Pig
Toxicokinetics
Electronic supplementary material

The online version of this article (https://doi.org/10.1007/s00204-018-2220-1) contains supplementary material, which is available to authorized users.

Introduction

Strategies for the prediction of in vivo kinetic data from in vitro biotransformation experiments are well established for pharmaceutical compounds (Obach et al. 1997; Iwatsubo et al. 1997; Ito and Houston 2005). Liver microsomes or hepatocytes of humans and animals are used to metabolise test compounds in vitro under conditions of the first-order kinetics, allowing in vitro-to-in vivo extrapolation (IVIVE) of the hepatic clearance and derived kinetic parameters with the help of species-specific conversion factors (Barter et al. 2007; Smith et al. 2008; Thörn et al. 2011). The determination of essential kinetic parameters such as clearance (CL), plasma half-life ($t_{1/2}$), volume of distribution (V_d), and oral bioavailability (f) is necessary for estimating the exposure and maximum plasma concentration after oral administration of a new drug, which is an important safety issue for the initial trial in human volunteers during phase-I of drug development. The resulting data describing the absorption, distribution, metabolism, and excretion (ADME) of a substance can then be applied as input parameters for *in silico* physiologically based pharmacokinetic (PBPK) modelling, which is used for the simulation of plasma concentration–time profiles (De Buck et al. 2007; Chen et al. 2012). The successful determination of IVIVE data is thus a prerequisite for all subsequent applications, and prediction accuracy within 0.5-fold to twofold of the observed values under consideration of the variation in the observed value is widely regarded as satisfactory (Jolivette and Ward 2005; De Buck et al. 2007; Abduljalil et al. 2014).

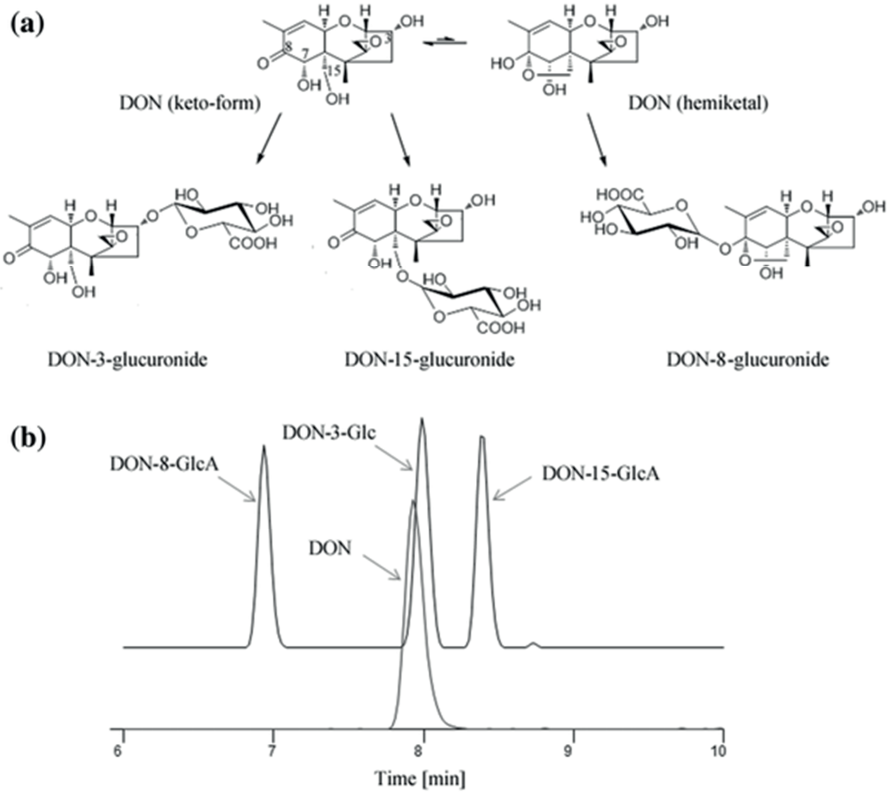
The combination of in vitro extrapolation data with in vivo data from animal studies can improve method predictability (Chiba et al. 2009). The empirical observation that anatomical, physiological, and biochemical characteristics in mammals are exponentially correlated with the individual bodyweights (BW) (Boxenbaum 1982) has been successfully employed for the allometric scaling of pharmacokinetic parameters in drug discovery. Data from at least two or three animal species are needed for the reliable extrapolation of, respectively, human V_d, or CL (Mahmood and Balian 1996), and their BW should span as broad a range as possible (Lindstedt and Schaeffer 2002). The same mechanisms apply for the extrapolation of oxidative cytochrome P450 enzymes-

catalysed phase-I transformations and phase-II UDP-glucuronosyltransferase (UGT) conjugation reactions, both for IVIVE (Soars et al. 2002; Naritomi et al. 2015) and allometric scaling of in vivo animal data (Deguchi et al. 2011).

Even if predictive pharmacokinetics has repeatedly proven its power and value, it has not been employed in toxicokinetic studies of natural toxins so far. The kinetics of the most important mycotoxins have been elucidated in a considerable numbers of in vivo studies in laboratory and domestic animals (Wu et al. 2010; Dänicke and Brezina 2013) and provide the opportunity for further data compilation. Several mycotoxins have been investigated in biotransformation assays, and main metabolites of hepatic metabolism have been identified (Maul et al. 2012). The combination of in vivo and in vitro results and their use in IVIVE and allometric scaling for exposure predictions and risk assessment in humans after unintentional uptake of mycotoxins in food is, however, unexplored. So far, human data are limited to collections of concentrations in urine, faeces, plasma, and breast milk (Waseem et al. 2014) and some in vitro metabolism assays (Warth et al. 2013).

Deoxynivalenol (DON) (Fig. 1a), a B-type trichothecene produced by field-growing *Fusarium* species, is the most prevalent mycotoxin in grain worldwide and occurs regularly in staple food and feed (Nagl and Schatzmayr 2015). The extent of contamination depends on the region of cereal production and is influenced by weather conditions (Bernhoft et al. 2013). Adverse health effects caused by DON in different animals include immunotoxicity, anorexia, damage to the intestinal barrier, neurotoxicity and reproductive toxicity at chronic low-level exposure, and abdominal distress, diarrhoea, and emesis under acute mycotoxicosis (Payros et al. 2016). In humans, several outbreaks of acute DON intoxication have been described with symptoms ranging from nausea, vomiting, and diarrhoea to fevers and bloody stool (Knutsen et al. 2017). Effects of chronical exposure are, however, not sufficiently documented. The main mode of action on the cellular level is the inhibition of protein synthesis by binding to the 60S ribosomal subunit (Pestka 2010). National and international food and feed safety authorities such as the European Food Safety Authority (EFSA) have assessed the risk from DON exposure and implemented maximum levels for DON in feed- and foodstuffs (Knutsen et al. 2017). The tolerable daily intake (TDI) of DON, including its derivatives 3-acetyl-DON, 15-acetyl-DON, and DON-3-glucoside, in humans was set to 1 µg/kg BW/day, and the acute reference dose ARfD to 8 µg/kg BW. Biomonitoring surveys have confirmed that more than 90% of the European population have measurable DON in urine, varying with population group, age, sex, and nationality (Meky et al. 2003; Waseem et al. 2014; Brera et al. 2015). Especially, small children can be exposed to doses that exceed the TDI several times (Sundheim et al. 2017; Knutsen et al. 2017).

Fig. 1
a Molecular structures of the keto and hemiketal conformations of DON and three DON-glucuronide isoforms, **b** UHPLC-HRMS (plasma method) extracted ion chromatograms for DON ([M-acetate]$^-$, *m*/*z* 355.1398) and DON-glucuronide ([M-H]$^-$, *m*/*z* 471.1508) standards



Animals and humans show different sensitivity to DON exposure (Dänicke and Brezina 2013), depending on species-specific biotransformation pathways. DON is eliminated mainly through conjugation to glucuronic acid and urinary as well as faecal excretion (Turner et al. 2009; Wu et al. 2010; Lattanzio et al. 2011; Warth et al. 2013). Major metabolites are DON 3-glucuronide (DON 3-GlcA), DON 15-glucuronide (DON 15-GlcA) (Fig. 1a), and in addition, glucuronidation products of hemiketal and isomeric DON-conformations such as, respectively, DON 8-glucuronide (DON 8-GlcA), iso-DON 3-glucuronide (iso-DON 3-GlCA), and iso-DON 8-glucuronide (iso-DON 8-GlCA) have been reported (Uhlig et al. 2016; Pestka et al. 2017). Ruminants show increased tolerance to DON due to intra-ruminal microbial detoxification to deepoxy-deoxynivalenol (DOM-1) (Wu et al. 2010), which is subject to further regioselective conjugation reactions. Birds extensively produce the sulphate-conjugated DON 3-sulphate and DON 15-sulphate, and

DON-sulphonates (DONS) have been detected in rat and mouse faeces (Wan et al. 2014; Pestka et al. 2017).

DON glucuronides can be produced in in vitro metabolism assays (Maul et al. 2012; Uhlig et al. 2016; Schwartz-Zimmermann et al. 2017). Data on DON toxicokinetics in humans that would allow the prediction of plasma concentrations and exposure for different doses are, however, not available.

It was, therefore, the aim of the present study to provide essential toxicokinetic parameters of this important mycotoxin in humans by combining IVIVE and allometric scaling. For this reason, we have conducted in vitro glucuronidation assays with substituted liver microsomes of different species under linear kinetic conditions and made a comprehensive search of published animal data. In addition, we have performed an in vivo kinetic study with oral and intravenous administration of DON in Norwegian-crossbred piglets, which allowed the direct comparison to results from our pig microsomal assay.

Materials and methods

Chemicals and biological materials

Deoxynivalenol (DON; 3α,7α,15-trihydroxy-12,13-epoxytrichothec-9-en-8-one; $C_{15}H_{20}O_6$; 296.32 g/mol) was obtained from Sigma-Aldrich Norway (Oslo, Norway). Deepoxy-DON (DOM-1) was purchased from Romer Labs (Tulln, Austria), while DON-3-*O*-D-glucuronide (DON-3-GlcA), DON-15-*O*-β-D-glucuronide (DON-15-GlcA), and DON-8-*O*-D-glucuronide (DON-8-GlcA) were available from earlier work (Uhlig et al. 2016). Acetonitrile, methanol, and water (Fisher Scientific, Fair Lawn, NJ, USA) were of Optima™ LC/MS quality. Uridine 5-diphosphoglucuronic acid, uridine 5-diphospho-N-acetylglucosamine, and methoxyamine hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO, USA) and UGT Reaction Mix Solution B containing 250 mM Tris–HCl, 40 mM $MgCl_2$, and 0.125 mg/mL alamethicin in water was from BD Biosciences (Woburn, MA, USA). Human liver microsomes (X008068, Lot SBM, mixed pool of 50 male and female donors), rat (pool of 20 male Wistar rats, M00021, Lot LTH), dog (pool of 6 male Beagle dogs, M00201, Lot HSN), and minipig (pool of 3 male Göttingen minipigs, M00061, Lot GNP) liver microsomes were obtained from Bioreclamation IVT (Baltimore, MD, USA) and stored in liquid nitrogen until use. The P450 content, enzyme activity, and total protein concentration had been determined by the manufacturer. In addition, self-prepared microsomes from female Wistar rats (Uhlig et al. 2016) and male chickens (Ivanova et al. 2014) were used.

Preparation of liver microsomes from Norwegian piglets

Livers were harvested from two weaned 6-week old male Norwegian-crossbred piglets (Landrace-Yorkshire/Duroc-Duroc); 13 and 15 kg body weight (BW) that had been

operated for hernia at the Norwegian University of Life Sciences (NMBU), Oslo, Norway, in accordance with the guidelines set by the Norwegian Animal Research Authority. After 2-day quarantine allowing the elimination of the drugs used in the hernia operation (azaperone, lidocaine, flunixin, and sulphadiazine–trimethoprim), the animals were euthanised by captive bolt pistol. The livers were flushed in situ with physiological saline solution (pH 7.0) by cannulation of the vena porta and washing out blood through an incision in the vena cava inferior. Subsequently, the livers were extricated, cut into pieces of about 100 g, and stored immediately at − 80 °C. Microsomes were prepared under cooling from minced livers by manual tissue homogenisation in ice-cold 0.1 M potassium phosphate buffer (PBS, pH 7.5) with a Potter Elvehjem homogeniser and subsequent differential centrifugation. Cell debris, cell nuclei, and organelles were precipitated twice at 16,000*g* (Beckman Instruments, Palo Alto, CA, USA) for 20 min at 4 °C. The resulting supernatants were centrifuged in polyallomer tubes at 100,000*g* in an ultracentrifuge (Beckman Instruments) for 1 h at 4 °C in an SW41Ti swing-out rotor. Microsomes were resuspended from the precipitate by manual homogenisation in 0.1 M PBS pH 7.5 and stored in aliquots at − 80 °C until further use. The total protein content was determined by Lowry Protein Assay (Bio-Rad Laboratories, Hercules, CA).

DON in vitro toxicokinetics with substituted liver microsomes of different species

Substrate depletion assays measuring the concentration–time course of DON were performed with commercially available rat (RLM), dog (DLM), minipig (MPLM), and human (HLM) liver microsomes under conditions of the first-order kinetics to determine assay half-life ($t_{1/2,\ assay}$). In addition, DON was metabolised with in-house made rat (RLMH), chicken (CLMH) and Norwegian-crossbred piglet (PLMH) microsomes. Microsomal protein (2 mg/mL in assay) from the different species was added to incubation buffer containing 7.4 mM uridine 5-diphosphoglucuronic acid, 50 mM Tris–HCl (pH 7.5), 8 mM $MgCl_2$, 0.3 mM uridine 5-diphospho-*N*-acetylglucosamine, and 25 µg/mL alamethicin in a total assay volume of 0.5 mL. After pre-incubation at 37 °C for 2 min, the reaction was started by adding DON in acetonitrile resulting in assay concentrations of 0.05–5 µM. The acetonitrile fraction in the microsomal incubation system did not exceed 0.9% (Busby et al. 1999). Metabolism reactions were performed at 37 °C in a shaking water bath, and 100 µL aliquots were withdrawn at 0, (2.5), 5, 10, 15, 30, and (60 and 90) min, immediately mixed with ice-cold acetonitrile (1:1) and stored on ice until centrifugation for 5 min at 20,000*g* (Eppendorf, Hamburg, Germany). The supernatant was stored at − 20 °C until analysis by UHPLC-MS/MS. Incubations without microsomes or without DON were included for control of compound stability or background noise, respectively.

DON in vivo toxicokinetics in Norwegian piglets

Norwegian-crossbred piglets, 5 weeks old, six of each sex, males castrated, about 20 kg BW, were obtained from a commercial breeder and allowed to acclimatise for 7 days in the production animal facility of the Faculty of Veterinary Medicine at NMBU. Animals were housed individually in boxes with socialisation-friendly openings on sawdust bedding and had free access to water (drinking cups-H_2O (49,014); Domino AS, Tørring, Denmark). The piglets were weighed and earmarked at study start. They had free access to conventional piglet feed via automatic feeders (K1, Domino AS) until 12 h before study start and 2 h after the application of DON. The feed contained less than 25 µg/kg DON and DON glucosides as measured by UHPLC-HRMS.

Six piglets of both sexes were dosed intravenously with 0.08 mg/kg BW DON in sterile physiological saline solution (0.2 mg DON/mL) via butterfly cannula in the ear vein. Blood was withdrawn at 0, 0.042, 0.083, 0.17, 0.33, 0.67, 1, 1.5, 2, 4, 6, 8, and 24 h from the jugular vein using a 21G cannulae and heparinised 3 mL vacutainers (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). The exact time of each blood withdrawal was recorded. Fresh blood samples (1–2 mL) were stored refrigerated until centrifugation at 1500*g* for 15 min (Eppendorf) and the obtained plasma was stored at − 20 °C until analysis by UHPLC-MS/MS.

Six piglets of both sexes were dosed orally with 0.125 mg/kg BW DON in water (0.1 mg DON/mL) by gavage via an infusion tube behind the tongue. Blood was withdrawn at 0, 0.083, 0.17, 0.33, 0.67, 1, 1.5, 2, 4, 6, 8, and 24 h, and centrifuged and stored as described above. At study end, the animals were euthanised after sedation with azoperone and ketamine by intravenous application of an overdose of pentobarbital.

The in vivo piglet study had been approved by the Norwegian Animal Research Authority.

Preparation of samples from in vitro and in vivo assays for analysis by UHPLC-HRMS

Aliquots from in vitro assays were centrifuged through Costar Spin-X centrifuge Tube Filters 0.22 µm (Corning INC, Corning, NY, USA) at 15,000*g* for 1 min (Eppendorf), transferred to 300 µL fixed- insert HPLC vials (Thermo Fisher Scientific, Waltham, MA, USA) and directly analysed by UHPLC-MS/MS.

Plasma samples (250 µL) were transferred into conical 15 mL plastic tubes (Corning Inc., Corning, NY, USA), mixed with 750 µL acetonitrile, vortexed for 15 s, and sonicated for 5 min. Proteins were precipitated by centrifugation at 2000*g* for 10 min at 4 °C (Beckman Coulter, Brea, CA, USA); supernatants were transferred to 10 mL conical glass tubes and evaporated to dryness at 60 °C using a gentle stream of nitrogen. Dried samples were stored refrigerated, or substituted with 200 µL water, vortexed for 15 s, sonicated for 5 min, transferred to HPLC vials (Thermo Fisher Scientific) and directly analysed by UHPLC-HRMS.

UHPLC-HRMS method for the quantitative analysis of DON and DON metabolites in plasma

Piglet plasma samples were analysed by ultra-high-pressure liquid chromatography high-resolution mass spectrometry (UHPLC-HRMS) on a Q-Exactive™ Hybrid Quadrupole-Orbitrap mass spectrometer equipped with a heated electrospray ion source (HESI-II) and coupled to a Dionex Ultimate 3000 UHPLC system (Thermo Fisher Scientific). The HESI-II interface was operated at 300 °C in the negative ionisation mode, and the parameters were adjusted as follows: spray voltage 4 kV, capillary temperature 250 °C, sheath gas flow rate 35 L/min, auxiliary gas flow rate 10 L/min, and S-lens RF level 55. Data were acquired in the selected ion monitoring (SIM)/data-dependent MS^2 (dd-MS^2) mode targeting $[M - acetate]^-$ ions of DON and deepoxy-DON (*m*/z 355.1387 and 339.1438, respectively) and $[M - H]^-$ ions of the DON glucuronides (*m*/*z* 471.1497) with a quadrupole isolation width of 2 *m*/*z*, and a mass resolution of 70,000 full-width half-maximum (FWHM) at *m*/*z* 200 for SIM. The presence of a target ion above the set threshold intensity of 5×10^3 triggered an MS^2 scan for analyte verification (dd-MS^2) using a normalised collision energy 35%. The mass resolution during dd-MS^2 was set to 17,500 FWHM. The automatic gain control (AGC) target was 5×10^5 ions including a maximum injection time of 250 ms during SIM, whereas, for dd-MS^2, the AGC target was 5×10^4 and the maximum injection time was 200 ms.
Chromatographic separation was achieved at 30 °C on a 100 × 2.1 mm i.d. Acquity UPLC HSS T3 column (1.8 µm; Waters, Milford, MA, USA) with 5 × 2.1 mm i.d. XSelect HSS T3 VanGuard pre-column (2.5 µm, 100 Å, Waters). The injection volume was 6 µL, and samples were eluted using a water (A)/acetonitrile (B) gradient, both phases containing 5 mM ammonium acetate and 0.1% acetic acid, with a flow rate of 0.5 mL/min. The column was eluted isocratically with 100% A for 1 min, and then, a linear gradient was applied increasing linearly to 15% B in 15 min, and to 100% B in 0.5 min. After flushing the column for 2.5 min with 100% B, the mobile phase composition was returned to the initial conditions, and the column was washed for 2.9 min. Xcalibur version 2.2 was used for data processing (Thermo Fisher Scientific). Buffer-based and matrix-matched calibration curves were constructed with standards in the range of 1.5 to 250 µg/L, and the LOD and LOQ were 0.1 and 0.3 µg DON/L plasma, respectively.
DON-related metabolites in piglet plasma were detected in full-scan (FS) mode with a mass resolution of 70,000 FWHM in both positive and negative ion modes using fast polarity switching (scan range m/z 150–1000 for both ion modes), and all-ion fragmentation (AIF) in the negative ion mode (scan range m/z 80–700). AIF was performed using stepped normalised collision energy set to 35 ± 20%. The AGC target was set to 3×10^6 and 1×10^6 during FS and AIF, respectively, including a maximum IT of 200 ms. Standards of DOM-1, DON-3-GlcA, DON-15-GlcA, and DON-8-GlcA allowed the unambiguous identification of the metabolites.

UHPLC-HRMS method for DON and DON metabolites in in vitro samples

Samples from in vitro assays were analysed using the same UHPLC-HRMS instrument as above with identical interface settings, but operated in full-scan (FS) mode with mass ranges of m/z 200–720 in both negative and positive modes. In FS mode, the AGC target was set to 5×10^5 ions with a maximum injection time of 250 ms, whereas, in dd–MS^2 mode, the AGC target was 1×10^5 and with a maximum injection time of 100 ms. The quadrupole mass filter was operated with an isolation window of m/z 2. Chromatographic separation was performed at 30 °C on a 150 × 2.1 mm i.d. Kinetex F5 column (2.6 µm; Phenomenex, Torrance, MA, USA) with KrudKatcherTM Ultra HPLC 0.5 µm in-line filter (Phenomenex). The injection volume was 1 µL, and samples were eluted using a water (A)/methanol (B) gradient, both containing 0.2% formic acid, with a flow rate of 0.25 mL/min. The column was eluted isocratically with 3% B for 1 min, then a gradient was applied increasing linearly to 15% B in 15 min, and finally to 97% B in 3 min. After washing the column for 2 min with 97% B, the mobile phase was returned to the initial conditions and the column was re-equilibrated for 3 min.

Determination of in vitro toxicokinetic parameters

Toxicokinetic parameters were derived from the substrate depletion rate constants (k_e) determined by the regression analysis of measured peak areas of DON versus time curves ($A_{DON}(t) = b + a \times e^{-ke \cdot t}$). Assay half-life ($t_{1/2,assay} = \ln 2/k_e$) and assay clearances ($CL_{assay} = V_{assay} \times k_e$) were calculated under consideration of the assay volume (V_{assay}). Disregarding potential protein binding of DON in the reaction mixture [assuming that the fraction unbound in the assay ($f_{u,assay} \sim 1$)], the determined assay clearances approximated the intrinsic assay clearances ($CL_{int,assay}$), which is a measure of enzyme activity and described by the Michaelis–Menten equation parameters' maximal velocity ($v_{max,assay}$) and reaction constant ($K_{M,assay}$) under the condition that the substrate concentration is well below the K_M value ($CL_{int,assay} = v_{max,assay}/K_{M,assay}$). The individual $K_{M,assay}$ were determined from depletion experiments with different initial DON concentrations by plotting the determined depletion rate constants versus the respective concentrations. The inflection point of the curve in a lin-log plot represented the K_M value, occurring when k_e is half of the theoretical maximum k_0 at infinitesimally low DON concentrations [$k_e = k_{0[DON]\to 0} \times (1 - [DON]/([DON] + K_M)$] (Obach and Reed-Hagen 2002).

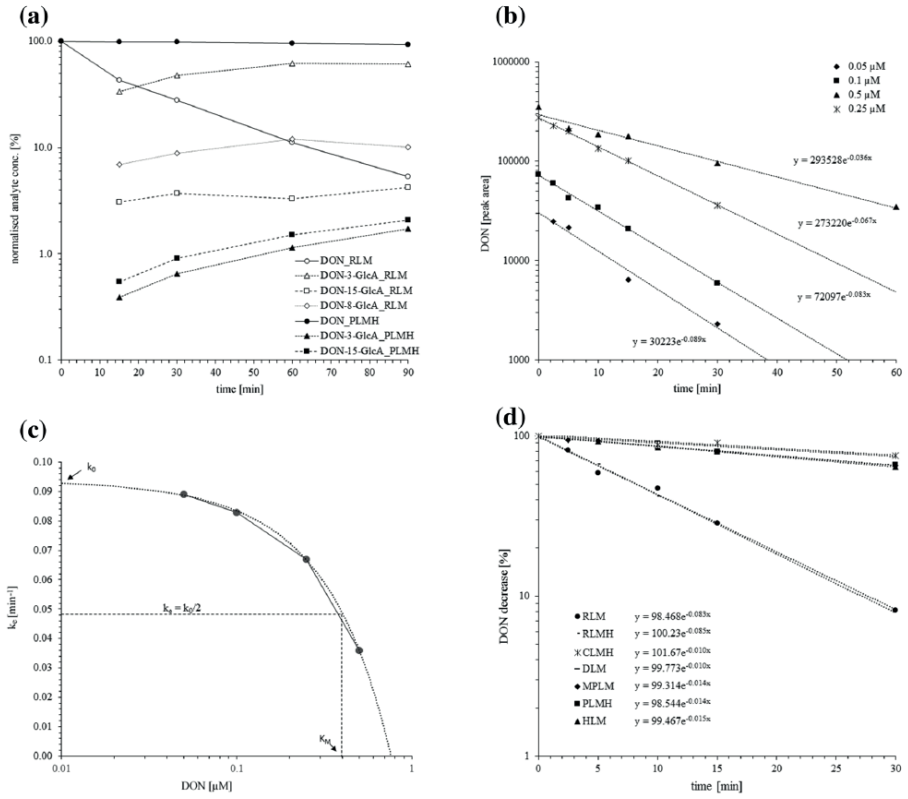
The $CL_{int,assay}$ for RLM, RLMH, CLMH, DLM, MPLM, PLMH, and HML were upscaled to the assay-independent, intrinsic liver clearances ($CL_{int} = CL_{int,assay} \times MRI \times RLW/Prot_{assay}$) by considering the amounts of microsomal protein in the assays ($Prot_{assay}$), species-specific relative liver weights (RLW), and microsomal recovery indexes (MRI) (Table 1). In vitro-to-in vivo extrapolation (IVIVE) was performed by applying the well-stirred liver model (Obach et al. 1997; Ito and Houston 2005) and calculating systemic blood clearances ($CL_{b,vitro}$) from the CL_{int}, while considering the hepatic blood flow (Q) of the different species [$CL_{b,vitro} = Q \times CL_{int} \times f_{u,b}/(Q + CL_{int} \times f_{u,b})$]. The fraction unbound ($f_{u,b}$) in blood of DON in pig, rat, and sheep was higher than 90% (Prelusky et al. 1987, 1988; Meky et al. 2003)

and was, therefore, not considered for the calculation of $CL_{b,vitro}$ ($f_{u,b} \approx 1$). The maximal bioavailability (f_{max}) after oral application was calculated under the assumption of complete absorption from the gastrointestinal tract ($f_a = 1$) as $f_{max} = 1 - CL_{b,vitro}/Q$ and used for estimating low (< 10%), intermediate (10–30%) and high (> 30%) exposure (Obach et al. 1997). Plasma clearances (CL_p) were calculated from the blood clearances ($CL_p = CL_{b,vitro} \times c_p/c_b$) using the blood–plasma partition coefficient (c_b/c_p) of DON, which is close to unity in pig (Prelusky et al. 1987). Maximum exposure after iv application was calculated as $AUC_{iv,norm,max} = 1/CL_p$, and maximum exposure after po application was calculated as $AUC_{po,norm,max} = f_{max}/CL_p$. Comparison of in vitro- and in vivo-derived toxicokinetic data allowed the evaluation of the prediction quality. Twofold differences between predicted and animal experimental clearances were considered as extrapolative inliers fulfilling the success criterion (Jolivette and Ward 2005).

Table 1
Toxicokinetic parameters of DON for different species derived from in vitro microsomal assays

Parameter	**RLM**[a]	**RLMH**[a]	**CLMH**[b]	**DLM**[c]	**MPLM**[d]	**PLMH**[e]	**HLM**[f]
k_e (min^{-1})[g]	0.083	0.085	0.010	0.010	0.014	0.014	0.015
$t_{1/2,assay}$ (min)	8.4	8.2	69	69	50	50	46
$K_{M,assay}$ (μM)[h]	0.40	0.46	1.12	0.13	0.73	0.48	0.21
$CL_{int,assay}$ (mL/min)	0.042	0.043	0.005	0.005	0.007	0.007	0.008
CL_{int} [L/(h × kg)]	6.08	6.22	0.37	0.53	0.41	0.46	0.39
$CL_{b,vitro}$ [L/(h × kg)][i]	2.48	2.51	0.33	0.42	0.35	0.37	0.30
f_{max} (%)	41	40	88	80	87	82	79
$AUC_{iv,norm,max}$ (h × kg/L)	0.40	0.40	3.07	2.36	2.82	2.70	3.33
$AUC_{po,norm,max}$ (h × kg/L)	0.17	0.16	2.66	1.90	2.49	2.21	2.63
[a]MRI = 61 mg/g; RLW = 40 g/kg bw; $Prot_{assay}$ = 1 mg; Q_{rat} = 4.2 L/(h × kg)							
[b]MRI = 35 mg/g; RLW = 35 g/kg bw; $Prot_{assay}$ = 1 mg; $Q_{chicken}$ = 2.7 L/(h × kg)							
[c]MRI = 55 mg/g; RLW = 32 g/kg bw; $Prot_{assay}$ = 1 mg; Q_{dog} = 2.1 L/(h × kg)							
[d]MRI = 41 mg/g; RLW = 24 g/kg bw; $Prot_{assay}$ = 1 mg; $Q_{minipig}$ = 2.6 L/(h × kg)							
[e]MRI = 34 mg/g; RLW = 32 g/kg bw; $Prot_{assay}$ = 1 mg; Q_{pig} = 2.1 L/(h × kg)							
[f]MRI = 40 mg/g; RLW = 22 g/kg bw; $Prot_{assay}$ = 1 mg; Q_{human} = 1.4 L/(h × kg)							
[g]Determined by regression of DON depletion plots (Fig. 2d)							
[h]Derived from depletion plots at different DON concentrations (Fig. 2b)							
[i]Equivalent to CL_p since $c_b/c_p \approx 1$							

Fig. 2
a Formation of DON glucuronides in rat (RLM) and pig (PLMH) liver microsome assays, **b** depletion of DON in rat liver microsomes (RLM), **c** determination of K_M in RLM assay, and **d** DON depletion kinetics in liver microsomes of different species



Determination of in vivo toxicokinetic parameters

Toxicokinetic analysis of the piglet study was performed by non-compartmental analysis using Phoenix 64 WinNonlin 7.0 (Pharsight, St. Louis, MO, USA). Parameters calculated for intravenous or extravascular administration of DON were area under the plasma concentration–time curve (AUC), plasma clearance (CL_p), volume of distribution (V_d), elimination half-life ($t_{1/2}$), time to maximal plasma concentration (t_{max}), and maximal plasma concentration (c_{max}). The absolute bioavailability was calculated from the dose-normalised AUCs as $f = AUC_{po,norm}/AUC_{iv,norm}$. Data were expressed as means of six animals per dose group ± standard deviations (SD). The exact sampling time points were used for each pig and slopes were adjusted individually for the calculation of elimination parameters. The AUCs for DON 3-GlcA and DON 15-GlcA were determined for all pigs, and means and SDs were calculated.

Compilation of toxicokinetic data of DON from published in vivo studies

A literature search was conducted to identify the published DON in vivo studies presenting toxicokinetic data. Using the following search string [deoxynivalenol (Title/Abstract)] AND (dose OR food OR feed OR diet) AND (half-life OR bioavailability OR exposure) in PubMed and Google Scholar numerous studies containing miscellaneous collections of toxicokinetic parameters were identified. In addition, several studies not captured by the search but found in reference lists of included studies, reviews or meta-analyses, or obtained by specific searching were considered. The search was not limited to recent years. All available toxicokinetic data from relevant studies were sorted by species and year of publication.

Allometric scaling and prediction of DON toxicokinetic data in humans

$CL_{b,vitro}$ of different species were correlated through BW-dependent allometric scaling (log CL ~ log BW) (Obach et al. 1997). The same was performed with the available clearance data from in vivo studies and the values were extrapolated to humans. The used BW were either acquired from the respective in vivo studies, or average BW of 0.2 kg for Wistar rats, 1.5 kg chicken, 11 kg for Beagle dogs, 19.6 kg for Göttingen minipigs, 25 kg for growing pigs, and 70 kg for humans were used for scaling of the in vitro data.
Human plasma clearance was calculated from the determined blood clearance using the blood–plasma partition coefficient (c_b/c_p) of DON in pig (Prelusky et al. 1988). Half-life in humans was calculated from the volume of distribution and the estimated plasma clearance, both, determined from IVIVE and from allometric scaling ($t_{1/2} = \ln 2 \times V_d/CL_p$). Tentatively, c_{max} after oral application ($c_{max} = f \times D \times V_d^{-1} \times e^{(-\ln 2 \times t_{max}/t_{1/2})}$) and exposure ($AUC_{po} = D \times AUC_{po,norm}$) were calculated using typical doses (D) in food and the predicted f, V_d and $t_{1/2}$. The concentration of total DON in 24 h urine ($c_{urine\_24\ (total\ DON)} = excretion_{urine\_24\ h} \times D \times BW/V_{urine\_24\ h}$) was estimated based on published renal excretion rates and mean daily urine excretion in adults. An excretion calculator (http://www.clinicalculator.com/english/nephrology/excrea/excrea.htm) was used for normalisation by creatinine content.

Results

Analysis of DON and DON glucuronides by UHPLC-HRMS

Samples from the in vitro kinetic assays and the in vivo pig study were analysed by high-resolution mass spectrometry. The baseline separation of the signals allowed the positive identification of DON and the metabolites DON-3-GlcA, DON-15-GlcA, and DON-8-

GlcA (Fig. 1b). The different microsomal preparations produced species-specific metabolite profiles. While all three DON glucuronides were detected in rat microsome incubations, both DON-3-GlcA and DON-15-GlcA were found in pig (Fig. 2a) and human microsomes, whereas dog microsomes produced only DON-3-GlcA. Small amounts of DON-3-GlcA and DON-15-GlcA were found in chicken microsomes (data not shown). The yields for the respective glucuronides varied with the species. In the rat microsome assay, DON-3-GlcA was the most important metabolite, in contrast to the human microsome assay, where DON-15-GlcA was dominating. In pig microsomes, both metabolites were produced with comparable rates.

Determination of in vitro toxicokinetic parameters of DON for different species

Depletion assays at different DON concentrations were performed with seven microsomal preparations and elimination constants (k_e) were determined by regression analysis for each incubation (Fig. 2b). Using the individual k_e, reaction constants, $K_{M,assay}$ were defined for each microsome species (Fig. 2c). The $K_{M,assay}$ of DON in the chicken glucuronidation assay was notably higher than for the other species and indicated lower enzyme affinity. The k_e from the in vitro assays run under optimised conditions and with DON concentrations below the K_M (Fig. 2d) were subsequently used for the calculation of species-specific kinetic parameters (Table 1). There was a clear difference between the metabolism rates in the rat microsome assays and the other assays. As a result, DON was predicted to be a substance with intermediate clearance in rats, and with low clearance in chickens, dogs, minipigs, pigs, and humans.

Determination of in vivo toxicokinetic parameters in piglets after iv and po application of DON

The toxicokinetic in vivo study in Norwegian-crossbred piglets was performed as a benchmark for the in vitro experiments. The pig liver microsomes had been prepared from animals of the same breed and age. The doses used for intravenous (iv) and oral (po) administration were chosen following published reports. The aim was achieving measurable plasma concentrations of DON and its metabolites, while at the same time avoiding symptoms of acute toxicosis. A single event of marginal emesis occurred at 0.5–1 h after po application in two of six piglets, and at 0.2–1.5 h after iv application in five of six piglets. Due to the negligible extent, it was considered insignificant for the outcome of the study, especially since further feed consumption was normal.

The plasma concentration–time profiles (Fig. 3a, b) were used for the calculation of toxicokinetic parameters by non-compartmental analysis (Table 2). The half-life in plasma was 2.6 h after iv administration. Plasma clearance was low and comparable to the value that was predicted in the in vitro study (Table 1). The volume of distribution of DON was approximately ten times the plasma volume in pig (Setiabudi et al. 1976), indicating moderate dispersion to lower body compartments and/or protein binding. The

maximal plasma concentration after po administration was reached after 3.5 h, and absolute bioavailability was determined as 53%.

Fig. 3
Plasma concentration–time profiles of DON and DON glucuronides in piglets (n = 6) after **a** intravenous application of 0.08 mg/kg BW or **b** oral application of 0.125 mg/kg BW DON

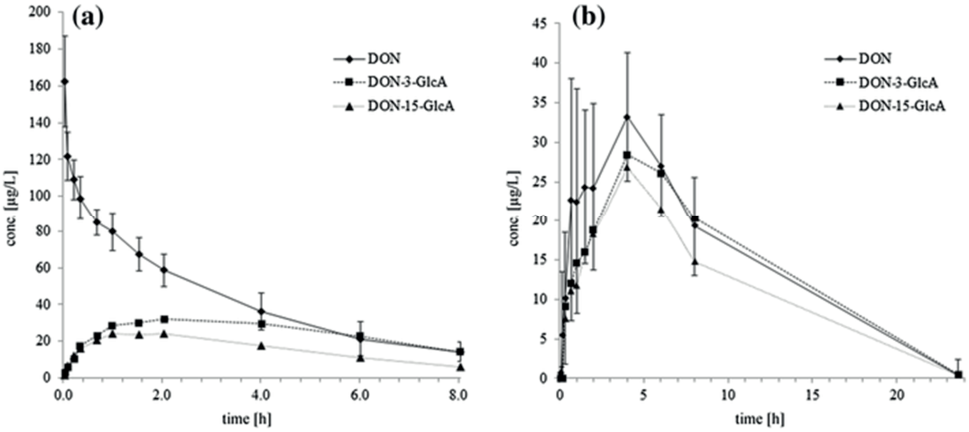


Table 2
Toxicokinetic parameters (means ± SD) of DON and the metabolites DON-3-GlcA and DON-15-GlcA in piglets after intravenous and oral application of DON

Parameter	po (n = 6)						iv (n = 6)					
	DON		DON-3-GlcA		DON-15-GlcA		DON		DON-3-GlcA		DON-15-GlcA	
		SD		SD		SD		SD		SD		SD
Dose (mg/kg)	0.125						0.08					
AUC_{iv} (µg × h/L)							407	97.1				
AUC_{po} (µg × h/L)	335	60	316	147	261	172			242	122	202	121
c_{max} (µg/L)	35.7	6.2	29.9	19.1	27.9	19.5			32.4	12.5	30.0	12.8
t_{max} (h)	3.5	1.7	4.3	0.7	4.0	1.2			1.8	1.0	1.3	0.4
$t_{1/2}$ (h)	3.8	1.0	3.9	1.2	3.7	1.3	2.6	0.6	2.7	0.9	3.3	1.5
CL_p [L/(h × kg)]							0.21	0.05				
V_d (L/kg)							0.70	0.05				
$AUC_{iv,norm}$ (h × kg/L)							5.09	1.21				
$AUC_{po,norm}$ (h × kg/L)	2.68	0.48										
f (%)	52.7											
AUC_{met}/AUC_{DON} (%)			94.3			77.9			59.5		49.6	

The DON metabolites DON-3-GlcA and DON-15-GlcA were detected with the same 10:8 ratio of exposures ($AUC_{DON-3-GlcA}$:$AUC_{DON-15-GlcA}$) both after iv and po application. In comparison to the measured DON exposure (AUC_{DON}), however, the exposure to the DON glucuronide metabolites (AUC_{met}) was 78–94% after po and 50–60% after iv application (Table 2).

Review of published in vivo toxicokinetic parameters of DON in different animal species

A thorough search of in vivo study reports containing toxicokinetic data was performed and the results were listed according to species and publication date (Table 3). In total, information on 13 animal species was collected. The available data were, however, rather fragmented, heterogeneous and incomplete. In many cases, only the excretion of total DON, i.e., the sum of DON and its metabolites, via the urine had been studied. The excretion ratios within 24 h after uptake ranged from < 18% in cows to > 90% in pigs. Measurable urine concentrations were detected in all species, depending strongly on the given dose and route of application. Maximum plasma concentrations after oral application appeared in mice, rats, and birds at less than 1 h, in sheep, pigs, cows, and horses at 2–4 h, and in fish at 8 h. Absolute bioavailabilities calculated from connected iv and po experiments were only determined in a few studies. The recorded values were in the range of < 3% in cow and 8% in sheep, 6–30% in chicken, turkey and pigeon, and 50–100% in pig and piglet. Plasma clearances have been reported for chicken, turkey, pigeon, pig, and sheep. In addition, we have calculated an approximated CL_p for mice from the given values for dose and AUC after intranasal instillation (Table 3). The clearances were low-to-intermediate in comparison with the respective species-specific liver blood flows. Half-lifes in plasma after iv administration ranged from about 0.3 h in birds to 1 h in sheep and 3 h in pig, while half-lifes were prolonged after oral uptake. The described volumes of distribution varied considerably between studies for some species. Mean values were at 0.2 L/kg in sheep ($n = 1$), 2.5 L/kg in chicken ($n = 3$), 3.9 L/kg in turkey ($n = 2$), 1.0 L/kg in piglet ($n = 4$), and 1.9 L/kg in pig ($n = 4$) (Table 3).

Table 3
Published toxicokinetics data of deoxynivalenol in different species

Species	Route	Dose (mg/kg BW) or (mg/kg diet/day)[b,c]	*F* (%) AUC_{po} (µg × h/L)	Excretion (%) CL_p [L/(h × kg)]	V_d (L/kg)	$t_{½}$ (h)	t_{max}[e] (h)	c_{max}[e] (µg/L) c_{urine}[g] (µg/L)	References[k]
Mouse	po	5[a]	600 (3 h)	–	–	7.6	0.5	1300	Azcona-Olivera et al. (1995)
	po	25	–	–	–	–	0.5	–	Yordanova et al. (2003)
	po	5	3100	–	–	–	0.25	1100[d]	Amuzie et al. (2008)

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	in	5	7200	0.69	–	–	0.5	3300[d]	Amuzie et al. (2008)
	po	25	–	–	–	11.8	5–15	12000[d]	Pestka et al. (2008)
	po	5	3150	–	–	4	0.25	1248[d]	Pestka and Amuzie (2008)
	ip	1	–	–	–	–	1	690–806[d]	Clark et al. (2015a)
	ip	1	–	–	–	–	1	770–1141[d]	Clark et al. (2015b)
	ip	1	–	79–90% (24 h)	–	–	–	–	Pestka et al. (2017)
Weanling	po	5	4380	–	–	4	0.25	2228	Pestka and Amuzie (2008)
Rat	po	6	–	15% (72 h)	–	–	–	–	Yoshizawa et al. (1983)
	po	10[a]	–	89% (72 h)[d]	–	–	–	–	Lake et al. (1987)
	po	10[a]	–	89% (72 h)[d]	–	–	–	–	Worrell et al. (1989)
	po	5[a]	–	37% (72 h)	–	–	8	291	Meky et al. (2003)
	Diet	3.57 /kg bw (4d)	–	–	–	–	–	1900–4900[g]	Lattanzio et al. (2011)
	po	2	–	28% (48 h)	–	–	–	–	Nagl et al. (2012)
	po	0.09	–	–	–	–	0.28	2.0	Versilovskis et al. (2012)
	po	0.5[a]/2.5[a]	–	75% (48 h)[d]	–	–	6	42/160[a]	Wan et al. (2014)
	po	2	–	75% (24 h)[d]	–	–	–	–	Schwartz-Zim. et al. (2014)
Fish									
Carp	Diet	1.0 (4w)	–	–	–	–	8	0.7	Pietsch et al. (2014)
Salmon	Diet	6 (8 w)	89.4 (8 h)	–	–	15.1	–	5.7	Bernhoft et al. (2017)
Birds									
Chicken	po	1.3–1.7[a]	< 1	99% (72 h)[d]	–	3.1	2.3	–	Prelusky et al. (1986a)
	po	0.7 (1d)	–	74% (24 h)[d]	–	–	–	–	Lun et al. (1988v
	Diet/po	5.8 (1w)/0.1[a]	–	75% (24 h)[d]	–	(1.5)	1	–	Lun et al. (1989)
	po	2.2[a]	–	79% (24 h)[d]	–	–	–	–	Prelusky et al. (1989)
	Diet	2.5 (35d)	–	–	–	–	–	< 2	Dänicke et al. (2007a)

Table 3
Published toxicokinetics data of deoxynivalenol in different species

Species	Route	Dose (mg/kg BW) or (mg/kg diet/day)[b,c]	*F* (%) AUC_{po} (µg × h/L)	Excretion (%) CL_p [L/(h × kg)]	V_d (L/kg)	$t_{½}$ (h)	t_{max}[e] (h)	c_{max}[e] (µg/L) c_{urine}[g] (µg/L)	References[k]
	Diet	0.095 /kg bw	–	–	–	3.8	2	0.9	Yunus et al. (2010)
	po/Diet	0.15/7.5 (1w)	–	–	–	–	0.7	6.5/3.9	De Baere et al. (2011)
	Diet	0.87/5.0 (5w)	–	–	–	–	–	< 0.7	Awad et al. (2011)
	po	2.8	–	–	–	–	1/5	27/2.6	Yunus et al. (2012)
	Diet	7.5 (1w)	–	–	–	–	–	4	Osselaere et al. (2012)
	po	0.75	–	–	–	0.7	0.6	8–14	Devreese et al. (2012a)
	iv	0.75	–	7.2	5.0	0.5	–	–	**Osselaere et al. (2013)**
	po	0.75	19%	–	–	0.6	0.5	26	**Osselaere et al. (2013)**
	po	2.5[a]	–	81% (24 h)[d]	–	–	6	210[a]	Wan et al. (2014)
	Diet	0.4–1 (70w)	–	–	–	–	–	0.2–0.6	Ebrahem et al. (2014)
	po	0.5	11%	–	–	–	–	–	Broekaert et al. (2014)
	iv	0.5	–	–	–	–	–	–	Broekaert et al. (2014)
	po	0.75	–	–	–	–	0.6	27	Devreese et al. (2015)
	iv	0.75	–	–	–	–	0.4	–	Devreese et al. (2015)
	iv	0.5	–	6.1	1.0	0.3	–	–	**Broekaert et al. (2015)**
	po	0.5	11%	–	–	–	0.5	4.2	**Broekaert et al. (2015)**
	Diet	1.7 (1d)	–	80% (24 h)[d]	–	–	–	–	Schwartz-Zim. et al. (2015)
	po	0.5	5.6%	–	–	–	0.5	7.4	**Broekaert et al. (2017)**
	iv	0.5	–	5.2	1.4	0.3	–	–	**Broekaert et al. (2017)**
Turkey	iv	1, (1[a])	–	0.41	0.3	0.7	–	–	Gauvreau (1991)
	po	1/5, (5[a])	1%	–	–	–	0.2/1	6.7/24	Gauvreau (1991)
	iv	5[a]	–	97% (48 h)[d]	–	–	–	–	Gauvreau (1991)
	Diet	(5.4 po) (2w)	–	–	–	–	–	< 2	Dänicke et al. (2007b)
	Diet	5.2 (12w)	–	–	–	–	–	3	Devreese et al. (2014)

Table 3
Published toxicokinetics data of deoxynivalenol in different species

Species	Route	Dose (mg/kg BW) or (mg/kg diet/day)[b,c]	*F* (%) AUC_{po} (µg × h/L)	Excretion (%) CL_p [L/(h × kg)]	V_d (L/kg)	$t_{½}$ (h)	t_{max}[e] (h)	c_{max}[e] (µg/L) c_{urine}[g] (µg/L)	References[k]
	iv	0.75	–	8.2	7.4	0.6	–	–	Devreese et al. (2015)
	po	0.75	21%	–	–	0.9	0.6	13	Devreese et al. (2015)
	Diet	1.5 (1d)	–	52% (24 h)	–	–	–	–	Schwartz-Zim. et al. (2015)
Pekin duck	Diet	6–7 (49d)	–	–	–	–	–	< 6	Dänicke et al. (2004b)
Pigeon	iv	0.3	–	12.5	5.7	0.3	–	–	**Antonissen et al. (2016)**
	po	0.3	30%	–	–	0.6	0.5	10	**Antonissen et al. (2016)**
Pig	Diet	2.8–5.9 (14w)	–	67% (5d)	–	–	–	–	Friend et al. (1986)
	po	1[a]	64%	–	–	–	3.8	367	**Prelusky et al. (1990)**
	iv	1[a]	–	0.19	1.1	4.1	–	–	**Prelusky et al. (1990)**
	iv	1	–	–	–	3.9	–	–	Prelusky and Trenholm (1991)
	Diet	0.23 (/BW; 16d)	–	–	–	–	–	580[g,h]	Razzazi et al. (2002)
	Diet	2.5 (2.5d)	–	–	–	2.4	2.8	52	Eriksen et al. (2003)
	Diet	4.2 (7d)	–	–	–	5.8	4.1	–	Dänicke et al. (2004a)
	Diet	1.2–4.6 (10w)	–	57% (7d)	–	–	–	14	Dänicke et al. (2004c)
	Diet	9.6 (35d)	–	–	–	–	(1.5)	22	Dänicke et al. (2005a)
	Diet	6.5 (12w)	–	–	–	–	(1.5)	18	Goyarts et al. (2005)
	Diet	5.7 (28d)	89%	–	–	6.3	1.6	22	Goyarts and Dänicke (2006)
	iv	0.053	–	0.23	3.8	4.2–31	–	–	**Goyarts and Dänicke (2006)**
	Diet	5.7 (0.07 po)	54%	–	–	5.2	1.7	15	**Goyarts and Dänicke (2006)**
	po	6.7 (12w)	–	–	–	–	2.2	13–30	Goyarts et al. (2007)
	Diet	9.6 (35d)	–	68% (5 h)[d]	–	–	1.0	9	Dänicke et al. (2007c)

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Species	Route	Dose (mg/kg BW) or (mg/kg diet/day)[b,c]	*F* (%) AUC_{po} (µg × h/L)	Excretion (%) CL_p [L/(h × kg)]	V_d (L/kg)	$t_{½}$ (h)	t_{max}[e] (h)	c_{max}[e] (µg/L) c_{urine}[g] (µg/L)	References[k]
	po	0.05	–	–	–	–	0.4	8.6	De Baere et al. (2011)
	iv (inf)	0.1/h (1 h)	–	0.71[f]	2.1	2.3	1.0	77	Dänicke et al. (2012)
	po	0.05	–	–	–	–	–	30	Devreese et al. (2012b)
	po	0.044	87%	–	–	3.7	1.8	17	Rohweder et al. (2013)
	Diet/inf	3.1(37d)/0.1/h	–	–	–	–	–	7.5/19	Dänicke et al. (2014)
	po	0.075	–	85% (24 h)[d]	–	–	–	–	Nagl et al. (2014)
	iv	0.05	–	0.22	0.6	2.0	–	–	**Paulick et al. (2015)**
	po (Diet)	0.07	99%	–	–	6.1	4.9	28.8	**Paulick et al. (2015)**
	Diet	1–5 (7d)	–	–	–	–	–	0.4–5.3[g,i,h]/c	Thanner et al. (2016)
Piglet	iv	0.5	–	< 57% (24 h)	–	2.1–3.7	–	–	Coppock et al. (1985)
	iv	0.3[a]	–	93%[d], 0.11	1.3	1.6	–	–	Prelusky et al. (1988)
	po	0.6[a]	48–65%	95% (14 h)[d]	–	–	0.8	135–322[a]	Prelusky et al. (1988)
	Diet	0.2–3.9 (35d)	–	–	–	–	–	0.4–12/56–380[g]	Döll et al. (2003a)
	Diet	0.2–3.9 (37d)	–	–	–	–	4.0	1–11/56–380[g]	Döll et al. (2003b)
	Diet	3.2 (12d)	–	–	–	–	–	15.5	Dänicke et al. (2005c)
	Diet	0.55–1.23 (11w)	–	–	–	–	–	5–7	Döll et al. (2008)
	Diet	2.3 (28d)	–	–	–	–	–	11	Dänicke et al. (2010)
	po	0.05	–	–	–	–	1.3	30	Devreese et al. (2014
	iv	0.036	–	0.91	1.2	2.9	–	–	**Broekaert et al. (2015)**
	po	0.036	100%	–	–	–	1.8	6.3	**Broekaert et al. (2015)**
	Diet/po	0.9 (10d)/0.3	–	–	–	–	2.0	168	Alizadeh et al. (2015)
	iv	0.25/0.75	–	–	–	–	0.5	9.2/26.8	Deng et al. (2015)

Table 3
Published toxicokinetics data of deoxynivalenol in different species

Species	Route	Dose (mg/kg BW) or (mg/kg diet/day)[b,c]	*F* (%) AUC_{po} (µg × h/L)	Excretion (%) CL_p [L/(h × kg)]	V_d (L/kg)	$t_{½}$ (h)	t_{max}[e] (h)	c_{max}[e] (µg/L) c_{urine}[g] (µg/L)	References[k]
	iv	0.036	–	0.32	0.6	2.7	–	–	**Broekaert et al. (2017)**
	po	0.036	81%	–	–	–	0.7	24	**Broekaert et al. (2017)**
	Diet	0.09/5.36 (6w)	–	–	–	–	–	< 0.2/31	Paulick et al. (2018)
	Diet	0.09/5.36 (6w)	–	–	–	–	–	3100[g,i,h]/C	Tran et al. (2018)
	iv	0.08	–	0.21	0.7	2.6	–	–	**Fæste et al. (2018[j])**
	po	0.125	53%	–	–	–	3.5	36	**Fæste et al. (2018[j])**
Sheep	iv	0.5	–	–	–	1.1	–	–	**Prelusky et al. (1985)**
	po	5	7.5%	–	–	1.9	4.7	–	**Prelusky et al. (1985)**
	iv	0.5	–	68% (7 h)[d]	–	–	–	–	Prelusky et al. (1986b)
	po	5	–	72% (14 h)[d]	–	–	–	–	Prelusky et al. (1986b)
	iv	4[a]	–	97% (24 h)[d]	–	–	–	–	Prelusky et al. (1987)
	iv	1[a]	–	0.12	0.2	1.1	–	–	Prelusky et al. (1990)
	Diet	0.01 po (7d)	–	–	–	–	1	0.4	Lohölter et al. (2012)
Cow	po	1.9	< 1%	–	–	(4.0)	4.1	90–200	Prelusky et al. (1984)
	Diet	66 (5d)	–	18% (72 h)	–	–	–	–	Côté et al. (1986)
	Diet	3.5 (28d)	< 3%	–	–	–	–	–	Dänicke et al. (2005b)
	Diet	8.2 (3w)	–	96% (24 h)[d]	–	–	2	< 0.5	Seeling et al. (2006)
	Diet	5.3 (8w)	–	–	–	–	3	3.5	Keese et al. (2008)
	Diet	2.6–5.2 (13w)	–	–	–	–	–	1.3/3.6	Winkler et al. (2014)
	Diet	2.6–5.2 (13w)	–	–	–	–	–	1.2/1.3	Winkler et al. (2015a)
	Diet	2.6–5.2 (13w)	–	–	–	–	–	131–242[g,i,h]/c	Winkler et al. (2015b)
	Diet	0.274 /kg bw (9w)	–	–	–	–	–	< 0.8	Dänicke et al. (2016)
Horse	Diet	12 (10d) (0.08 po)	–	–	–	–	7.3	38	Setyabudi et al. (2012)

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Published toxicokinetics data of deoxynivalenol in different species

Species	Route	Dose (mg/kg BW) or (mg/kg diet/day)[b,c]	*F* (%) AUC_{po} (µg × h/L)	Excretion (%) CL_p [L/(h × kg)]	V_d (L/kg)	$t_{½}$ (h)	t_{max}[e] (h)	c_{max}[e] (µg/L) c_{urine}[g] (µg/L)	References[k]
	Diet	7.9 (21d) (0.05 po)	–	–	–	–	3	5.2	Schulz et al. (2015)
Human	Diet	0.3–1.4 × 10^{-3}/d/kg	China	–	–	–	–	12[g,h,d]	Meky et al. (2003)
	Diet	1.1–7.4 × 10^{-3}/d/kg	China	–	–	–	–	37[g,h,d]	Meky et al. (2003)
	Diet	26 g cereals	UK	–	–	–	–	0.6[g,i,h,d]/c	Turner et al. (2008a)
	Diet	322 g cereals	UK	–	–	–	–	7.2[g,i,h,d]/c	Turner et al. (2008a)
	Diet	0.3 × 10^{-3} in 197 g	UK	–	–	–	–	8.9[g,i,h,d]/c	Turner et al. (2008b)
	Diet	107–300 g cereals	UK	–	–	–	–	5.4–9.3[g,i,h]/c	Turner et al. (2008c)
	Diet	180 g cereals	UK	–	–	–	–	7.5[g,h,d]	Turner et al. (2009)
	Diet	20 × 10^{-3} in 400 g	France	–	–	–	–	0.5–28.8[g,d]	Turner et al. (2010a)
	Diet	11 × 10^{-3} in 206 g	UK	72% (24 h)[d]	–	–	–	10[g,i,h,d]	Turner et al. (2010b)
	Diet	206 g cereals	UK	–	–	–	–	2.4[g,h]	Turner et al. (2011a)
	Diet	45 g cereals	China	–	–	–	–	5.9[g,i,h,d]/c	Turner et al. (2011b)
	Diet	0.5–1 × 10^{-3}/d/kg	Austria	–	–	–	–	30[g,h,d]	Warth et al. (2011)
	Diet	–	Italy	–	–	–	–	3.0–8.0	Lattanzio et al. (2011)
	Diet	–	Italy	–	–	–	–	3.7[g,h]	Solfrizzo et al. (2011)
	Diet	–	Iran	–	–	–	–	1.5[g,i,h,d]/c	Turner et al. (2012)
	Diet	0.2/2.4 × 10^{-3}/d/kg	UK	–	–	–	–	0.5–43[g,i,d]/c	Hepworth et al. (2012)
	Diet	ca. 200 g cereals	Austria	–	–	–	–	22[g,h,d]	Warth et al. (2012)
	Diet	–	Egypt	–	–	–	–	0.5–59.9[g,i]/c	Piekkola et al. (2012)
	Diet	–	Portugal	–	–	–	–	16.3[g,h,d]	Cunha and Fernandes (2012)
	Diet	–	Belgium	–	–	–	–	0.7–68.3[g,i]/c	Ediage et al. (2012)
	Diet	2.3 × 10^{-3}/d/kg	Austria	68% (24 h)[d]	–	–	–	8–11, 39[g,d]	Warth et al. (2013)

Table 3
Published toxicokinetics data of deoxynivalenol in different species

Species	Route	Dose (mg/kg BW) or (mg/kg diet/day)[b,c]	*F* (%) AUC_{po} (μg × h/L)	Excretion (%) CL_p [L/(h × kg)]	V_d (L/kg)	$t_{½}$ (h)	t_{max}[e] (h)	c_{max}[e] (μg/L) c_{urine}[g] (μg/L)	References[k]
	Diet	$2.5–5.4 \times 10^{-3}$/d/kg	Sweden	–	–	–	–	0.5–178[g,i,d]/c	Wallin et al. (2013)
	Diet	$0.9–33 \times 10^{-3}$/d/kg	Croatia	–	–	–	–	7–903[g,i,d]/c	Šarkanj et al. (2013)
	Diet	$0.8–33 \times 10^{-3}$/d/kg	S. Africa	–	–	–	–	20.4[g,i,h,d]/c	Shepard et al. (2013)
	Diet	$0.2–2.6 \times 10^{-3}$/d/kg	Cameroon	–	–	–	–	6.0[g,i,h,d]/c	Abia et al. (2013)
	Diet	$0.2–0.6 \times 10^{-3}$/d/kg	Europe	–	–	–	–	> 1[g] (0–5%)	EFSA (2013)
	Diet	$0.2–0.5 \times 10^{-3}$/d/kg	UK	–	–	–	–	7.2–20[g,i]/c	Gratz et al. (2014)
	Diet	0.3×10^{-3}/d/kg	Nigeria	–	–	–	–	2.0[g]	Ezekiel et al. (2014)
	Diet	< 4.2 μg/kg maize	Ivory Coast	–	–	–	–	< 0.8–10[g,d]	Kouadio et al. (2014)
	Diet	0.5×10^{-3}/d/kg	Germany	68% (24 h)[d]	–	–	–	21[g,i,h,d]/c	Gerding et al. (2014)
	Diet	0.4×10^{-3}/d/kg	Spain	–	–	–	–	15–32[g,i,d]/c	Rodriguez-Carr. et al. (2014)
	Diet	0.6×10^{-3}/d/kg	Italy	–	–	–	–	12[g,h]	Solfrizzo et al. (2014)
	Diet	–	Thailand	–	–	–	–	7.2[g,i,h,d]/c	Warth et al. (2014)
	Diet	49×10^{-3}/d/kg	Spain	72% (24 h)	–	–	–	17.1[g,i,h]/c	Rodriguez-Carr. et al. (2015)
	Diet	$0.3–4.4 \times 10^{-3}$/d/kg	Haiti	–	–	–	–	20.2[g,h,d]	Gerding et al. (2015)
	Diet	111–135 g cereals	Sweden	–	–	–	–	4.4[g,i,h]/c	Wallin et al. (2015)
	Diet	397–456 g maize	Tanzania	–	–	–	–	40–49[g,i,d]/c	Gong et al. (2015)
	Diet	–	Europe	–	–	–	–	4.6–39[g,i,d]/c	Brera et al. (2015)
	Diet	$0.06–10 \times 10^{-3}$/d/kg	Belgium	–	–	–	–	6.1[g,i,h]/c	Heyndrickx et al. (2015)
	Diet	–	Belgium	–	–	–	–	59[g,h,d]	Huybrechts et al. (2015)
	Diet	$0.05–7.2 \times 10^{-3}$/d/kg	Bangladesh	–	–	–	–	0.2–7.2[g,d]	Ali et al. (2015)
	Diet	–	Germany	–	–	–	–	1.1–13.4[g,i]/c	Föllmann et al. (2016)
	Diet	–	Sweden	–	–	–	–	0.5–135[g,d]	Turner et al. (2016)
	Diet	–	UK	–	–	–	–	38–40[g,i,h,d]/c	Wells et al. (2016)

Table 3
Published toxicokinetics data of deoxynivalenol in different species

Species	Route	Dose (mg/kg BW) or (mg/kg diet/day)[b,c]	*F* (%) AUC_{po} (µg × h/L)	Excretion (%) CL_p [L/(h × kg)]	V_d (L/kg)	$t_{½}$ (h)	t_{max}[e] (h)	c_{max}[e] (µg/L) c_{urine}[g] (µg/L)	References[k]
	Diet	6×10^{-3}/d/kg	Bangladesh	–	–	–	–	0.2–1.8[g]	Ali et al. (2016)
	Diet	$268–975 \times 10^{-3}$/d/kg	Germany	–	–	–	–	0.2–38.4[g]	Ali et al. (2016)
	Diet	$0.4–1.5 \times 10^{-3}$/d/kg	Spain	–	–	–	–	1.1[g,i,h]/c	Vidal et al. (2016)
	Diet	–	UK	–	–	–	–	12.7–40[g,i,h,d]/c	Wells et al. (2017)
	Diet	$0.5–1.0 \times 10^{-3}$/d/kg	UK	–	–	–	–	19–25[g,i,h,d]/c	Papageorgiou et al. (2018)
	Diet	$1.0–1.4 \times 10^{-3}$/d/kg	China	–	–	–	–	27.8–45.1[g,h,d]	Deng et al. (2018)
	Diet	0.3×10^{-3}/d/kg	Nigeria	–	–	–	–	0.1–6.2[g]	Šarkanj et al. (2018)
	Diet	1 µg/kg BW bolus	Belgium	45–73%(24 h)[d]	–	–	–	–	Vidal et al. (2018)
Children									
	Diet	$0.1–2.9 \times 10^{-3}$/d/kg	Cameroon	–	–	–	–	0.1–77[g]	Ediage et al. (2013)
	Diet	$0.5–1.0 \times 10^{-3}$/d/kg	Europe	–	–	–	–	> 1[g] (0.4–46%)	EFSA (2013)
	Diet	$0.1–0.3 \times 10^{-3}$/d/kg	Tanzania	74% (24 h)[d]	–	–	–	2.3–5.7[g,h,d]	Srey et al. (2014)
	Diet	0.8×10^{-3}/d/kg	Spain	–	–	–	–	28[g,i,h,d]/c	Rodriguez-Carr. et al. (2014)
	Diet	257 g maize	Tanzania	–	–	–	–	48[g,i,h,d]/c	Gong et al. (2015)
	Diet	–	Europe	–	–	–	–	14–42[g,i,h,d]/c	Brera et al. (2015)
	Diet	$0.1–20 \times 10^{-3}$/d/kg	Belgium	–	–	–	–	5.5[g,i,h]/c	Heyndrickx et al. (2015)
	Diet	$1.0–2.0 \times 10^{-3}$/d/kg	UK	–	–	–	–	32–56[g,i,h,d]/c	Papageorgiou et al. (2018)
	Diet	$2.1–3.1 \times 10^{-3}$/d/kg	China	–	–	–	–	63–73[g,h,d]	Deng et al. (2018)
	Diet	0.3×10^{-3}/d/kg	Nigeria	–	–	–	–	1.5–5.0[g]	Šarkanj et al. (2018)

[a]Radiolabelled (^{14}C)-DON, total radioactivity measured

[b]Units for po or iv studies: mg/kg BW; units for DON application via the diet (feeding studies): mg/kg feed/d; units for human studies: g food/d or mg DON/ food or estimated daily exposure (PMTDI): mg DON/d/kg BW

[c]Duration of feeding studies: d = days, w = weeks

[d]Total amount (all DON species)

[e]Plasma concentration, mean of study individuals

[f]Endotoxaemic pigs

[g]Concentration in urine

[h]Mean concentration (of n study participants) in urine: first-voided morning, 24 h-collected or 48 h-collected urine

[i]ng DON/mg creatinine in urine (/c)

Table 3
Published toxicokinetics data of deoxynivalenol in different species

Species	Route	Dose (mg/kg BW) or (mg/kg diet/day)[b,c]	*F* (%) AUC_{po} (µg × h/L)	Excretion (%) CL_p [L/(h × kg)]	V_d (L/kg)	$t_{½}$ (h)	t_{max}[e] (h)	c_{max}[e] (µg/L) c_{urine}[g] (µg/L)	References[k]
[j]This manuscript									
[k]References are listed in Supplement; references in bold type include toxicokinetic studies with complete data sets									

Comparison of in vitro predicted and in vivo determined plasma clearances

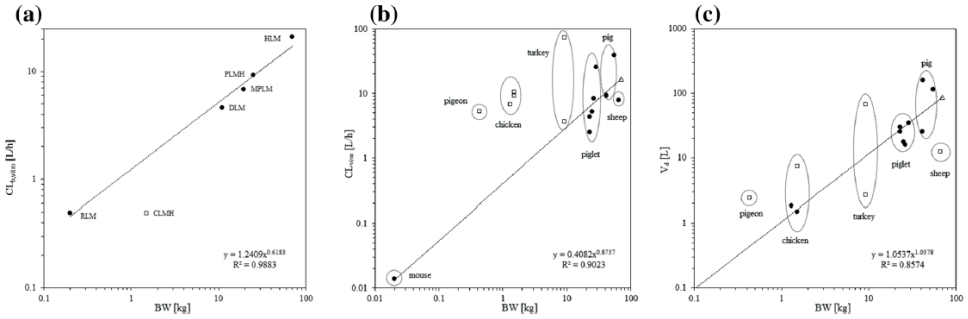
The heterogeneity of the publishedin vivo data (Table 3) and the limitation of the in vitro data to available liver microsomes (Table 1) was a handicap for the comparison. Nevertheless, the performance of the piglet toxicokinetics study (Table 2) in the present project allowed the direct alignment with the predicted parameters from incubations with microsomes prepared from the same pig breed. The in vitro $CL_{b,vitro}$ = 0.37 L/(h × kg), equal to the $CL_{p,vitro}$ under consideration of $c_p/c_p \approx 1$, was within the 0.5-fold to twofold range of the in vivo CL_p = 0.21 L/(h × kg) (Jolivette and Ward 2005). This match demonstrated the applicability of the substituted liver microsomal assay and the IVIVE computations to imitate DON biotransformation in pigs, which gives reason to expect a similar outcome for other species with glucuronidation as the main metabolisation pathway of DON. In contrast, the prediction of the clearance in chicken was unsuccessful. While the in vitro assay predicted a low $CL_{b,vitro}$ = 0.33 L/(h × kg) (Table 1), DON was cleared in vivo very efficiently with CL_p = 6.12 L/(h × kg) (mean of n = 3 studies) (Table 3). This mismatch indicated that glucuronidation is not the predominant metabolism pathway of DON in chicken.

Extrapolation of human DON toxicokinetics using in vitro and in vivo data from different species

The DON depletion data obtained in the human microsome assay were used for the direct prediction of human clearance [$CL_{b,vitro}$ = 0.30 L/(h × kg)] and maximal bioavailability (f_{max} = 79%) by IVIVE (Table 1). Allometric scaling of the in vitro predicted $CL_{b,vitro}$ from six species (Fig. 4a) showed good correlation (R^2 = 0.9883) with the exception of the chicken value, which was consequently excluded. This divergence pointed at alternative biotransformation pathways in this species, in line with the detected lower affinity (increased $K_{M,assay}$, Table 1) of DON to chicken glucuronosyltransferases and the lack of correspondence between predicted in vitro and measured in vivo clearance.

Fig. 4
Allometric scaling of published DON: **a** in vitro clearances, **b** in vivo clearances, and **c** in vivo volumes of distribution. Filled circles indicate values that were included into the

correlation, unfilled squares show unconsidered data, and the unfilled triangle represents the predicted human value



The allometric scaling of plasma clearances from in vivo studies (Table 3) showed a similar deviation for chicken, turkey, and pigeon (Fig. 4b). When the data from the three published chicken studies were considered, the correlation coefficient dropped to $R^2 = 0.5119$ (data not shown). We decided, therefore, to exclude the chicken, pigeon and turkey clearances, the latter coming from two each other contradicting studies. This approach resulted in $R^2 = 0.9023$ and a predicted human $CL_p = 0.24$ L/(h × kg) (Fig. 4b; Table 4) that was close to the CL_p predicted by IVIVE. The allometric scaling of the published volumes of distribution resulted in a human $V_d = 1.24$ L/kg with correlation $R^2 = 0.8574$ (Fig. 4c; Table 4). It was performed with all available data (Table 3), excluding, respectively, one chicken, turkey, pigeon, and sheep study.

Table 4
Predicted DON toxicokinetics in humans and exposure from grain consumption

Parameter	Humans	Sources
CL_b [L/(h × kg)]	0.30/0.24	$CL_{b,vitro}$ (Table 1)/allom. scal. (Fig. 4b)
c_b/c_p (blood/plasma coeff.)	1.01	From pig (Prelusky et al. 1988; Table 3)
CL_p [(L/(h × kg)]	0.30/0.24	Calculated from CL_b (Table 4)
V_d (L/kg)	1.24	Allom. scal. (Fig. 4c)
$t_{1/2}$ (h)	2.9/3.6	Calculated from CL_p and V_d (Table 4)
t_{max} (h)	2.0	Estimated from pig (Table 3)
f (%)	50–90	f_{max} (Table 1) and estimated from pig (Table 3)
$AUC_{po,norm(,max)}$ (h × kg/L)	2.63/2.08–3.75	CL_p, f_{max} (Table 1)/CL_p allom. scal., f (Table 4)
$excretion_{urine\_24\ h}$ (%, total DON)	70	Exposure studies (Table 3)
dose (mg/kg)	0.2-2.0 × 10^{-3}	Exposure studies (Sundheim et al. 2017; Table 3)

Table 4
Predicted DON toxicokinetics in humans and exposure from grain consumption

Parameter	Humans	Sources
c_{max} (µg/L)	0.1-1.0	Calculated from $t_{1/2}$, f, dose, V_d, t_{max} (Table 4)
c_{max_ARfD} (µg/L)	2.0-5.8	Calculated from $t_{1/2}$, f, ARfD[a], V_d, t_{max} (Table 4)
$AUC_{po,max}$ (µg × h/L)	0.5–5.4	Dose (Table 4), $AUC_{po,norm,max}$ (Table 1)
AUC_{po} (µg × h/L)	0.4–7.5	Dose (Table 4), $AUC_{po,norm}$ (Table 4)
$c_{urine\_24\ h\ (total\ DON)}$ (µg/L)	4.9–49	Dose, $excretion_{urine\_24\ h}$, V_{urine_24h}[b], BW (Table 4)
$c_{urine\_24\ h\ (total\ DON)}$/c (ng/mg creatinine)	3.0–30	$c_{urine\_24\ h\ (total\ DON)}$, creatinine $excretion_{\_24\ h}$[c] (Table 4)
[a]8 µg/kg/day (Knutsen et al. 2017)		
[b]2.0 L/day, mean daily urine excretion for adult		
[c]1610 mg/day for adult male, 70 kg BW		

Prediction of human exposure from consumption of cereals

The determination of the fundamental toxicokinetic parameters CL_p and V_d by IVIVE and allometric scaling was the prerequisite for the prediction of depending parameters such as $t_{1/2}$ and c_{max} (Table 4). Using both values for human CL_p and V_d, half-life in human plasma was estimated as 2.9 to 3.6 h, which is comparable to $t_{1/2}$ in pig studies (Table 3). The absolute bioavailability of DON in humans was assumed to be high and in the range of f = 50% to 90%, in congruence with the in vitro determined maximal bioavailability f_{max} = 79%. The combination of the different values for CL_p and $f_{max)}$ yielded a dose-independent AUC after oral uptake in the range of $AUC_{po,norm(,max)}$ = 2.08–3.75 h × kg/L (Table 4).

Human exposure (AUC_{po}) to DON from the consumption of cereal-based food products was estimated using doses derived from mean low and mean high intake in a risk assessment on DON for different age groups in Norway that was based on typical occurrence and food consumption data (Sundheim et al. 2017; Table 3). The predicted exposures were AUC_{po} = 0.4–0.5 µg × h/L for low consumers and AUC_{po} = 5.4–7.5 µg × h/L for high consumers of cereals (Table 4). Accordingly, maximum DON concentrations in plasma were calculated as c_{max} = 0.1 – 1.0 µg/L, under consideration of two doses, the predicted range for $t_{1/2}$ and an estimated t_{max}. Using the acute reference dose, ARfD = 8 µg/kg, as intake resulted in c_{max_ARfD} = 2.0–5.8 µg/L.

The excretion of total DON via the urine within 24 h after low and high intake via the diet was predicted for Norwegian adults using a mean excretion ratio (70%) that was derived from published human biomonitoring studies (Table 3). Both, the calculated absolute $c_{urine\_24\ h\ (total\ DON)}$ = 4.9–49 µg/L and the creatinine content-normalised $c_{urine\_24h\ (total\ DON)}$/c = 3.0–30 ng/mg (Table 4) were in the range of data observed in human studies in Europe (Table 3; Brera et al. 2015).

Discussion

Mycotoxins are omnipresent in food and feed, posing an immanent risk for human and animal health. Occurrence and toxic potential of the most prevalent mycotoxins have been studied, and levels for maximum concentrations in consumables have been implemented by authorities in many countries. Tolerable daily intakes (TDI) are based on animal toxicity data and the application of a safety margin (Knutsen et al. 2017). Toxicokinetic data that would directly link intake, exposure and toxic effects, are, however, not available for humans and cannot be determined in vivo due to ethical reasons.

This gap can be bridged by the application of in vitro-to-in vivo extrapolation (IVIVE) and allometric scaling, which are well-established concepts for the prediction of kinetic parameters in humans in preclinical drug discovery (Iwatsubo et al. 1997; Chiba et al. 2009; Chen et al. 2012). In the present study, we have, therefore, ventured to transfer this approach to mycotoxins. Using the extensively studied deoxynivalenol (DON), the most common mycotoxin in cereals, as model compound, we could draw on experiences and data from numerous in vivo animal experiments. They delivered the database for the allometric scaling, while we developed the format for in vitro metabolism assays under kinetic conditions for IVIVE for this project.

Since glucuronidation has been shown to be the major biotransformation pathway for DON in most species (Payros et al. 2016), we decided to base our IVIVE approach on this phase-II conjugation reaction and established an incubation system with liver microsomes substituted with UDP-glucuronosyltransferase (UGT) co-substrates. The use of microsomes instead of primary hepatocytes in in vitro metabolism studies has the advantage of greater availability, practicability (storage), and comparability (reduced inter-individual variability through pooled livers from several individuals) but reduces the number of observable conversions. The formation of DON-sulphates, as reported in birds, depends on cytosolic sulphotransferases and was, therefore, not detectable in the microsomal, membrane-bound system. Likewise, the production of DOM-1 through reductive de-epoxidation by microbiota was not considered. In vivo DON sulphonation, which has been observed in rodents (Wan et al. 2014; Pestka et al. 2017), is a rare metabolic pathway with unclear mechanism. It was potentially associated with the addition of glutathione to an unsaturated ketoaldehyde in the substrate, and was thus not covered by thein vitro experiments in the present study.

The hepatic glucuronidation pattern of DON varies between species, which has been shown consistently in in vivo (Lattanzio et al. 2011; Schwartz-Zimmermann et al. 2017) and in vitro (Maul et al. 2012; Uhlig et al. 2016) studies. In addition, there are differences related to sex, age, health status, diet, or environment (Pestka et al. 2017; Chen et al. 2016). The DON-metabolising UGTs belong to an enzyme superfamily that is found ubiquitously in living organisms and has a widely ranging spectrum of endogenous and exogenous substrates. Genetic polymorphism is known for several UGTs and isoforms

are expressed tissue-specifically (Guillemette 2003). Using recombinant human UGTs, it was demonstrated that DON-15-GlcA is predominantly produced by UGT2B4 and DON-3-GlcA by UGT2B7, one of the most important hepatic UGTs (Maul et al. 2015). Both enzymes occur in different variants in Caucasian, Hispanic, African, and Asian populations (Guillemette 2003), which might be one reason for the observed geographical variability in DON metabolism (Chen et al. 2016). However, considering this diversity, the data on animal and human DON glucuronides from different reports are astonishingly congruent, and were also confirmed in this study by our in vitro and in vivo experiments. DON-3-GlcA is the main glucuronidation product in mouse, rat, fish, and dog, whereas DON-15-GlcA is predominant in humans, and the levels of both metabolites are similar in pig (Maul et al. 2012; Nagl et al. 2014; Uhlig et al. 2016; Schwarz-Zimmermann et al. 2017). In contrast, only traces of DON-3-GlcA have been found in chicken and turkey, where the main biotransformation product is DON-3-sulphate (Maul et al. 2012; Devreese et al. 2015).

Although we followed the formation of DON glucuronides in the different in vitro metabolism assays, with equivalent outcome as in a previous study (Maul et al. 2015), our main objective was the determination of species-specific elimination constants k_e as basis for the IVIVE calculations. The assays were run under linear conditions with DON start concentrations below the individual $K_{M,assay}$, preventing an underestimation of the intrinsic enzyme activities, i.e., the assay clearances $CL_{int,assay}$, which is especially critical for high-affinity (low $K_{M,assay}$) substrates (Iwatsubo et al. 1997). The $K_{M,assay}$ were comparable for all species with the exception of chicken, indicating a lower affinity of DON to avian UGTs, and reflecting the in vivo situation. The inclusion of microsomes from two separate preparations of, respectively, male and female Wistar rats in the experiment showed that there was no sex-related difference in the in vitro DON-elimination rates. Similarly, in a mouse study, the slight sex-dependant disparity observed in the formation of individual DON glucuronides became insignificant, when the sum of all metabolites was considered (Pestka et al. 2017).

The depletion half-life in the liver microsomal assays were used for the prediction of hepatic clearances by IVIVE using well-established parameters and models for the upscaling of data from enzyme activity in the assay to the intact organism (Iwatsubo et al. 1997; Ito and Houston 2005; Naritomi et al. 2015). The extrapolated blood clearances ($CL_{b,vitro}$) were low for chicken, dog, minipig, pig, and human, and intermediate for rat. Consequently, the predicted maximum bioavailabilities after oral uptake were very high for all species aside from rat. Reviews on the success rate of extrapolations from human hepatic microsomes in drug discovery, however, have shown a systematic underprediction of in vivo clearances that is particularly relevant for substances with low $CL_{b,vitro}$ (Iwatsubo et al. 1997; Soars et al. 2002; Chiba et al. 2009; Naritomi et al. 2015). Factors contributing to the bias are on one hand assay-related such as non-specific binding, enzyme inhibition, or loss of metabolic activity, and, on the other hand, dependent on variations of CL_b in humans due to genetic polymorphism, dietary habits,

smoking, alcohol consumption, or medication. Prediction accuracy can also be compromised if a substance is extensively metabolised in extrahepatic tissues or is substrate to multiple biotransformation pathways. Nevertheless, we considered DON as a good choice for exploring the applicability of IVIVE in mycotoxinology, because the molecule is hydrophilic, binds little to plasma proteins (Prelusky et al. 1987, 1988), and eliminates mainly through glucuronidation. Furthermore, the existence of toxicokinetic data from in vivo animal studies allowed direct comparison of predicted and measured parameters for several species without being restricted to humans.

In this context, the in vivo study in Norwegian-crossbred piglets was performed to allow direct comparison with results from the in vitro assay that was carried out with microsomes prepared from exactly the same breed. In this way, we excluded several of the factors threatening success in IVIVE. Comparison of the results showed agreement of the predicted and measured clearances, proving the suitability of the approach (De Buck et al. 2007; Chen et al. 2012; Abduljalil et al. 2014). The toxicokinetic data determined in the present experiment fitted well to findings in previous pig studies (Dänicke and Brezina 2013). DON is highly bioavailable ($f > 50\%$) and cleared slowly ($CL_p < 0.5$ L/(h × kg); $t_{1/2} > 2.5$ h), with small differences between piglets and adult pigs. The considerable conformity between studies performed in several European countries indicated that DON toxicokinetics in pigs was little influenced by the different breeds used in the respective experiments. Assuming that the same is valid for in vitro metabolism methods, this could imply extended applicability of IVIVE for DON.

The comprehensive survey of animal and human studies containing in vivo toxicokinetic parameters of DON was performed to generate a database for comparison with IVIVE data and for interspecies allometric scaling. Regarding the reported high-clearance values in several chicken studies, it was evident that the prediction of $CL_{b,vitro}$ from the chicken microsomal assay had failed. This was, however, not surprising, since, in avian species, DON is cleared mostly as DON sulphate (Devreese et al. 2015). In this sense, the missed match between results from in vitro glucuronidation and in vivo sulphation confirmed the specificity and reliability of the microsomal UGT assay. Allometric scaling of the IVIVE-predicted $CL_{b,vitro}$ from different species confirmed that DON is metabolised differently in chicken. Whereas correlation was good for rat, dog, minipig, pig, and human data, the predicted chicken $CL_{b,vitro}$ did not fit.

Allometric scaling of the compiled in vivo CL_p from different species showed that the data for chicken, turkey, and pigeon had to be removed to achieve good correlation. Including only animals with glucuronidation as predominant elimination pathway for DON, we were able to extrapolate to human clearance using the standard power-law equation $CL_p = a \times BW^b$ with the allometric exponent $b = 0.87$. This value is in the expected range, since metabolism-related body functions such as oxygen consumption, cardiac output, and minute ventilation at rest have been shown to scale in average with $b = 0.75$ in species with bodyweights from 4 g to 4000 kg (Lindstedt and Schaeffer 2001). Allometric scaling of clearances takes advantage of the finding that fundamental

physiological mechanism in mammals are size-dependent, so that human data can be derived (Boxenbaum 1982; Mahmood and Balian 1996; Deguchi et al. 2011). The good allometric fit of the DON in vivo clearances was remarkable, because the underlying data set actually fulfilled the criteria of including at least three species but was clearly imbalanced by the great number of pig studies. The predictability of a substance's human CL_p from allometry can be obstructed by several determinants, including high lipophilicity (octanol–water partition coefficient, ClogP > 2) and great differences in plasma binding between species (Jolivette and Ward 2005; Tang and Mayersohn 2006). Both factors are, however, unproblematic in case of DON. Moreover, even if the DON in vivo clearance values were low, the CL_p correlation was apparently not compromised, although allometric scaling works generally better for high clearance than for low clearance substances.

The allometrically determined human CL_p for DON [0.24 L/(h × kg)] was close to the $CL_{p,vitro}$ predicted by IVIVE [0.30 L/(h × kg)], fulfilling the success criterion of < twofold deviation (De Buck et al. 2007; Abduljalil et al. 2014). We decided, therefore, to stay with the basic BW-based allometric scaling and to not include correction factors such as brain weight (BrW), maximum lifespan potential (MLP), or glomerular filtration rate (GFR), which have been applied in different studies with varying outcomes (Nagilla and Ward 2004).

The prediction of a substance's volume of distribution in humans is based on animal V_d allometry or on in silico physiologically based modelling under consideration of in vitro determined tissue–plasma partitioning coefficients (Mahmood and Balian 1996; De Buck et al. 2007; Chen et al. 2012). V_d can be divided into small < 0.7 L/kg, moderate 0.7–3.5 L/kg, and large > 3.5 L/kg categories (Jolivette and Ward 2005), reflecting to which extent the substance is dispersed into the different body compartments. Since distribution is mainly dependent on the substance's physical properties, resulting in typical tissue binding properties, it correlates with tissue mass and body weight, and it is unaffected from potential differences in the metabolism. Thus, V_d categorisation is generally stable in different species, and the exponent of the allometric equation for V_d is close to unity ($b \approx 1$) (Mahmood and Balian 1996; Lindstedt and Schaeffer 2001).

Regarding the published in vivo studies on DON toxicokinetics, the reported V_d in pigs and chicken were of moderate size. Allometric scaling predicted likewise a moderate human V_d (1.24 L/kg) with the allometric exponent $b = 1.04$, fulfilling the condition of unity. The number of species included met the minimum requirement (Mahmood and Balian 1996), and the correlation was sufficient, although pig data were dominant. Sheep, pigeon, and turkey V_d had to be excluded from the allometry as they were either inconclusive (two diametrically different values for turkey) or disproportionally high (pigeon) and low (sheep), indicating dissimilar binding properties of DON in these species. However, pigs are regarded as the most suitable animal model for DON exposure in humans (Nagl et al. 2014), which supported our approach and gave confidence in the predicted V_d.

Surveys in different human populations in Europe have shown that the amount of cereal consumption is the strongest significant determinant of urinary DON levels (Turner et al. 2009; Brera et al. 2015). In consequence, DON levels in urine have been used to estimate exposure from food by applying different dietary models (Heyndrickx et al. 2015). Due to the lack of human toxicokinetic data on DON, there are, however, many uncertainties regarding the assessment of risk from dietary intake. Using our predicted values for human CL_p and V_d, we have calculated plasma half-life and bioavailability after oral uptake of DON. Furthermore, we estimated total exposure (AUC_{po}) and the maximum plasma concentration under consideration of a range of typical DON concentrations in grain-based food products (Sundheim et al. 2017; Knutsen et al. 2017). The resulting figures were in the same range as parameters reported in pigs that had received comparable doses; reference values from human studies have never been recorded. Applying the same dose range and the mean urinary excretion rate from human biomonitoring experiments, we calculated the expected DON concentrations in adult urine, which fitted well to observed values.

In conclusion, the combination of IVIVE and allometric upscaling of in vivo animal data allowed the prediction of important human toxicokinetic parameters of DON that were successfully applied to calculate plasma and urine concentrations after oral dietary exposure.

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Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical standards

This article does not contain clinical studies or patient data. This article does not contain any studies with human participants performed by any of the authors. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution at which the studies were conducted.

Electronic supplementary material

Below is the link to the electronic supplementary material.
Supplementary material 1 (DOCX 35 KB)

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Supplement

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Errata

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