On the metabolism and toxicokinetics of deoxynivalenol and zearalenone in endotoxaemic pigs

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To my family

Table of Contents

List of Abbreviations

(Accounts for chapters 1. Background, 2. Aims of Study, and 6. General Discussion and Conclusion)

°C	Degree Celsius
ACCP/SCCM	American college of chest physicians/society of critical care medicine
ALP	Alkaline phosphatase
ALT	Alanin-aminotransferase
APP	Acute phase proteins
APR	Acute phase reaction
AST	Aspartate transaminase
α-ZAN	Alpha-zearalanol
α-ZEN	Alpha-zearalenol
BW	Body weight
β-ZAN	Beta-zearalanol
β-ZEN	Beta-zearalenol
DOM-1	De-epoxy-deoxynivalenol
DON	Deoxynivalenol
EFSA	European Food Safety Authority
FTH	Iron-sequestering ferritin H chain
γGT	Gamma-glutamyl transferase
GIT	Gastrointestinal tract
Hg	Mercury
Ht	Hematocrit
i.m.	Intramuscular
i.p.	Intraperitoneal
i.v.	Intravenous

IgA	Immunoglobulin A
IgM	Immunoglobulin M
IL	Interleukin
kPa	Kilo Pascal
Kyn/Try	Kynurenine/tryptophan ratio
LD	Lactate dehydrogenase
LD100	Lethal doses
LD50	Median lethal dose
LPS	Lipopolysaccharides
MAP	Major acute phase protein
MAPKs	Mitogen-activated protein kinases
MCH	Mean corpuscular hemoglobin
MCV	Mean corpuscular volume
MRI	Max Rubner-Institute
NF-κB	Nuclear factor-κB
PAMP	Pathogen associated molecular patterns
RBC	Red blood cells
SIRS	Systemic inflammatory response syndrome
SRD	Swine respiratory disease
Teer	Transepithelial electrical resistance
TLR	Toll-like receptors
TNF	Tumor necrosis factor
UDP-GT1-A1	Uridine 5'-diphospho glucuronosyltransferase 1 A1
WBC	White blood cells
ZAN	Zearalanone
ZEN	Zearalenone

Summary

The contamination of grains with the mycotoxins deoxynivalenol (DON) and zearalenone (ZEN) constitutes a threat to human and animal health since these mycotoxins frequently occur in relevant quantities in harvested grains. Due to prevention strategies prior to harvest aimed at minimizing the risk of grain contamination by theses toxins chronic exposure at low doses is a typical practical scenario rather than cases of acute intoxications. Moreover, large species differences are observed and especially pigs are highly sensitive. Low-dose chronic exposure to DON and ZEN possibly results in decreased feed-intake and disturbed reproduction, respectively, inflicting economical losses. Furthermore, in pig production different infectious and non-infectious diseases are observed. In many of these diseases an acute-phase-reaction (APR) as a strong innate immune response pattern is initiated whereby the liver as a central detoxifying and metabolizing organ plays a pivotal role. Therefore, under practical circumstances the co-occurrence of a chronic mycotoxin exposure with DON and ZEN, and an APR, due to for example a bacterial infection, is a possible scenario. However, only little is known on the interactions of these pathomechanisms, as well as about the role of the liver in this scenario. Therefore, a project was conducted where the clinical, systemic, metabolic, and immunological response, and the alterations in the toxicokinetics of DON and ZEN were investigated collectively during a lipopolysaccharide (LPS)-induced APR in pigs which where chronically exposed to a diet comprising naturally DON containing maize co-contaminated by low levels of ZEN and further mycotoxins. In the context of toxic effects, the role of DON is putatively predominant in the applied experimental setup since it exerts a much higher cytotoxicity compared to ZEN and is discussed to interact with the sequence and magnitude of the APR besides its generally higher level in the diet. In the experimental design a special focus was laid on the role of the liver as main detoxifying organ.

This thesis arose out of this holistic project, focusing on two aspects: 1. the systemic response to an endotoxin challenge under the influence of a chronic DON and ZEN exposure, and *vice versa*; and 2. the metabolism of DON and ZEN in the organism during an endotoxin challenge.

The project involved one central trial including 44 ten weeks old barrows (German Landrace with an initial mean body weight (BW) of 25.8 ± 3.7 kg) which were divided in two feeding groups over a period of 29 days. One group was fed with an almost DON-free diet (CON) while the other group received a mycotoxin contaminated diet (4.59 mg DON and 0.22 mg ZEN per kg feed; daily feed allowance was increased continuously over the course of the trial to attain a voluntary intake of

1400 g feed per day in both feeding groups). During experimental days 24 until 29, barrows were housed in metabolism crates and on day 27 surgically equipped with indwelling catheters in *Vena jugularis externa* (post-hepatic) and *V. lienalis* (pre-hepatic) for infusion, and *V. jugularis interna*, *V. portae hepatis* and *Arteria carotis communis* for simultaneous blood sampling from different blood vessels and parallel infusion. Together with the classification according to the levels of dietary and both infusion sites (post- or pre-hepatic) with LPS or NaCl infusion alternately, the complete study comprised six experimental groups. On the experimental day (day 29) the animals were either infused with LPS (7.5 µg/kg BM dissolved in 0.9% NaCl, *Escherichia coli* O111:B4) or a control solution (NaCl 0.9%) 15 min after feeding for one hour. Blood sampling occurred 10x in the period of 180 min after start of the infusion. Thereafter at minute 195 the animals were sacrificed, and different organ weights were recorded, and various specimens collected.

Blood samples were analyzed for blood gases, electrolytes, glucose, pH, lactate and red hemogram. The red hemogram and electrolytes were not affected by DON and LPS. DON-feeding solely decreased portal glucose uptake. The LPS infusion induced an APR which was indicated by a decreased blood partial oxygen pressure (pO_2), pH and base-excess and an increase in blood lactate, which was furthermore mirrored in the clinical signs. The lactic acidosis was more pronounced in the DON-fed, jugular LPS infused group than in CON-fed counterparts. It was therefore concluded that the DON-feeding aggravated the porcine acid-base balance in response to a subsequent immune stimulus dependent on its exposure site (pre- or post-hepatic).

The metabolism of DON and ZEN in the organism during an endotoxin challenge was investigated in two different studies, one focusing on the pre-absorptive (intestinal) and the other on the post-absorptive (systemic) metabolism of DON and ZEN. Upon dissection, in all LPS-infused animals higher amounts of dry matter were recovered in the small intestines irrespective of LPS-entry site suggesting a reduced gastric emptying and a decreased gastrointestinal motility under endotoxaemic conditions. DON metabolism in the gastrointestinal tract (GIT) remained unaltered by treatments and included an increase in the proportion of DOM-1 along the GIT, particularly from distal small intestine to rectum. Variables describing ZEN metabolism suggest a stimulated biliary release of ZEN and its metabolites in LPS-infused groups. In conclusion, the GIT metabolism of ZEN was markedly influenced in endotoxaemic pigs whereby a jugular induction of an APR was more effective than portal LPS infusion hinting at a strong hepatic first-pass effect.

On the systemic side, DON concentrations in jugular and portal blood decreased in both LPS-infused groups, whereas the ZEN concentrations increased, regardless of the treatment site. In liver tissue, a decrease of both toxin concentrations was observed in endotoxaemic pigs as well as a drop in hepatic conjugation, regardless of LPS-entry site. In contrast to the central hypothesis of the project, DON and ZEN were not differently altered depending on the LPS-entry site. Neither the absorption nor the accumulation of DON and ZEN in different tissues differed significantly between animals which were infused with LPS via either the jugular or portal vein.

Placing these results in the findings of the other studies conducted within the scope of the entire research project, it can be concluded that the symptoms of an LPS induced APR were altered in animals chronically exposed to DON and ZEN, as well as aggravated when LPS was infused systemically. The results confirm the hypotheses, that the systemic response to an endotoxin challenge is pronounced differently according to the entry route of LPS (pre- or post-hepatic), due to the “first-pass-effect” of the liver, and that a chronic mycotoxin exposure has an influence on this pathogenesis. Furthermore, the results confirm the toxicokinetics-altering nature of an APR, especially in case of ZEN.

On a practical level, these results imply that a chronic exposure to DON and ZEN alters the initial inflammatory response and that the enrichment of ZEN and metabolites in the enterohepatic cycle could possibly enhance the negative effects of an inflammatory response due to a triggered release of ZEN in the systemic circulation upon an APR.

Zusammenfassung

Die Kontamination von Getreide mit den Mykotoxinen Deoxynivalenol (DON) und dem meist gleichzeitig auftretenden Zearalenon (ZEN) stellt nach wie vor ein ernst zu nehmendes Risiko für die Gesundheit von Menschen und Tieren dar, da diese Toxine regelmäßig in relevanten Konzentrationen in Getreideernten vorzufinden sind. Akute Intoxikationen kommen aufgrund verschiedener Regelungen, Kontrollmechanismen und verbesserten Verfahrenstechniken in der Nutztierfütterung jedoch äußerst selten vor. Es besteht ein starker Unterschied in der Sensibilität zwischen den verschiedenen Tierarten. Vor allem bei Schweinen werden immer wieder ökonomische Verluste verzeichnet, da bei einer Verfütterung von geringeren als den maximal empfohlenen Konzentrationen zwar kein relevanter Übertrag von DON und ZEN ins Lebensmittel (Fleisch) geschieht, es beim Tier jedoch zu einer Reduktion der Futteraufnahme (bei DON) und einer verringerten Fruchtbarkeit (bei ZEN) kommen kann. Deswegen stellen auch eine chronische Intoxikation mit geringen Mengen DON und ZEN ein mögliches Risiko für die Tiergesundheit dar. Zusätzlich sieht sich das Tiergesundheitsmanagement in der Schweineproduktion regelmäßig mit verschiedenen Infektionskrankheiten konfrontiert. In der Pathophysiologie dieser Erkrankungen spielen die Akute-Phase-Reaktion (APR), die Aktivierung des Immunsystems und die Leber als zentrales detoxifizierendes Organ eine wichtige Rolle. Es kann davon ausgegangen werden, dass ein zeitgleiches Auftreten einer chronischen Intoxikation mit den Myktoxinen DON und ZEN und einer APR, welche beispielsweise durch eine bakterielle Infektion ausgelöst wurde, ein realistisches regelmäßig vorkommendes Szenario darstellt. Jedoch sind die möglichen Interaktionen auf der Ebene des Immunsystems, der Toxinmetabolisierung und des Krankheitsverlaufes noch unzureichend erforscht. In einem groß angelegten Forschungsprojekt sollte die klinische, systemische, metabole und immunologische Antwort und Veränderung in der Toxikokinetik der Mykotoxine DON und ZEN unter Einfluss einer APR in Schweinen, welche einer chronischen Mykotoxin kontaminierten Diät ausgesetzt wurden, untersucht werden. Im Versuchsaufbau wurde dabei die Rolle der Leber im Speziellen berücksichtigt.

Diese Dissertation bearbeitet Teilaspekte aus diesem ganzheitlichen Projekt, und beinhaltet zwei Hauptthemen: 1. Die systemische Antwort auf eine Endotoxin-Challenge, unter dem Einfluss einer chronischen DON- und ZEN-Fütterung; und komplementär dazu: 2. Der Einfluss einer Endotoxin-Challenge auf die Metabolisierung von DON und ZEN.

Hierfür wurde im Rahmen des Projektes ein groß angelegter *in vivo* Versuch durchgeführt. Vierundvierzig Eber (10 Wochen alt, Deutsche Landrasse, Lebensmasse (LM): 25,8 ± 3,7 kg) wurden während mehrerer Versuchsperioden, welche jeweils 29 Tage betrugen, randomisiert in zwei Fütterungsgruppen unterteilt. Eine Gruppe erhielt eine nahezu Mykotoxin unbelastete Ration (CON), wohingegen die andere Gruppe mit einer natürlich kontaminierten Ration (4,59 mg DON and 0,22 mg ZEN pro kg Futter) versorgt wurde. Die tägliche verfügbare Menge Futter wurde kontinuierlich über den Versuch gesteigert, um eine freiwillige Aufnahme von mindestens 1400 g pro Tag zu erreichen. An den Versuchstagen 24 bis 29 wurden die Tiere in Stoffwechselkäfigen gehalten. Am Tag 27 wurden operativ verschiedene Verweilkatether eingesetzt: in der *Vena jugularis externa* (post-hepatisch) und der *V. lienalis* (pre-hepatisch) zur Infusion und in der *V. jugularis interna*, der *V. portae hepatis* und der *Arteria carotis communis* für die simultane Blutprobenahme aus verschiedenen Blutgefäßen bei paralleler Infusion. Durch die Unterteilung in zwei Fütterungsgruppen, zwei Infusionslokalisationen (pre- oder posthepatisch) mit wechselweise Lipopolisaccharid-, Kontrollinfusion oder in beide Lokalisationen nur Kontrollinfusion, beinhaltete die Studie sechs Versuchsgruppen. Am Versuchstag (Tag 29) wurden die Tiere 15 min nach der Fütterung entweder mit LPS (7.5 µg/kg LM gelöst in 0.9% NaCl, *Escherichia coli* O111:B4) oder einer Kontrolllösung (NaCl 0,9 %) während 60 min infundiert. In der gesamten Zeit, von 30 min vor dem Infusionsstart bis 180 min danach, wurden 10 Blutproben entnommen. Final, zum Zeitpunkt 195 min (nach Infusionsstart), wurden die Tiere euthanasiert und verschiedene Organe/Proben entnommen.

Um die systemische Antwort auf eine Endotoxin-Challenge unter dem Einfluss einer chronischen DON- und ZEN-Fütterung zu untersuchen, wurden Blutgase, Elektrolyten, Glukose, pH, Laktat und das rote Blutbild in den Blutproben bestimmt. Das rote Blutbild und die Elektrolyten wurden weder durch die Mykotoxinfütterung noch durch die LPS-Infusion beeinflusst. Die DON-Fütterung führte jedoch generell zu einer geringeren Glukose-Absorption. Die LPS-Infusion induzierte erfolgreich eine APR, was sich durch eine verringerte Blutsauerstoffsättigung (pO_2), eine Absenkung sowohl des Blut-pHs als auch des Basenüberschusses („base-excess“), sowie einen Anstieg im Blut-Laktat und im klinischen Erscheinungsbild der Tiere äußerte. Die Laktat-Azidose war jedoch ausgeprägter in den DON-gefütterten, jugular LPS-infundierten Tieren gegenüber ihren Pendants, welche eine Kontrollration erhielten. Dies führte zu der Schlussfolgerung, dass eine chronische Fütterung mit DON und ZEN die negative Verschiebung des Säure-Base-Haushalt

unter einem Immunstimulus abhängig von der Ausgangslokalisation (pre- oder post-hepatisch) unterschiedlich verschärft.

Die Metabolisierung von DON und ZEN im Organismus unter Einfluss einer Endotoxin-Challenge wurde in zwei einzelnen Manuskripten betrachtet, welche sich jeweils auf den pre-absorptiven (intestinalen) sowie den post-absorptiven (systemischen) Metabolismus von DON und ZEN fokussierten. Während der Sektion der Tiere wurde bei allen LPS-infundierten Tieren ein höherer Anteil Trockensubstanz im Magen festgestellt, was auf eine reduzierte Magenentleerung und gastrointestinale Motilität schließen lässt. Der Metabolismus von DON über den Verlauf des gastrointestinalen Traktes (GIT) unterschied sich nicht zwischen den einzelnen Versuchsgruppen, mit einer Zunahme von De-epoxy-DON über den Verlauf des GIT, vor allem zwischen Dünndarm und Rektum. Die verschiedenen Merkmale, welche den GIT-Metabolismus von ZEN beschreiben, weisen auf eine erhöhte Entleerung von biliärem ZEN und Metaboliten in den LPS-infundierten Tieren hin. Zusammengefasst konnte die Schlussfolgerung gezogen werden, dass der GIT Metabolismus von ZEN in endotoxämischen Tieren stark beeinflusst war, wobei eine stärkere Ausprägung in den jugular-infundierten Tieren auf einen starken „first-pass“-Effekt der Leber deutet. Systemisch wurde eine Reduktion der DON-Konzentration und gleichzeitig ein Anstieg der ZEN-Konzentration in beiden LPS-infundierten Gruppen beobachtet. In der Leber nahm die Konzentration beider Mykotoxine und deren Konjugation unter Einfluss von LPS ab. Im Gegensatz zur zentralen Hypothese des Projektes konnte kein Unterschied zwischen pre- und post-hepatisch infundierten Tieren beobachtet werden. In keinem der untersuchten Organe bzw. Gewebe konnte eine unterschiedliche Absorption und/oder Akkumulation von DON und ZEN unter jugularem oder portalem LPS-Einfluss aufgezeigt werden.

Bringt man diese Ergebnisse in den größeren Kontext des Projektes, bzw. setzt man diese in Zusammenhang mit den Ergebnissen aus den weiteren Studien, welche Aspekte der Klinik, Immunologie und Zellfunktion näher betrachtet haben, so kann zusammenfassend die Schlussfolgerung gezogen werden, dass die Symptome einer LPS-induzierten APR durch eine chronische Exposition von DON und ZEN verändert und durch eine post-hepatische Infundierung deutlich verschlimmert werden. Die Ergebnisse bestätigen die Hypothese, dass die systemische Antwort auf eine Endotoxin-Challenge sich anhand der Expositionsroute (pre- oder post-hepatisch), aufgrund des „first-pass“ Effektes der Leber, unterscheidet und dass eine chronische Mykotoxin Fütterung einen Einfluss auf diese Pathogenese hat. Des Weiteren bestätigen die

Ergebnisse, dass sich unter einer APR die Toxikokinetik von Mykotoxinen verändert. Dies konnte vor allem für ZEN und seine Metaboliten gezeigt werden.

Für die Praxis bedeutet dies, dass eine chronische Fütterung mit DON und ZEN kontaminiertem Futter die Prozesse einer initialen Entzündungsreaktion verändert und dass möglicherweise die plötzliche systemische Anflutung von ZEN und dessen Metaboliten während einer APR den Verlauf und die negativen Auswirkungen einer Entzündungsreaktion verstärken könnte.

1. Background

1.1. General Introduction

Although in the last decades the planting, harvesting, processing, and the storage of cereal grains were improved by continuous technological development in agriculture, the contents of mycotoxins in relevant quantities still represents a concern in human and animal nutrition (EFSA, 2013, 2017). Among the different mycotoxins found in food and feed deoxynivalenol (**DON**) is of major importance and owed to its co-occurrence also zearalenone (**ZEN**) is considered relevant (EFSA, 2004a, 2004b). In 2017 the Max Rubner-Institute (**MRI**) in Detmold has reported on average 149 (<1-6,395; min-max) µg/kg DON in wheat and 164 (<1-2,959) µg/kg DON in rye samples from Germany. Also ZEN was found in detectable amounts in 90% of the wheat and in 75% of the rye samples (BLE, 2018).

Both mycotoxins, DON and ZEN, are very stable, and are therefore not easily eliminated during feed processing (Döll and Dänicke, 2011). The main sites for absorption and/or disappearance are the stomach and the small intestines, and both toxins are metabolized by micro-organisms to either non-toxic or toxic metabolites (see chapter 1.2.1.). Therefore, major differences in mycotoxin metabolism between monogastric and polygastric animals are observed (Dänicke and Winkler, 2015; Prelusky et al., 1994).

Especially in pig production the mycotoxin contamination of feed is of large relevance due to the high sensitivity of pigs to DON and ZEN (Dänicke and Brezina, 2013; Dänicke and Winkler, 2015). Their impact mainly concerns economic losses, due to performance decreasing effects by reducing feed intake (DON) and disturbing reproduction (hormonal effect of ZEN; Dänicke and Winkler, 2015; Wu and Munkvold, 2008; see chapter 1.2.2.). There is, however, only little transfer (carry-over) of DON and ZEN residues from the feed to the animal product. Therefore, the contamination of the feed itself does not indirectly constitute a risk to human health (Dänicke et al., 2010; Dänicke and Winkler, 2015; EFSA, 2017; Zinedine et al., 2007). A regulation ordering the discard of feed comprising DON and ZEN concentrations exceeding a limiting threshold would imply, due to its common occurrence, a regular disposal of unreasonable amounts of grain. Therefore, the European Commission has issued a concentration guideline for these mycotoxins, but not a limiting threshold, implying that contaminated feed may still be diluted among uncontaminated feed (European Commission, 2006). Consequently, in practice low concentrations of DON and ZEN are regularly encountered in animal feed due to the blending of contaminated with uncontaminated

cereals. Hence, even though acute effects of these toxins are barely observed, chronic effects of a long-time low-dose exposure are possible due to the general occurrence at low levels of these mycotoxins in feedstuffs and need to be investigated.

The liver as main detoxifying organ holds a central role in keeping the body in homeostasis in relation to xenobiotic substances (Gibaldi et al., 1971; Jirillo et al., 2002). This role may be affected by an immune response and the immune response itself could be affected by a mycotoxin long- or short-term exposure *vice versa* (Döll et al., 2009b; Döll et al., 2009c). In several studies DON and ZEN were found to affect the immune system (Dänicke et al., 2013; Döll et al., 2009a; Döll et al., 2009c; Marin et al., 2010; Marin et al., 2011; Pestka and Smolinski, 2005; Stanek et al., 2012, see chapter 1.2.2.). Moreover, physical traumata, stress, and infections by different viruses or bacteria are known to induce immune responses such as an acute phase reaction (**APR**, see chapter 1.3) in pigs (Martínez-Miró et al., 2016; Sabourin et al., 2002; Zimmerman et al., 2012). However, the interrelations between chronic mycotoxin exposure, and inflammatory and detoxifying processes are only beginning to be understood (Stanek et al., 2012).

Therefore, we conducted a large project in which different aspects of a chronic oral DON and ZEN exposure in the presence of an APR, induced by lipopolysaccharides (**LPS**) infusions, were investigated. Especially the role of the liver was considered in the study design. The project involved the following main working hypotheses:

1. The liver as the main detoxifying organ plays a central role in the metabolism and elimination of the substances which are coming from the intestinal tract through the portal vein. Besides the detoxifying function, the liver itself is also a target of toxic substances.
2. A pre-exposure of the organism to DON and ZEN leads to a priming of the immune system which is causing a less pronounced reaction to a subsequent stimulus. As an immunologically active organ the liver constitutes a specific role in this context.
3. DON and/or ZEN affect the epithelial metabolism, cell cycles and the intestinal barrier. They therefore alter the amount of toxic substances and organisms entering the liver.

The present thesis has resulted from this holistic project. It mainly focuses on the metabolic aspects investigated in the respective trail, such as the effects of a chronic mycotoxin exposure on blood gas level during an acute LPS challenge and the mycotoxin digestion, distribution, and excretion during such a challenge (see chapter 2. Aims of Study).

1.2. The Mycotoxins Deoxynivalenol (DON) and Zearalenone (ZEN) and their Metabolites

Systematically, DON belongs to the Type B trichothecenes, produced by different fungi such as *Fusarium*. DON is one of the most common mycotoxins in cereal production and was first isolated in Japan and described by Morooka et al. (1972). Even in temperate climate zones DON is ubiquitously found in grain (Gajęcka et al., 2018). Especially wheat, triticale, and maize are vulnerable for *Fusarium* infections (Döll and Dänicke, 2011).

Zearalenone (or F-2 toxin) is a nonsteroidal estrogenic mycotoxin biosynthesized through a polyketide pathway. It is formed by a variety of *Fusarium* fungi, including *F. graminearum (Gibberella zeae), F. culmorum, F. cerealis, F. equiseti, F. crookwellense* and *F. semitectum*. They occur as common soil fungi in temperate and warm countries, and they are regular contaminants of cereal crops worldwide (Bennett and Klich, 2003). Even if they are mainly produced before harvest, their formation is likely to continue under poor storage conditions (Rohweder et al., 2011).

1.2.1. Physico-chemical Attributes and Toxicokinetics

The pure solid DON or "12,13-epoxy-3α,7α,15-trihydroxy-trichothec-9-ene-8-one" builds colorless fine needles and it is soluble in polar organic solvents (e.g., aqueous methanol, ethanol, chloroform, acetonitrile, ethyl acetate) and water. At 151-153 °C DON starts to melt. It exhibits a molecular weight of 296.32 and the chemical formula is $C_{15}H_{20}O_6$ (Rotter et al., 1996).

DON owns its toxicity mainly to its very stable 12,13-epoxide-group. During the metabolism of DON this epoxide group is lost which leads to the metabolite de-epoxy-deoxynivalenol (**DOM-1**) causing an extensive reduction in toxicity (Fig.1, Eriksen et al., 2004).

https://doi.org/10.1007/s00216-013-6792-6

Fig. 1: Chemical structures of deoxynivalenol (DON) and de-epoxy-deoxynivalenol (DOM-1).
Copied from Li et al. (2013). *Springer Nature: Analytical and Bioanalytical Chemistry; Water-based slow injection ultrasound-assisted emulsification microextraction for the determination of deoxynivalenol and de-epoxy-deoxynivalenol in maize and pork samples.; Songqing Li et al.; Feb 5, 2013*

DON is rapidly absorbed in the proximal parts of the swine´s small intestine. Fifteen to twenty minutes after ingestion it is detected in plasma with a maximal concentration after 0.8-4.1 h (Dänicke et al., 2004; Goyarts and Dänicke, 2006; Rohweder et al., 2013). The small molecular size (296.31 g/mol) and amphipathic properties allow DON to cross cell membranes passively, and it can therefore be easily absorbed via the system (McCormick et al., 2011). Additionally, DON affects the intestinal absorption by altering the membrane permeability, the barrier function and the transepithelial electrical resistance (**TEER;** Pinton and Oswald, 2014). The absorption commences in the stomach and the proportion DON/DOM-1 is continuously altered throughout the gastro-intestinal system from 100% DON in the stomach to nearly 100% DOM-1 in the colon (Dänicke et al., 2004). DOM-1 is mainly formed by microbiota in the intestine and to a smaller degree in the liver (Dänicke et al., 2013; Kollarczik et al., 1994). The majority (53-62%) of the ingested DON is directly excreted via urine (mainly as DON) and less than 0.1% of the ingested DON is excreted via feces (mainly as DOM-1; Dänicke et al., 2004). The rest, of up to 40%, was not recovered and might include undetected further DON-metabolites (Dänicke and Brezina, 2013). For mammals, the conjugation with glucuronic acid by the liver was described as the major pathway of phase II metabolism(Gareis et al., 1987; Ritter, 2000). In blood of fattening pigs, the proportion of free DON to conjugated DON was 65% to 35% (Dänicke et al., 2014).

In contrast to DON, ZEN or "6-(10-hydroxy-6-oxo-trans-1-undeceny) β-resorcylic acid lactone" is not water soluble but may be solubilized in aqueous alkali and various organic solvents like acetone or ethanol. It exhibits a white color, a crystalline structure and is melting at 164-165 °C. The chemical formula of this mycotoxin is $C_{18}H_{22}O_5$ and it has a molecular weight of 318.36 (EFSA, 2004b).

Fig. 2: Chemical structures a. zearalenone (ZEN), b. α-zearalenol (α-ZEL), c. β-zearalenol (β-ZEL), d. zearalanone (ZAN), e. α-zearalanol (α-ZAL) and f. β-zearalanol (β-ZAL; from Metzler, 2011). *Springer Nature: Mycotoxin Research; Proposal for a uniform designation of zearalenone and its metabolites.; Manfred Metzler; Nov 25, 2010*

The possible pathways available for ZEN biotransformation relate mainly to the hydrolysis of the lactone ring, the reduction of the ketonic carbonyl group, the modification of the hydroxyl groups (i.e., sulfation and glycosylation), and the reduction of the carbon-carbon double bond, resulting in the metabolites α-zearalenol (**α-ZEL**), β-zearalenol (**β-ZEL**), zearalanone (**ZAN**), α-zearalanol (**α-ZAL**, zeranol), and β-zearalanol (**β-ZAL**, taleranol, Fig. 2; details in Metzler, 2011).

In general, intestinal mucosa and liver are the main sites of absorptive and post-absorptive modifications of ZEN resulting in reductive and oxidative metabolites, as well as in conjugated forms with some species differences observed (Dänicke and Winkler, 2015; Warth et al., 2013). For example, several *in vitro* experiments with rumen fluid have shown that beside ZEN its main metabolites α-ZEL and β-ZEL occur in the forestomach system of cattle due to the microbial metabolism in this site, whereas in swine α-ZEL is the main metabolite and the enterohepatic circle takes the primary role in the metabolism (Biehl et al., 1993; Binder et al., 2017; Dänicke and Winkler, 2015; Malekinejad et al., 2006). Shin et al. (2009b) describe in a study with rats detectable amounts of ZEN and its metabolites in arterial and venous blood, lung, liver, spleen, kidneys, heart, testes, brain, muscle, adipose, stomach, small intestine after i.v. infusion, with the highest ZEN concentration found in small intestine, followed by kidneys, liver, adipose tissue, and lung and concentrations in stomach, heart, brain, spleen, muscle, and testes being lower than those found in

serum. It was furthermore shown that terminal elimination half-life of ZEN was significantly prolonged after oral administration compared to after i.v. injection in mice (Shin et al., 2009a).

1.2.2. Toxic Effects

The striking effect of DON is the name giving vomiting ("vomitoxin", emesis) if highly contaminated feedstuff is consumed (Vesonder et al., 1973). Other symptoms of an acute DON intoxication include abdominal distress, increased salivation, malaise, diarrhea and anorexia (Forsyth et al., 1977; Friend et al., 1982; Pestka et al., 1987; Prelusky and Trenholm, 1993; Young et al., 1983; reviewed in Pestka, 2007). Its toxicity is low compared to other mycotoxins, such as for example aflatoxins or ergot alkaloids (Pestka and Smolinski, 2005) and under practical circumstances mainly comprises a decrease in feed intake (Abbas et al., 1986; Forsyth et al., 1977; Rotter et al., 1994; Trenholm et al., 1984; Young et al., 1983; reviewed in Pestka, 2007). This effect is, however, already observed at low doses. In young swine anorexia was observed from a concentration of 1-2 mg DON/kg feed (Prelusky, 1996). The underlying mechanisms have not fully been elucidated yet, but it is assumed that the observed nausea and vomiting are caused by a central-nervous effect (Bonnet et al., 2012; Flannery et al., 2012).

The minimal oral emetic dose in pigs is indicated between 0.05-0.2 mg DON/kg BW (Larsen et al., 2004). Additionally, depending on the dosage used (10-1000 mg/kg BW of DON) various toxic signs ranging from necrosis of the intestinal tract, bone marrow, lymphoid tissues to kidney and heart lesions were observed in mice (Forsell et al., 1987). Acute high doses can produce shock-like death. For example, after oral administration in mice and broiler chicks the median lethal dose (**LD 50**) was observed at 46-78 mg/kg and 140 mg/kg body weight (**BW**), respectively (Huff et al., 1981; Yoshizawa and Morooka, 1974).

At the level of the immune system, several authors describe an increased expression of different cytokines and chemokines *in vitro* and *in vivo* upon low level of trichothecene exposure, involving transcriptional and post-transcriptional mechanisms (Dong et al., 1994; Efrat et al., 1984; Holt et al., 1988; Miller and Atkinson, 1986; Pestka et al., 2004; Warner et al., 1994; reviewed in Pestka, 2007). Furthermore, DON was repeatedly shown to inhibit the protein synthesis through binding to the 60S sub-unit of eukaryotic ribosomes and the resulting interference with the peptidyltransferase (Döll et al., 2009c; Ehrlich and Daigle, 1987; Feinberg and McLaughlin, 1989). Different studies also describe the activation of mitogen-activated protein kinases (**MAPKs**) inducing apoptosis through the so called "ribotoxic stress response" (Iordanov et al., 1997; Laskin

et al., 2002; reviewed in Pestka, 2007). It is assumed that these mechanisms are the reason for its immune suppressive action when applied at higher doses (Pestka, 2007). However, also other toxic mechanism such as impaired membrane function or altered intercellular communication may also play a role in this pathogenesis (Rotter et al., 1996).

Macrophages are specifically sensitive immune cells regarding to DON, due to the possible higher potential of DON to enter these cells, leading to an increased rate of apoptosis (Wang et al., 2012). Additionally, alterations of the macrophage functions like reduced phagocytosis, bacterial killing and cytokine production also inhibit the macrophages to play their role as antigen-presenting cells, thereby inhibiting the activation of B- and T-cells (reviewed in Maresca, 2013 and Pestka, 2007). Apart from the alterations of the macrophages the proliferation and function of lymphocytes, including B-, T- and natural killer cells is impaired by DON (Pestka et al., 1994). On a histological level this is mirrored in apoptosis in the Peyer´s patches, thymus, and bone marrow (reviewed in Pestka, 2007). As a result of this immune suppression an increased susceptibility to infection, reactivation of latent infections and decreased vaccine efficiency are observed (reviewed in Maresca, 2013).

The acute toxicity of ZEN is relatively low. After oral administration in guinea pigs the LD50 varied between < 5,000 and up to > 10,000 mg/kg BW. After systemic administration (intraperitoneal) in rats and mice the LD50 varied between > 500 and 10,900 mg/kg BW (Hidy et al., 1977). Orally given ZEN is less toxic than when injected intraperitoneally or intravenously (Shin et al., 2009a; Zinedine et al., 2007). The estrogen-like structure of ZEN and its metabolites allows their binding to the estrogen receptors in various species, resulting in hyperestrogenism and reproductive disorders (Alm et al., 2006; Dänicke and Winkler, 2015). Young female pigs are especially sensitive to estrogenic imbalance through ZEN and its metabolites (Bauer et al., 1987). In contrast to DON, the metabolites of ZEN have also a toxic potential. Besides the estrogenic effects, ZEN and its metabolites also exhibit a negative effect on RNA and protein synthesis (Döll and Dänicke, 2011).

At the cellular level ZEN is targeting mitochondria and/or lysosomes, it induces lipid peroxidation, apoptosis, it is genotoxic by forming DNA adducts and it inhibits protein and DNA synthesis (reviewed by Zinedine et al., 2007). In rats (intraperitoneally treated once with 1.5, 3.0, and 5.0 mg/kg), mice (treated orally with a single dose of 40 mg/kg) and rabbits (given orally 100 µg/kg BW daily for 14 d) ZEN was reported to exhibit hemotoxic and hepatotoxic effects, respectively,

indicated by the alteration in the hemogram and biochemical markers (e.g. **AST**, **ALT**, **ALP**, **γGT**, **LD**, serum creatinine and bilirubin; Abbes et al., 2006; Conkova et al., 2001; Maaroufi et al., 1996). But the data on hematological variables are not consistent. In another study on rats with 3.0 mg/kg BW daily for 28 d no alterations were found on **RBC**, **WBC**, **Ht**, **Hg**, **MCH** and **MCV** (Hueza et al., 2014).

The immunotoxicity of ZEN is similar to the observed toxicity by high circulating estrogen concentrations. ZEN modulates different aspects of the immune response and impairs lymphoid organ function (Hueza et al., 2014). An alterations of thymus and spleen lymphocyte phenotypes, thymus atrophy, the impairment of the T-cell dependent humoral response, and a decrease in peroxide production by peritoneal macrophages was observed under the influence of ZEN (Hueza et al., 2014). In another study ZEN had no effect to organ weights, white blood cells, serum immunoglobulin (**Ig)**M or IgA, and also no synergistic effect was found here (Forsell et al., 1986).

A detailed review of the literature regarding the influence of an exposure to DON and ZEN on the immediate response of the immune system to an infection (acute phase reaction) is given in chapter 1.4.

1.2.3. Interactions between DON and ZEN

Due to its higher toxicity and occurrence in relevant concentrations DON is generally acknowledged as the toxin of major importance in naturally *Fusarium* contaminated diets (EFSA, 2004a, 2013, 2017). Therefore, these diets are mostly described as "DON contaminated" throughout the literature. However, due to their co-occurrence, also ZEN and other mycotoxins of minor importance are often encountered in these feedstuffs (EFSA, 2004b). By reason of the similar cellular targets and mode of actions, especially on immunological level, it can be expected that DON and ZEN interact in their toxic effects (Tiemann and Dänicke, 2007).

Studies working with naturally contaminated feed sources showing effects on clinical traits and growth are for example Bergsjo et al. (1993); Goyarts et al. (2006); Prelusky et al. (1994); unanimously describing a decreased feed intake and weight gain in the animals receiving the DON contaminated rations and heterogenous results concerning alterations in blood parameters. Interestingly, Prelusky et al. (1994) observed a significant difference between groups receiving either the purified DON toxin or a naturally contaminated diet with 3 mg/kg DON in a study with a feeding period of 32 days. Whereas the weight gains of pigs fed the pure DON diet recovered

after several days, the values for pigs fed the naturally contaminated diet remained depressed over the course of the study. The authors conclude that it is possible that these observations reflected the presence of other unidentified toxic compounds in the naturally contaminated grain.

Furthermore, Alm et al. (2006) describe a significantly reduced oocyte quality in gilts fed a *Fusarium* contaminated diet throughout 35 days, possibly contributing to reproductive failure. In the same setting, Tiemann et al. (2006) report histopathological changes in liver including glycogen decrease, hemosiderin enhancement, interlobular collagen uptake, and increased fatty and autophagic vacuoles.

Dänicke et al. (2014); Goyarts et al. (2006); Kullik et al. (2013); Lessard et al. (2015); Nossol et al. (2013) have investigated different aspects of naturally contaminated diets on immune function in swine. Lessard et al. (2015) and Nossol et al. (2013) both showed, in *in vitro* and *in vivo* settings, that DON impairs the intestinal barrier and immunological function at the basal membrane of the intestinal epithelium. Goyarts et al. (2006) studied, simultaneously in an *in vivo* and *in vitro* setting, the influence of DON on immunoglobulin concentrations and proliferation of porcine blood lymphocytes, describing a decrease in both traits due to the mycotoxin exposure (with differences in effect size depending on trait and method). Noteworthy is, that in the studies applying and comparing *in vitro* and *in vivo* methods (Goyarts et al., 2006; Nossol et al., 2013), purified DON was used in case of *in vitro* methods, whereas in the *in vivo* models naturally contaminated diets were fed. A debilitating or negative effect of DON on the immune response was furthermore demonstrated by Stanek et al. (2012), describing an alleviating effect of DON feeding on the impacts of an LPS infusion on histopathology and blood chemistry levels in swine exposed to a naturally contaminated diet. Contrary to this, in a study of Kullik et al. (2013) the feeding of DON did not have any influence on the degree of an LPS-induced APR in terms of protein synthesis of acute phase proteins, cytokines and metabolic activity of peripheral blood mononuclear cells. An influence of DON on the immune response was furthermore shown in a study by Pinton et al. (2008) reporting an altering effect on immune globulin production and lymphocyte proliferation after vaccination.

Also, the bioavailability and metabolism of DON were investigated in different settings involving naturally contaminated diets, for example in Dänicke et al. (2014); Goyarts and Dänicke (2006); Goyarts et al. (2007); Paulick et al. (2015).

However, in these studies applying naturally contaminated diets, the attribution of effects to one or both mycotoxins, and to synergistic or even antagonistic effects, represents a challenge. Only very few published experiments compare the toxicity of the different separate mycotoxins, as well as their possible interactions. In an *in vivo* model in mice Liang et al. (2015) investigated the nephrotic toxicity of DON and ZEN. Both toxins induced apoptosis, dysfunction, and oxidative stress, which were dose- and time-dependent. The combination of the two mycotoxins, however, led to a sub-additive nephrotoxic effect. A similar observation was made in an *in vitro* study by Bensassi et al. (2014) using human colon carcinoma cells (HCT116). The measurements of cell viability, cell cycle analysis, mitochondrial transmembrane potential determination and permeability transition pore opening showed decrease of cell viability in a dose-dependent manner for both mycotoxins. Combined DON and ZEN, however, reduced all the toxicities observed with the mycotoxins separately. These results stand in contrast with an *in vitro* study of Wan et al. (2013) showing only an additive effect of the mycotoxins DON, ZEN, nivalenol and fumonisin B1, and their combinations, on a normal porcine jejunal epithelial cell line (IPEC-J2). A possible reason for these contradictory results was later delivered in a study by Kachlek et al. (2017) investigating the effects of the combination of different mycotoxins (DON, ZEN and fumonisin B1) in porcine lymphocytes. At the height of cytotoxicity, the authors observed antagonism between the different mycotoxins. At the level of genotoxicity, however, where different mycotoxin concentrations were applied in the comet assay, the results showed a heterogenous picture of antagonism and synergism depending on mycotoxin combination and concentrations.

This overview of the current state of literature regarding the toxic effects and the interaction of the two mycotoxins shows that, due to the possible multi-layered effects, the ascription of effects observed *in vivo* to a single mycotoxin is clearly hampered when naturally contaminated diets are applied. Furthermore, *in vivo* studies are lacking that investigate the possible effects of each mycotoxin separately or combined and at different levels of contamination.

1.3. Acute Phase Reaction

1.3.1. Pathomechanisms

The APR is a non-specific complex systemic early-defense immune response, activated by trauma, infection, stress, neoplasia and inflammation with the goal of reestablishing homeostasis and promoting healing (Cray et al., 2009).

The APR is a core part of the innate immune response and it is presumed that it is present in all animal species. The innate immune response includes physical barriers, phagocytes, the complement reaction, and toll-like receptors (**TLR**) with the task to prevent infections, eliminate potential pathogens and initiate the inflammatory process. The induced innate response depends on the cytokines which are formed by activated cells including monocytes, macrophages, fibroblasts, endothelium, platelets, keratinocytes and T-cells (Janeway et al., 2001). Cytokines like interleukin (**IL**)-1, IL-6, and tumor necrosis factor (**TNF**)-α are acting as messengers between the local site of injury and the hepatocytes synthesizing the acute phase proteins (Petersen et al., 2004). They affect the hepatocytes which modulate the protein synthesis. This cascade has numerous effects like leukocytosis, complement activation, protease inhibition, clotting and opsonization throughout the body (Murata et al., 2004).

Upon an inflammatory reaction the formation of the negative acute phase protein (**APP**) albumin and subsequently its concentration in blood is decreased and the amino acids at disposal are utilized for the formation of positive APP (Kaneko, 1997). In pigs of substantial importance is the positive APP haptoglobin, which is reducing oxidative damage associated with hemolysis, and α1-acid glycoprotein, having an LPS binding and inhibiting function (Parra et al., 2006). Furthermore, a porcine specific APP is the porcine- or pig major acute phase protein (**MAP)** which has been described to inhibit trypsin activity in swine (Grau-Roma et al., 2009).

On the local site the cytokines and other inflammatory mediators affect the local blood vessels. This leads to increased blood vessel permeability and an increased local blood flow together with a decreased velocity of blood flow in small blood vessels which is altogether resulting in leakage of fluid, heat, redness and swelling (Janeway et al., 2001). Cytokines and complement fragments cause a migration and adhesion of leukocytes such as neutrophils to the site of infection. The local action of these immune cells causes pain. After the influx of neutrophils, monocytes are following which rapidly differentiate into macrophages (Janeway et al., 2001). The blood monocyte or tissue macrophage generally triggers the APR cascade (Baumann and Gauldie, 1994). On the systemic

level the complement system initiates a number of potent inflammatory events through the action of a large number of complement proteins, the zymogenes, which are widely distributed throughout body fluids and tissues (Janeway et al., 2001).

The systemic inflammatory response syndrome (**SIRS**) is the sum of the clinical manifestations of the complex metabolic processes during an APR (Nystrom, 1998). For humans SIRS was defined in 1991 at the American College of Chest Physicians/Society of Critical Care Medicine (**ACCP/SCCM**) consensus conference. The response is manifested by two or more of the following conditions: temperature >38°C or <36°C; heart rate >90 beats/min; respiratory rate >20 breaths/min or P_aCO_2 <32 torr (<4.3 kPa); WBC >12,000 cells/mm^3, <4,000 cells/mm^3 or >10% immature (band) forms (Bone et al., 1992). Up to now such clinical guidelines have not been published for pigs but they are necessary in order to establish better defined animal models for inflammatory processes.

1.3.2. Lipopolysaccharides (LPS) as Model Substance

Lipopolysaccharides (syn. endotoxins) are constituents of the cell wall of Gram-negative bacteria such as *Escherichia* coli and *Salmonella enterica* and are released during bacterial growth or lysis (Fig. 3). In animal farming Gram-negative bacteria are present ubiquitously in high concentrations in the environment, such as in feedstuff, on surfaces in the handling system or even in the air. Bacterial infections in pig production are the cause for substantial economic losses and reduced animal welfare, due to decreased growth rate, morbidity, mortality, and medication costs. Major pathogens involved in swine respiratory disease (**SRD**) or postweaning diarrhea in pigs are caused by many different bacteria (Fairbrother et al., 2005; Rose et al., 2013).

The LPS released during a bacterial infection acts as one of the most effective stimulators of the innate immune system and in higher doses it can induce an endotoxic shock (Wyns et al., 2015). It belongs to the well investigated pathogen associated molecular patterns (**PAMP;** Mogensen, 2009) and it is therefore often used as a model substance to induce an APR. Especially in porcine experimental models it is widely applied to simulate endotoxemia (Wyns et al., 2015). In contrast to a bacterial infection model, an LPS model is more standardized and reproducible, less expensive to develop and validate, and properly accessible (Myers et al., 2003; Olson et al., 1995; Schrauwen et al., 1986). Different reviews and studies have, however, pointed out, that LPS is a suitable model substance for endotoxemia and to induce an APR, but misses to depict the whole complexity of a sepsis (Dyson and Singer, 2009; Freise et al., 2001; Kingsley and Bhat, 2016; Rittirsch et al., 2007).

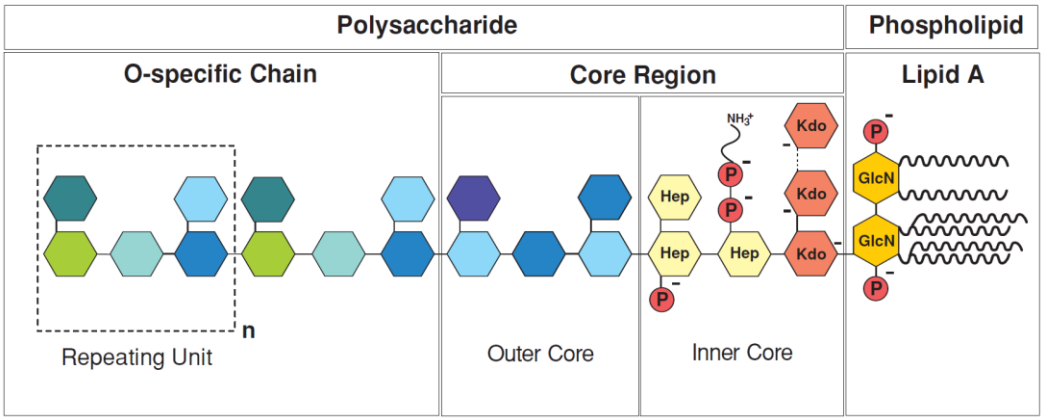

Fig. 3: General chemical structure of LPS from Gram-negative enterobacteria. All forms of LPS known to date consist of the membrane-anchoring lipid A domain and a covalently linked polysaccharide or oligosaccharide portion. In the depicted general scheme of the LPS architecture from S-type enterobacteria, the polysaccharide domain is composed of the lipid A proximal core region and the terminal O-specific chain formed by up to 50 repeating units. According to preferential carbohydrate compositions in the core structure, an inner and an outer core region are commonly distinguished. In mammalian species the lipid A domain represents the primary immunostimulatory centre of LPS determining endotoxicity. In the inner core region and the lipid-A domain phosphate and ethanolamine residues are additionally indicated. Abbreviations of monosaccharide residues: GlcN: glucosamine; Kdo: '2-keto-3-deoxyoctulosonic acid' (3-deoxy-D-*manno*-octulosonic acid); Hep: D-glycero-D-*manno*-heptose. Copied from Alexander and Rietschel (2001). *Permission granted by SAGE publishing under the open access policy.* *http://dx.doi.org/10.1177/09680519010070030101*

The functional site of LPS (Lipid A domain) binds to TLR-2 and TLR-4. These receptors are activating intracellular signal-transduction pathways that lead to the activation of the cytosolic nuclear factor-κB (**NF-κB**). The NF-κB moves from the cytoplasm to the nucleus and binds to transcription sites, with the result of activating sets of genes. This leads thereafter to forming of cytokines and costimulatory molecules inducing the proinflammatory response (Fig. 4; Janeway et al., 2001).

The usage, dosage and effect of LPS challenges in pigs and other species was reviewed by Wyns et al. (2015) and will be briefly described in the following section. An LPS challenge can be performed either as an intravenous (**i.v.**), intraperitoneal (**i.p.**) or intramuscular (**i.m.**) single dose administration, or as a continuous i.v. infusion. Each mode of application is characterized by different advantages and disadvantages. While a single LPS bolus administration is more suitable to represent the sequence of events after an LPS challenge, a continuous LPS infusion is more accurately reflecting a clinical endotoxemia/septicemia (Fink and Heard, 1990; John et al., 2008;

Olson et al., 1995). Furthermore, depending on whether a systemic or a local infection is to be simulated, the mode of application may be varied. However, up to date only a few studies have investigated the difference in systemic reaction between a pre-hepatic and post-hepatic LPS infusion. Such a mode of application forms an ideal model to investigate the difference between a systemic (post-intestinal barrier / post-hepatic) and a local / intestinal (pre-intestinal barrier / pre-hepatic) infection (Piccone et al., 1980; Reynolds et al., 1988).

Between species a large difference in endotoxin sensitivity and clinical symptoms is reported (Redl et al., 1993). Pigs and cattle are relatively sensitive to LPS administration at direct application into the body tissue or blood system whereas poultry and rodents are not (Olson et al., 1995; Poli-de-Figueiredo et al., 2008; Schmidhammer et al., 2006). High doses of LPS in pigs and cattle, inducing clear clinical symptoms, are amounting to 25 and 2.5 µg/kg BW, respectively. In broiler chickens and rodents 100 up to 10,000 times higher doses need to be applied to achieve clinical symptoms (2,500 and 20,000 µg/kg BW; de Boever et al., 2010; Gerros et al., 1993; Plessers et al., 2015; Purswani et al., 2002; Zhang et al., 2008). The lethal dose (**LD100**) in pigs was reported to be at 500 and 2,500 µg/kg BW (Schrauwen and Houvenaghel, 1984, 1985; Schrauwen et al., 1986).

After LPS administration the first clinical signs in pigs occur after 15 min. Symptoms such as intermittent coughing, salivation, chewing movements, retching, and vomiting recurrently were observed. After this immediate reaction upon the LPS challenge a state of depression follows for certain hours, as manifested by general sickness, lethargy, somnolence, and sternal or lateral decubitus. Respiratory difficulties develop varying from panting to severe dyspnea. Sporadically, shivering, generalized rubor, cyanosis, and even necrotic lesions have been described (Dänicke et al., 2013; Johnson and von Borell, 1994; Kanitz et al., 2002; Leininger et al., 2000; Monshouwer et al., 1996; Myers et al., 2003; Peters et al., 2012; Schrauwen and Houvenaghel, 1985; Schrauwen et al., 1988). Also, a direct LPS associated, dose-dependent reduction in feed consumption and activity after a challenge in pigs has been documented (Frank et al., 2005; Johnson and von Borell, 1994; Monshouwer et al., 1996; Myers et al., 2003; Warren et al., 1997; Wright et al., 2000).

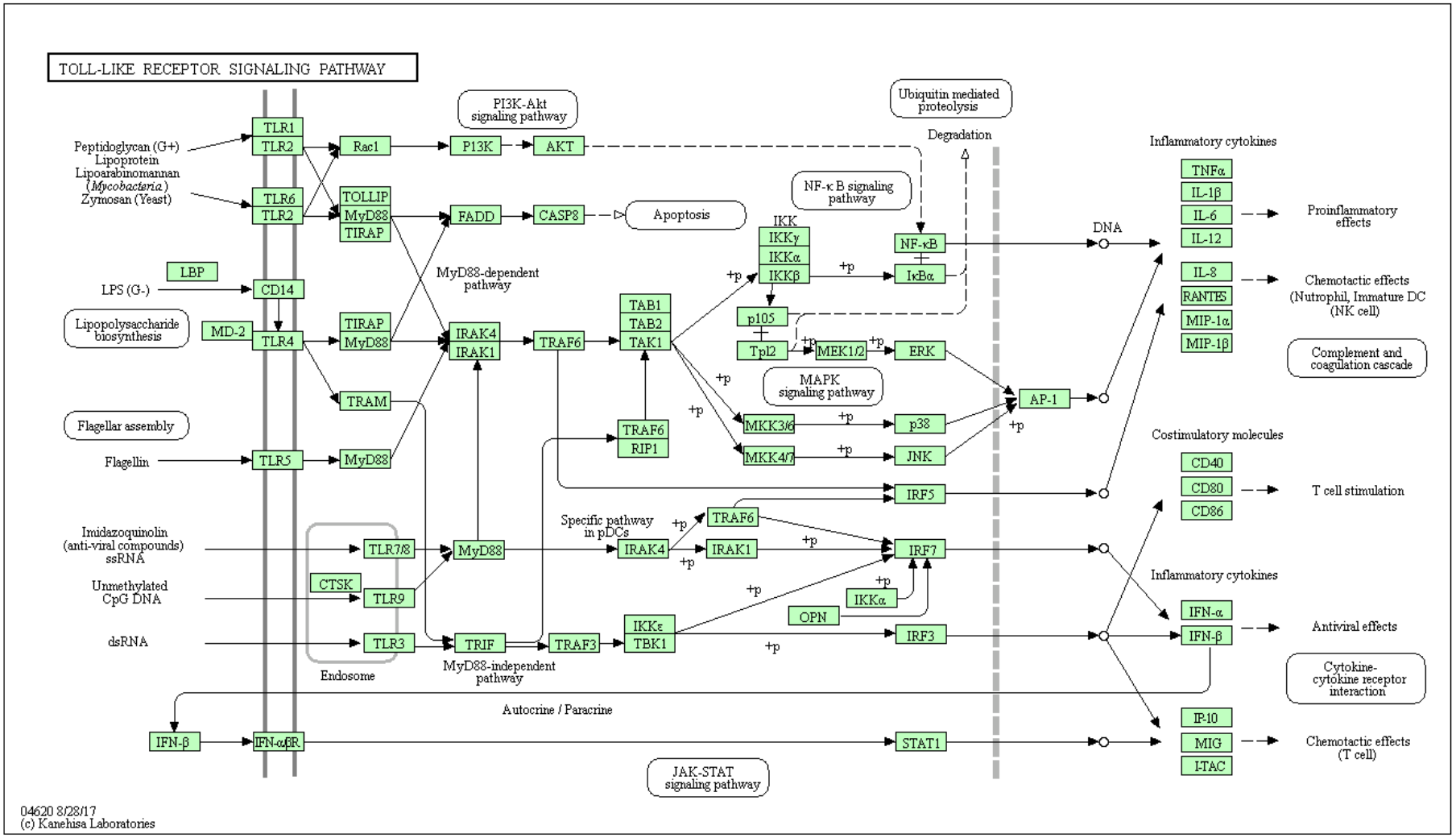

Fig. 4: The Toll-like receptor signaling pathway. Copied from the Kyoto Encyclopedia of Genes and Genomes: *https://www.kegg.jp/kegg-bin/show_pathway?map04620* (KEGG, 2017). *Permission granted by Kanehisa Laboratories*.

1.4. Interactions between DON / ZEN and LPS

The toxins DON and LPS may exhibit a direct encounter in the gastrointestinal tract (**GIT**) since DON is ingested via feed and LPS is released upon the degeneration of commensal and/or pathogenic bacteria (Andreasen et al., 2008; Döll and Dänicke, 2011; Goyarts and Dänicke, 2006). An increase in absorption into the portal blood circulation of both toxins is expected upon the disturbance of the blood-intestinal barrier. However, different possible modes of interaction were observed. In case of a stand-alone exposure of intestinal epithelial cells to DON a dose-dependent effect can be observed. At high doses DON reduces cell function and impairs the intestinal barrier while at low doses it exerts a cell proliferation stimulus by influencing gene activation *in vitro* (Diesing et al., 2011). Also, an increased mucosal uptake of nutrients and other substances in the jejunum, but not in the ileum was found under the influence of DON *in vitro* (Halawa et al., 2013). But in contrast to the presumption a direct interaction of DON and LPS at the intestinal level were not described in previous studies. Neither a higher translocation of LPS nor of DON was observed upon simultaneous exposure to both toxins (Dänicke et al., 2012a; Halawa et al., 2012). Studies have, however, shown that the intestinal uptake and the distribution of DON as well as the renal elimination are decreased due to the LPS induced systemic inflammatory response which includes centralization or hemodynamical dysfunction (Kreimeier et al., 1993).

Different studies revealed that when DON is applied i.v. an LPS challenge causes higher systemic DON levels, presumably due to a delayed elimination from the system, whereas when DON is applied orally, an LPS challenge causes a decrease in DON plasma levels. Between both DON-entry routes (i.v. or orally) no differences between the proportion of total and conjugated DON were observed, which lead the authors to conclude that the conjugation of DON itself is not affected (Dänicke et al., 2012b; Dänicke et al., 2014).

Due to its detoxifying function, the liver forms for LPS as well as DON the first target organ after entry into the circulatory system. In different studies with different species interrelating effects between DON and LPS were found. As mentioned in chapter 1.2.3., it was shown that pigs receiving a DON-contaminated diet exhibited an attenuation of hepatic histopathological lesions in contrast to their control fed counterparts after LPS exposure (Stanek et al., 2012), but contrary to this, Kullik et al. (2013) did not observe any influence of the feeding of DON on protein synthesis of acute phase proteins, cytokines and metabolic activity of peripheral blood mononuclear cells. In cultures of porcine pulmonary alveolar macrophages and Kupffer cell enriched hepatocytes, DON

and LPS synergistically cause an increase of TNF-α protein levels and higher mRNA expressions compared to a single toxin exposure (Döll et al., 2009a, 2009b). An increase in concentrations of IL-6, TNF-α and splenic mRNA level was also found in mice exposed to both toxins (Zhou et al., 1999) as well as increased apoptosis and lymphoid atrophy in the thymus, Peyer´s patches, bone marrow, spleen, and liver (Islam and Pestka, 2003; Zhou et al., 2000). The data is however not definite, since opposite to this, Dänicke et al. (2013) showed in pigs either infused with DON and LPS alone or together a significantly attenuated TNF-α peak, and no or little influence on the cytokine production and clinical symptoms, respectively. Furthermore, priming with LPS obviously sensitizes the organism to DON, which is shown in a shortened onset time and simultaneously increasing magnitude and duration levels of a cytokine response (IL-6, TNF-α serum proteins) and splenic mRNA observed in an *in vivo* experiment in mice (Islam and Pestka, 2006). In murine cell lines LPS priming potentiates the DON induced increase in IL-6 and TNF-α mRNA expression (Pestka and Zhou, 2006).

The research on interaction between ZEN and LPS is rare. As described in chapter 1.2.3. ZEN is mostly concurrently investigated with DON, due to their natural co-occurrence. The main interface of ZEN and LPS lays most likely in their effect on the immune system, which has been shown in several studies. For example, in LPS stimulated mice ZEN alters the production of regulatory cytokines and antibodies (Salah-Abbes et al., 2010). Furthermore, ZEN was found to activate the ROS-mediated NLRP3 inflammasome and, in turn, contributes to the caspase-1-dependent activation of the inflammatory cytokines IL-1β and IL-18 (Fan et al., 2018). Also, a study investigating the effects of ZEN exposure on pro- and anti-inflammatory cytokines in the murine macrophage cell line showed that ZEN has a dose dependent ability to modulate important pro- and anti-inflammatory cytokines like IL-6, IL-10 and TNF-α (Loftus et al., 2016). Moreover, Obremski (2014) showed an alteration in T- and B-cell sensitivity *in vitro* LPS stimulated Peyer´s patches, which had previously been exposed *in vivo* to ZEN. In this experiment, ZEN inhibited IL-2 and interferon (**IFN**)-γ production, and stimulated IL-4 and IL-10 secretion by T helper (**Th)**1 and Th2 cells (Obremski, 2014).

This short overview of the current state of literature shows that several studies indicate a potential interaction between the mycotoxins DON and ZEN, as well as the pathomechanisms observed during an APR. However, in dept-analyses using a sophisticated *in vivo* model including the role of the liver as central detoxifying organ have not been conducted so far.

2. Aims of Study

As elaborated in the introduction, this work is part of a large, diverse project where altogether the role of the liver as main detoxifying organ during an LPS challenge is investigated in organisms which where chronically exposed to the mycotoxins DON and ZEN.

In this thesis the focus lays on two aspects: the metabolism of DON and ZEN in the organism during an endotoxin challenge, and *vice versa* the systemic response to an endotoxin challenge under the influence of a chronic DON and ZEN exposure; with two central hypotheses:

1. The systemic response to an endotoxin challenge is pronounced differently depending on the entry route of LPS (pre-hepatic (portal, first-pass hepatic exposure) vs. post-hepatic (jugular, second pass hepatic exposure) and during a chronic DON/ZEN exposure. Here, metabolic and hematological traits were used as response criteria.
2. The metabolism and the kinetics of the mycotoxins DON and ZEN is modulated under the influence of an LPS challenge, depending on the LPS-entry site.

For the investigation of these and other hypotheses the project involved one central trial including 44 ten-weeks-old barrows (German Landrace with an initial mean BW of 25.8 ± 3.7 kg) which were divided in two feeding groups over a period of 29 days. One group was fed with an almost DON-free diet (CON) while the other group received a naturally mycotoxin contaminated diet (4.59 mg DON and 0.22 mg ZEN per kg feed; daily feed allowance was increased continuously over the course of the trial to attain a voluntary intake of 1400 g of feed per day). During experimental days 24 until 29, barrows were housed in metabolism crates and on day 27 surgically equipped with indwelling catheters in *Vena jugularis externa* (post-hepatic) and *V. lienalis* (pre-hepatic) for infusion, and *V. jugularis interna*, *V. portae hepatis* and *Arteria carotis communis* for simultaneous blood sampling.

Together with the classification depending on the dietary levels (mycotoxin exposure, CON vs DON) and on both infusion sites (post- or pre-hepatic: ju vs po) with LPS or NaCl infusion (CON vs LPS) alternately, the complete study comprised six experimental groups: CON_CONju-CONpo, CON_CONju-LPSpo, CON_LPSju-CONpo, DON_CONju-CONpo, DON_CONju-LPSpo, DON_LPSju-CONpo (illustrated in Fig. 5).

On the experimental day 29 the animals were 15 min after feeding either infused with LPS (7.5 µg/kg BM dissolved in 0.9% NaCl, *Escherichia coli* O111:B4, product number L2630, Sigma-

Aldrich, Taufenkirchen, Germany) or a control solution (NaCl 0.9 %) for one hour (Fig. 5). Blood sampling occurred 10x in the period of 30 min before and 180 min after start of infusion. Thereafter at minute 195 the animals were sacrificed, and different organ weights and samples were collected (detailed description in Chapter 3-5).

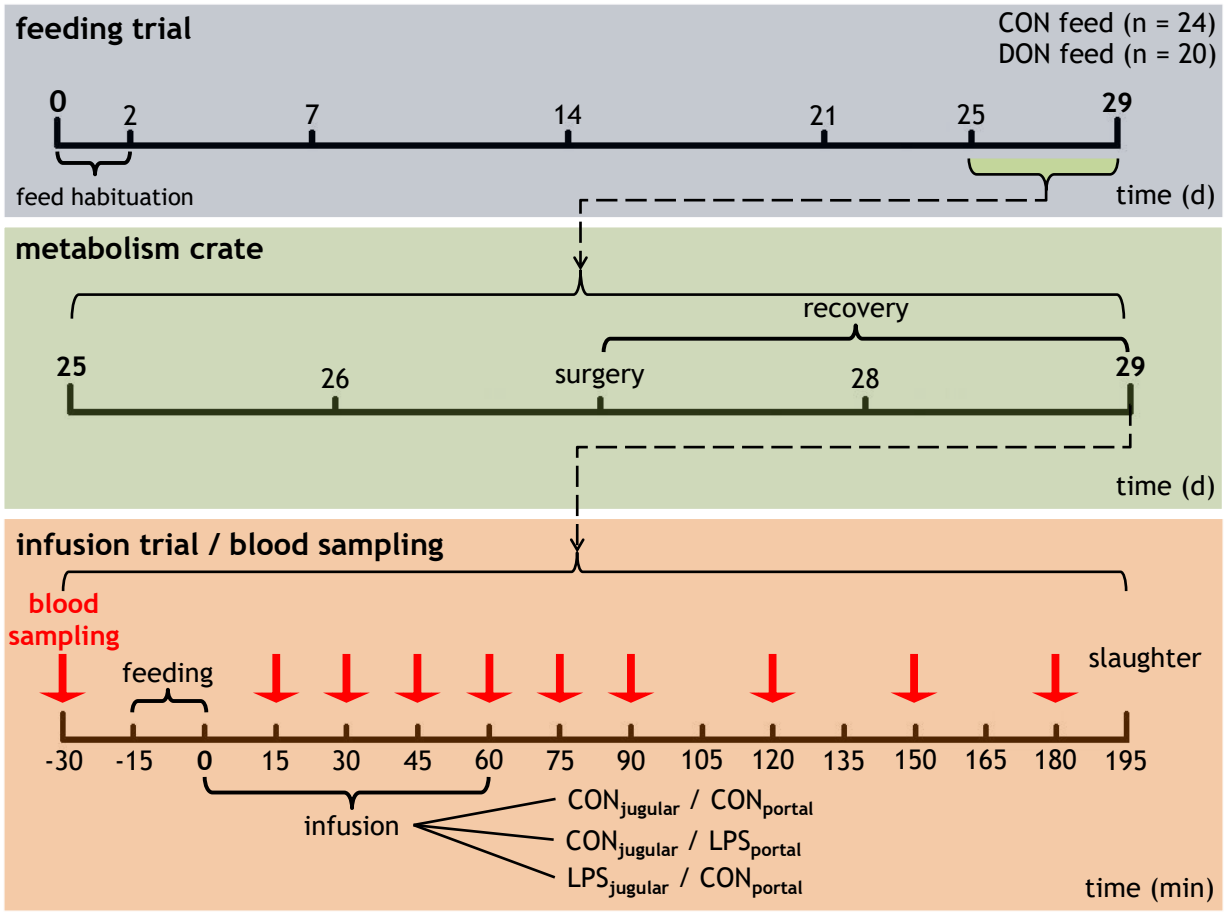

Fig. 5: Experimental design

3. Metabolic and Hematological Consequences of Dietary Deoxynivalenol Interacting with Systemic *Escherichia coli* Lipopolysaccharide

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3.1. Abstract

Previous studies have shown that chronic oral deoxynivalenol (DON) exposure modulated *Escherichia coli* lipopolysaccharide (LPS)-induced systemic inflammation, whereby the liver was suspected to play an important role. Thus, a total of 41 barrows was fed one of two maize-based diets, either a DON-diet (4.59 mg DON/kg feed, $n = 19$) or a control diet (CON, $n = 22$). Pigs were equipped with indwelling catheters for pre- or post-hepatic (portal *vs.* jugular catheter) infusion of either control (0.9% NaCl) or LPS (7.5 µg/kg BW) for 1h and frequent blood sampling. This design yielded six groups: CON_CON$_{jugular\text{-}CON_{portal}}$, CON_CON$_{jugular\text{-}LPS_{portal}}$, CON_LPS$_{jugular\text{-}CON_{portal}}$, DON_CON$_{jugular\text{-}CON_{portal}}$, DON_CON$_{jugular\text{-}LPS_{portal}}$ and DON_LPS$_{jugular\text{-}CON_{portal}}$. Blood samples were analyzed for blood gases, electrolytes, glucose, pH, lactate and red hemogram. The red hemogram and electrolytes were not affected by DON and LPS. DON-feeding solely decreased portal glucose uptake ($p < 0.05$). LPS-decreased partial oxygen pressure (pO_2) overall ($p < 0.05$), but reduced pCO_2 only in arterial blood, and DON had no effect on either. Irrespective of catheter localization, LPS decreased pH and base-excess ($p < 0.01$), but increased lactate and anion-gap ($p < 0.01$), indicating an emerging lactic acidosis. Lactic acidosis was more pronounced in the group DON_LPS$_{jugular\text{-}CON_{portal}}$ than in CON-fed counterparts ($p < 0.05$). DON-feeding aggravated the porcine acid-base balance in response to a subsequent immunostimulus dependent on its exposure site (pre- or post-hepatic).

Keywords: swine; deoxynivalenol; *E. coli* lipopolysaccharides; endotoxin; sepsis; blood gas; metabolism; glucose; inflammatory response

3.2. Introduction

Due to its dependency on moderate climate conditions and its resistance to processing the Fusarium toxin, deoxynivalenol (DON) can be often found in toxicologically relevant concentrations in cereals in temperate climate zones [1]. It is of special importance in pig production due to the high susceptibility of pigs, causing reduced feed intake and live weight gain, resulting in considerable economic losses [2-4].

Several studies indicate that DON influences the systemic inflammatory response. The toxin exerts immune modulatory effects on blood leukocytes depending on the dose and frequency of exposure. Different studies have shown that a low dose exposure to Fusarium toxins has an immune-stimulating effect due to an upregulation of transcriptional and post-transcriptional expression of cytokines, chemokines and inflammatory genes, whereas a high dose exposure has an immune-suppressive effect (reviewed in [5]). It has further been shown that exposure to DON causes an altered immune response [6-8] and liver cell metabolism [9,10] to a subsequent lipopolysaccharide (LPS) challenge *in vitro* and *in vivo*.

Lipopolysaccharides form the major component of the outer cell membrane of Gram-negative bacteria and are responsible for the onset of an inflammatory response in the case of systemic LPS-entry [11]. Triggering similar (immune biological) pathways, a variety of infectious pathogens, such as Gram-positive and Gram-negative bacteria, viruses and fungi, leads to identical clinical sequelae commonly described with the term sepsis [12,13]. Since infections with Gram-negative bacteria contribute to a substantial part of the sepsis cases worldwide, LPS-induced systemic inflammation is a well-established sepsis model in animals and humans [11,14-16].

On a systemic level, the recognition of LPS by the immune system causes the release of pro-inflammatory cytokines. This leads to inflammation, apoptosis, causing endothelial dysfunction, and microcirculation thrombosis, resulting in perfusion heterogeneity and microcirculatory failure [12]. Clinically, these alterations manifest themselves in a variety of symptoms, such as hypothermia or hyperthermia, tachycardia, tachypnea, edema, central nervous dysfunction, leukocytosis and leukopenia [11,16,17]. In concurrence with a pronounced inflammatory response, blood analysis reveals often lactic acidosis [18-20], along with either dysglycemia depending on the stage of disease [17,18,21].

As DON can be ubiquitous in cereals and pigs might be sub-acutely exposed on the one hand, while LPS is always present in the environment and commensal intestinal microbiota, on the other hand, pigs might be often co-exposed to both toxins at the same time. The liver possesses a central role in LPS detoxification [22,23] or in metabolic and immunological homeostasis [24] of, and it has been shown before that chronic exposure to DON leads to altered liver cell metabolism [9,10]. We hypothesized that liver metabolism and hematological variables are altered in chronically-DON-fed animals during a subsequent LPS stimulus. An increase in systemic LPS can either be a consequence of a systemic infection [17] or an increased passage from portal-drained viscera [25]. In order to simulate these pathways of systemic LPS entrance and the consequences of a possible hepatic first-pass effect, we infused LPS pre- or post-hepatically. Arterial, jugular and portal blood metabolic variables were assessed to evaluate the role of the liver in this pathogenesis as a consequence of LPS infusion.

3.3. Results

3.3.1. Red Hemogram

Generally, neither DON nor LPS treatment influenced red hemogram variables irrespective of infusion site, and all values were always in their respective physiological range [26] (Table 1). Slight fluctuations combined with small variances in all measures contributed to significant time, catheter and group × catheter × time effects (Table 1).

Table 1.: Effect of chronic enteral deoxynivalenol (DON) exposure and pre- or post-hepatic *E. coli* lipopolysaccharide (LPS) infusion on arterial, venous or portal red hemogram in pigs.

Parameter	Overall Mean	PSEM	p-Values			
			Group	Catheter	Time	G*C*T
RBC (x10^6 cells/µl)	5.57	0.24	0.306	0.299	< **0.001**	0.467
Hct (%)	30.44	1.40	0.629	**0.006**	< **0.001**	0.415
Hgb (g/dl)	10.82	0.49	0.618	0.073^T	< **0.001**	0.279
MCV (fl)	54.78	1.26	0.632	< **0.001**	0.981	**0.036**
MCH (g/dl)	19.48	0.52	0.602	0.144	< **0.001**	0.080^T
MCHC (g/dl)	35.56	0.52	0.554	**0.001**	< **0.001**	0.429

Notes: RBC = red blood cells (reference: 5.5 - 8.5 x10^6 cells/µl[1]); Hct = hematocrit (reference: 33 - 45 %[1]); Hgb = hemoglobin (reference: 10.16 - 14.03 g/dl[1]); MCV = mean corpuscular volume (reference: 50 - 65 fl[1]); MCH = mean corpuscular hemoglobin (reference: 17 - 21 g/dl[1]); MCHC = mean corpuscular hemoglobin concentration (reference: 30 - 35 g/dl[1]); [1] = [26]; references in venous blood; [T] = trend ($P \leq 0.10$); PSEM = pooled standart error of Means. Barrows were either fed a DON contaminated ration (DON; 4.59 mg/kg feed) or control feed (CON) during 29 days. Infusion groups were divided as follows: pre-hepatic LPS infusion (CON_$CON_{jugular}$-LPS_{portal}, n = 7 and DON_$CON_{jugular}$-LPS_{portal}, n = 6), post-hepatic LPS infusion (CON_$LPS_{jugular}$-CON_{portal}, n = 8 and DON_$LPS_{jugular}$-CON_{portal}, n = 6), and control infusion (CON_$CON_{jugular}$-CON_{portal}, n = 7 and DON_$CON_{jugular}$-CON_{portal}, n = 7). Infusion from time 0 until 60 min with 7.5 µg LPS/kg LW in 0.9 % saline. Feed was offered during 15 min prior to infusion start. Blood samples were collected at times: -30, 15, 30, 45, 60, 75, 90, 120, 150, 180 min.

3.3.2. Blood Gas Analysis

Partial Oxygen Pressure

Arterial, jugular and portal partial oxygen pressures (pO2) are illustrated in Figure 1. On average, partial O2 pressures of the control group were 92.42 mmHg in arterial, 36.07 mmHg in jugular and 43.08 mmHg in portal blood (SEM = 1.21), respectively. The arterial pO2 was near the physiological reference value of 98 mmHg [26]. Between 15 and 60 min, a general decrease in pO2 in all LPS-infused groups (compared to their control groups) was observed at all infusion sites ($p < 0.05$), which started to return to base level at 60 min. The decrease was most pronounced in arterial blood. Thereafter, a subsequent increase in pO2 in all LPS-infused groups until 180 min was observed ($p < 0.05$). At jugular and portal sampling sites, pO2 decreased after 90 min, again below the control group level, and decreased thereafter until 120 min in jugular ($p < 0.05$) and 180 min in portal ($p < 0.05$) blood. A significant effect of DON exposure on arterial pO2 was observed at 60 min with pre-hepatic LPS-infused control-fed pigs starting to return to base level earlier than their DON-fed counter parts ($p < 0.01$). No effect of DON treatment was observed in post-hepatic

infused animals. No DON effects were observed on jugular and portal pO2. Portal pO2 pressures were subjected more to fluctuations compared to arterial and portal pO2.

Partial Carbon Dioxide Pressure

Partial CO2 pressure (pCO2) of control group was 37.80 mmHg in arterial, 47.59 mmHg in jugular and 52.42 mmHg in portal blood (SEM = 0.75) on average. At the arterial and jugular sampling site, a pCO2 below the physiological reference range (50 mmHg) [26] was observed during the entire course of the trial and in all groups. Only arterial pCO2 was influenced by LPS treatment (Figure 2). From 120-180 min, a steady decrease was observed compared to the control group ($p < 0.05$). A slight portal pCO2 increase was observed from −30 min until 180 min in all groups (Figure 2). Chronic oral exposure to DON had no impact on pCO2 irrespective of LPS infusion site. In jugular, as well as portal blood samples, undirected fluctuations were observed.

3.3.3. Electrolytes

No significant effects of DON and LPS were observed on electrolytes (Na+, Cl−, K+, iCa2+). However, catheter site and collecting time significantly influenced electrolyte concentrations (Table 2). Slight fluctuations were observed for Na+, K+ and iCa2+ concentrations at different times, combined with minimal variation of the data, contributing to significant time and group × catheter × time effects. A significant catheter site effect was detected due to the generally higher jugular concentrations compared to arterial and portal levels at all groups. Electrolytes did not deviate from their respective physiological values [26,27] (Table 2).

Table 2.: Effect of chronic enteral deoxynivalenol (DON) exposure and pre- or post-hepatic *E. coli* lipopolysaccharide (LPS) infusion on arterial, venous or portal blood electrolytes in pigs.

Parameter	Overall Mean	PSEM	p-Values			
			Group	Catheter	Time	G*C*T
Na^+ (mmol/l)	142.90	1.15	0.449	**< 0.001**	**< 0.001**	0.629
Cl^- (mmol/l)	105.45	1.16	0.702	**< 0.001**	**< 0.001**	0.67
K^+ (mmol/l)	4.28	0.16	0.585	**< 0.001**	**< 0.001**	**< 0.001**
iCa^{2+} (mmol/l)	1.37	0.03	0.985	**< 0.001**	**< 0.001**	**0.006**

Notes: Na^+ = sodium (reference: 140 - 160 mmol/l[1]); Cl^- = chloride (reference: 102 - 106 mmol/l[1]); K^+ = potassium (reference: 4.0 – 5.0 mmol/l[1]); iCa^{2+} = calcium iones (reference: 0,87 – 1,45 mmol/l[2]); [1] = [26]; [2] = [27]; references in venous blood; [T] = trend ($P \leq 0.10$); PSEM = pooled standart error of Means. Barrows were either fed a DON contaminated ration (DON; 4.59 mg/kg feed) or control feed (CON) during 29 days. Infusion groups were divided as follows: pre-hepatic LPS infusion ($CON\_CON_{jugular}$-LPS_{portal}, n = 7 and $DON\_CON_{jugular}$-LPS_{portal}, n = 6), post-hepatic LPS infusion ($CON\_LPS_{jugular}$-CON_{portal}, n = 8 and $DON\_LPS_{jugular}$-CON_{portal}, n = 6), and control infusion ($CON\_CON_{jugular}$-CON_{portal}, n = 7 and $DON\_CON_{jugular}$-CON_{portal}, n = 7). Infusion from time 0 until 60 min with 7.5 µg LPS/kg LW in 0.9 % saline. Feed was offered during 15 min prior to infusion start. Blood samples were collected at times: -30, 15, 30, 45, 60, 75, 90, 120, 150, 180 min.

3.3.4. Glucose

A post-prandial increase in glucose until 15-30 min and a subsequent decrease until time 120 min to the base level was observed in all groups (Figure 3), most pronounced at the portal sampling site. The control (CON)-fed animals generally exhibited higher glucose levels at the portal sampling site at 30-45 min (depending on group) than DON-fed animals (Figure 3). The control group (CON_CONjugular-CONportal) maintained elevated post-prandial glucose levels for nearly the entire time course (significantly higher than other groups at 60 min, 90 min and 120 min; $p < 0.05$ at 120 min).

3.3.5. Acid-Base Balance

In all LPS-infused pigs, lactic acidosis was induced, and the acid-base balance variables were altered accordingly (Figures 4-8). Compared to the control group (CON_CONjugular-CONportal), pH (Figure 4), bicarbonate (Figure 6) and base excess (BE; Figure 7) decreased and lactate (Figure 5) and anion-gap (AG; Figure 8) increased significantly ($p < 0.05$). The control group stayed in the physiological range [26] for pH (arterial), lactate (venous) [28], HCO3 (arterial) and AG (venous) [27], but BE (arterial) was above the reference range over the course of the trial with individual variations (5.23 mmol/L ± 0.79; mean ± SE). DON treatment had no effect on control and pre-hepatic LPS-infused groups, but a significant DON effect was observed in post-hepatic LPS-infused animals. Lactic acidosis was most pronounced in post-hepatic LPS-infused DON-fed pigs

(DON_LPSjugular-CONportal) at different times in comparison to their control-fed counterparts (CON_LPSjugular-CONportal) and the pre-hepatic LPS-infused groups ($p < 0.05$) (shown in the post hoc tables, Figures 4-8). The DON_LPSjugular-CONportal group had a different time course compared to the other LPS groups for all variables of acid-base balance. This was most apparent in lactate, with a significant increase compared to the control already at 75-180 min at all catheters in contrast to the other LPS groups, which were only significantly different from the control at 150 min and 180 min. Additionally, we observed a chronological sequence of lactic acidosis variables compared to the control: pO2, $HCO3^-$ and BE already changed at 30-45 min, followed by pH at 60 min, and as the last variable, lactate rose significantly at 75 min.

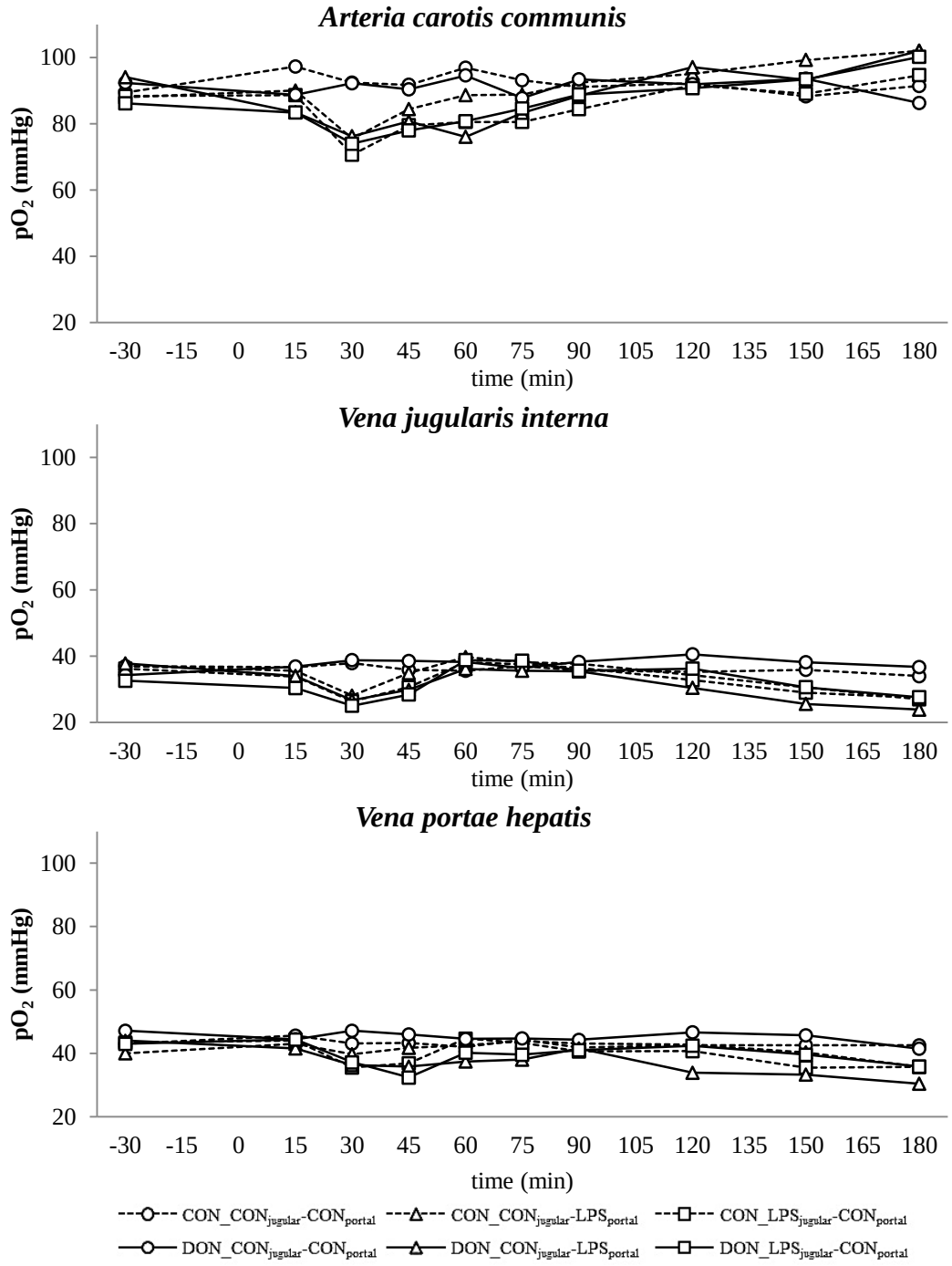


Fig. 1: Effect of chronic enteral *Fusarium* toxin deoxynivalenol (DON) exposure and pre- or post-hepatic *E. coli* lipopolysaccharide (LPS) infusion on arterial, jugular or portal blood partial oxygen pressure (pO_2) in pigs. Reference value: 98 mmHg in arterial blood [26]. Barrows were either fed a DON contaminated ration (4.59 mg/kg feed) or control feed during 29 days. Infusion groups were divided as follows: pre-hepatic LPS infusion ($CON\_CON_{jugular}$-LPS_{portal}, n = 7 and $DON\_CON_{jugular}$-LPS_{portal}, n = 6), post-hepatic LPS infusion ($CON\_LPS_{jugular}$-CON_{portal}, n = 8 and $DON\_LPS_{jugular}$-CON_{portal}, n = 6), and control infusion ($CON\_CON_{jugular}$-CON_{portal}, n = 7 and $DON\_CON_{jugular}$-CON_{portal}, n = 7). Infusion from time 0 until 60 min with 7.5 µg LPS/kg LW in 0.9 % saline. Feed was offered during 15 min prior to infusion start. LSMeans. PSEM = 2.89. Significance: Group (G): $p = 0.003$; Catheter (C): $p \leq 0.001$; Time (T): $p \leq 0.001$; G*C*T: $p \leq 0.001$.

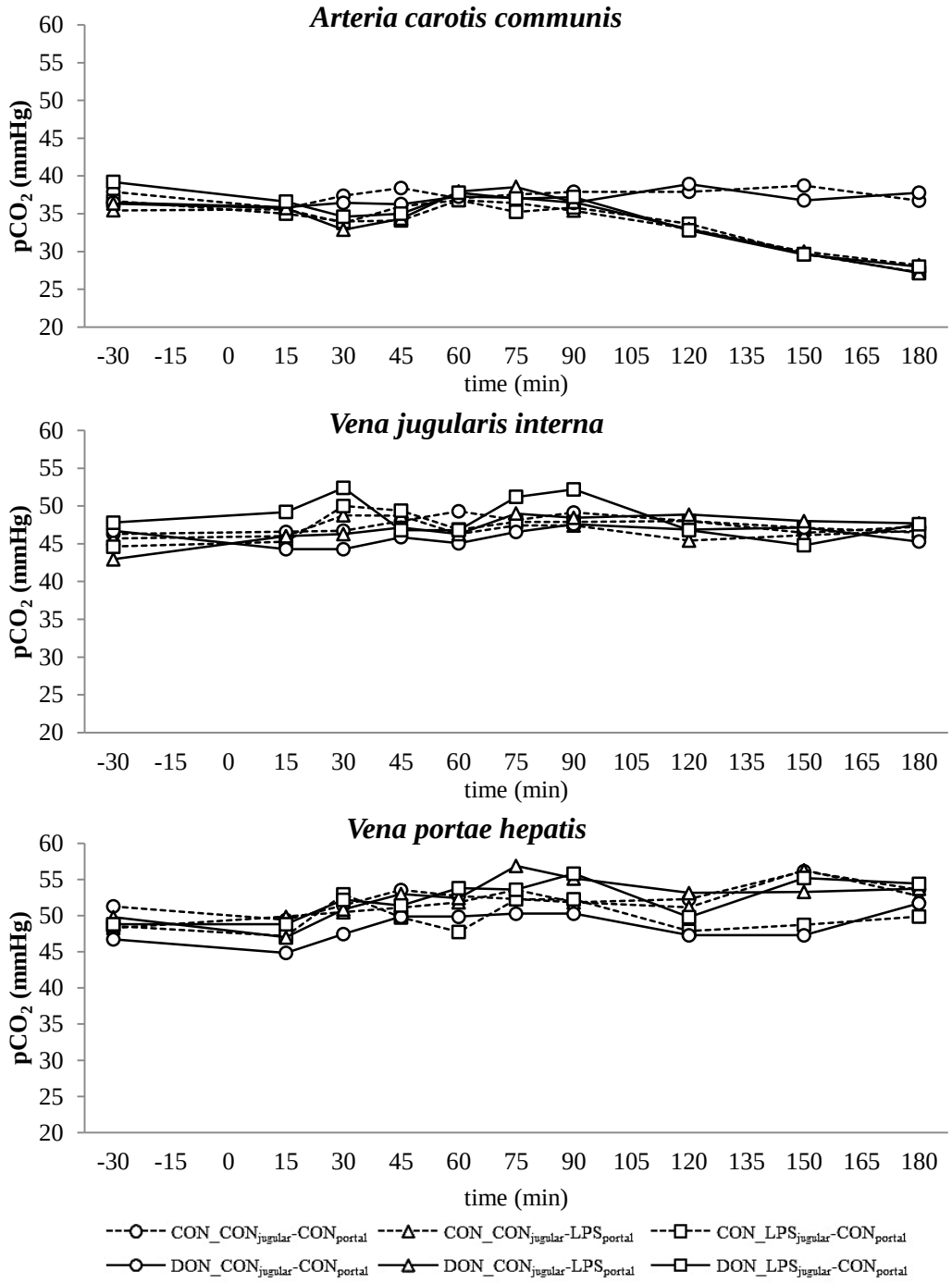
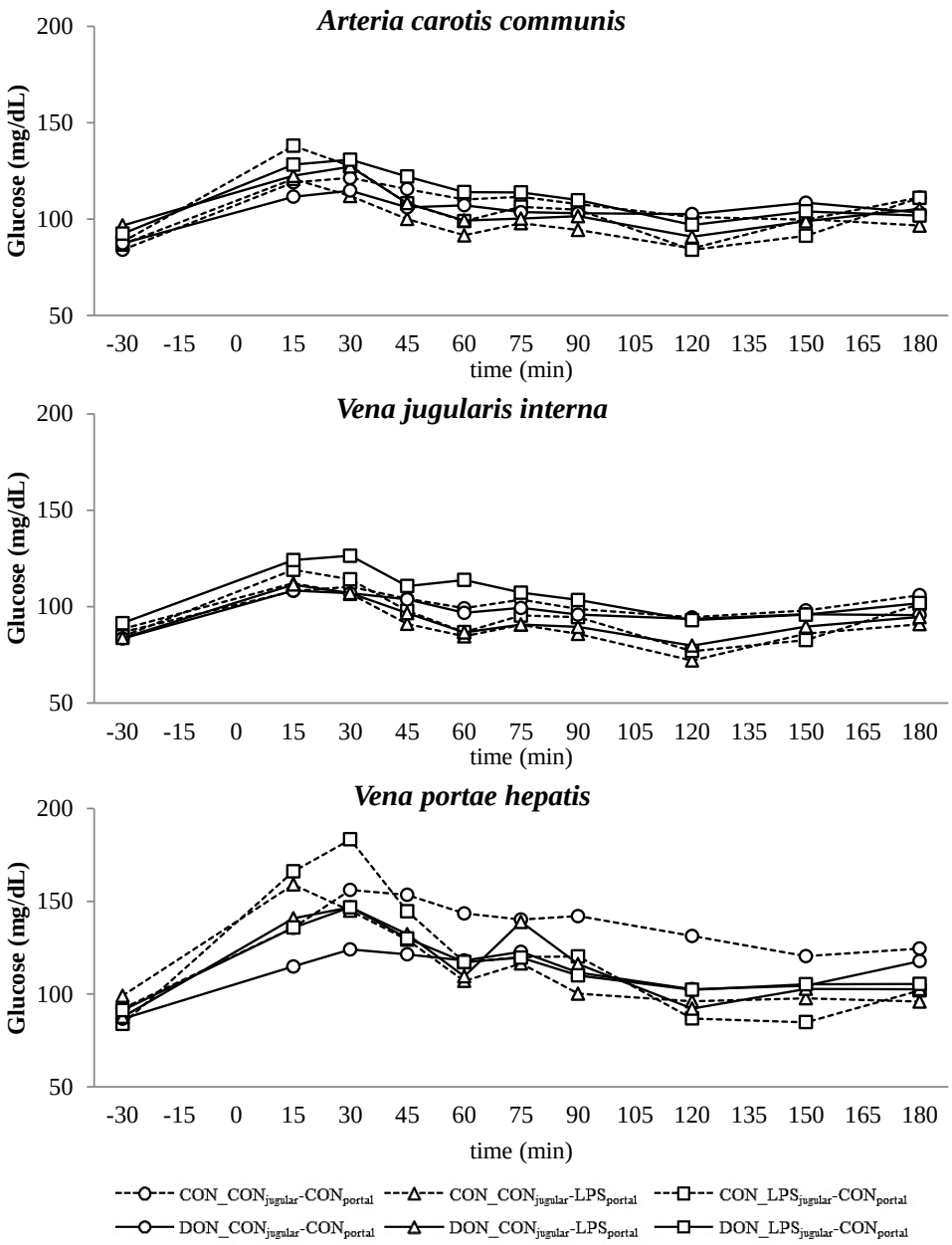


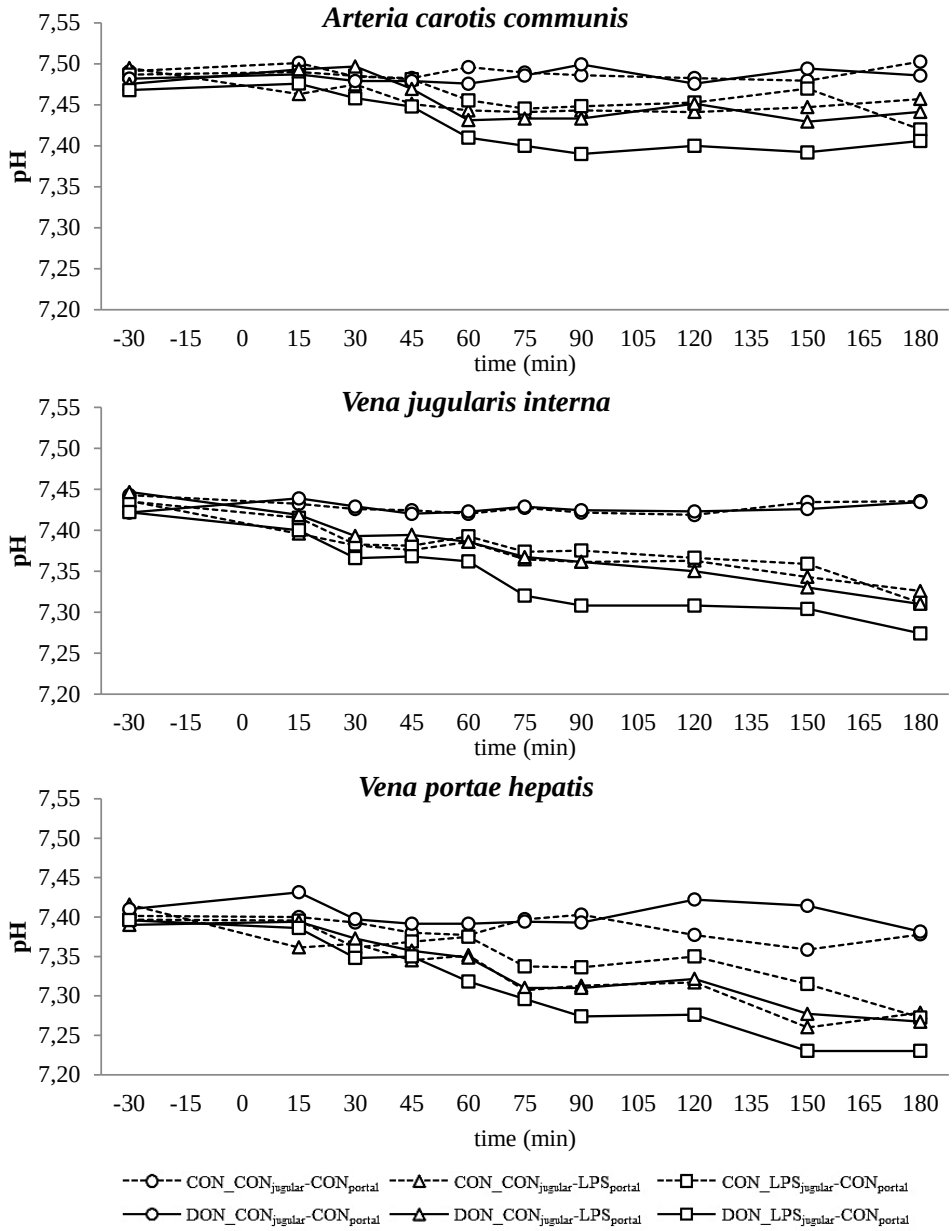
Fig. 2: Effect of chronic enteral *Fusarium* toxin deoxynivalenol (DON) exposure and pre- or post-hepatic *E. coli* lipopolysaccharide (LPS) infusion on arterial, jugular or portal blood partial carbon dioxide pressure (pCO_2) in pigs. Reference value: 50 mmHg in arterial blood [26]. Barrows were either fed a DON contaminated ration (4.59 mg/kg feed) or control feed during 29 days. Infusion groups were divided as follows: pre-hepatic LPS infusion (CON_$CON_{jugular}$-LPS_{portal}, n = 7 and DON_$CON_{jugular}$-LPS_{portal}, n = 6), post-hepatic LPS infusion (CON_$LPS_{jugular}$-CON_{portal}, n = 8 and DON_$LPS_{jugular}$-CON_{portal}, n = 6), and control infusion (CON_$CON_{jugular}$-CON_{portal}, n = 7 and DON_$CON_{jugular}$-CON_{portal}, n = 7). Infusion from time 0 until 60 min with 7.5 µg LPS/kg LW in 0.9 % saline. Feed was offered during 15 min prior to infusion start. LSMeans. PSEM = 1.75. Significance: Group (G): $p = 0.28$; Catheter (C): $p \leq 0.001$; Time (T): $p \leq 0.001$; G*C*T: $p \leq 0.001$.



***post hoc* test (p-value) CON_CON-CON vs. DON_CON-CON**

time (min)	-30	15	30	45	60	75	90	120	150	180
A.carotis	0.819	0.599	0.652	0.518	0.833	0.587	0.745	0.912	0.546	0.587
V.jugularis	0.812	0.991	0.811	0.983	0.865	0.756	0.836	0.950	0.885	0.465
V.portae	0.686	0.131	**0.024**	**0.021**	0.066^{T}	0.207	**0.028**	**0.043**	0.247	0.632

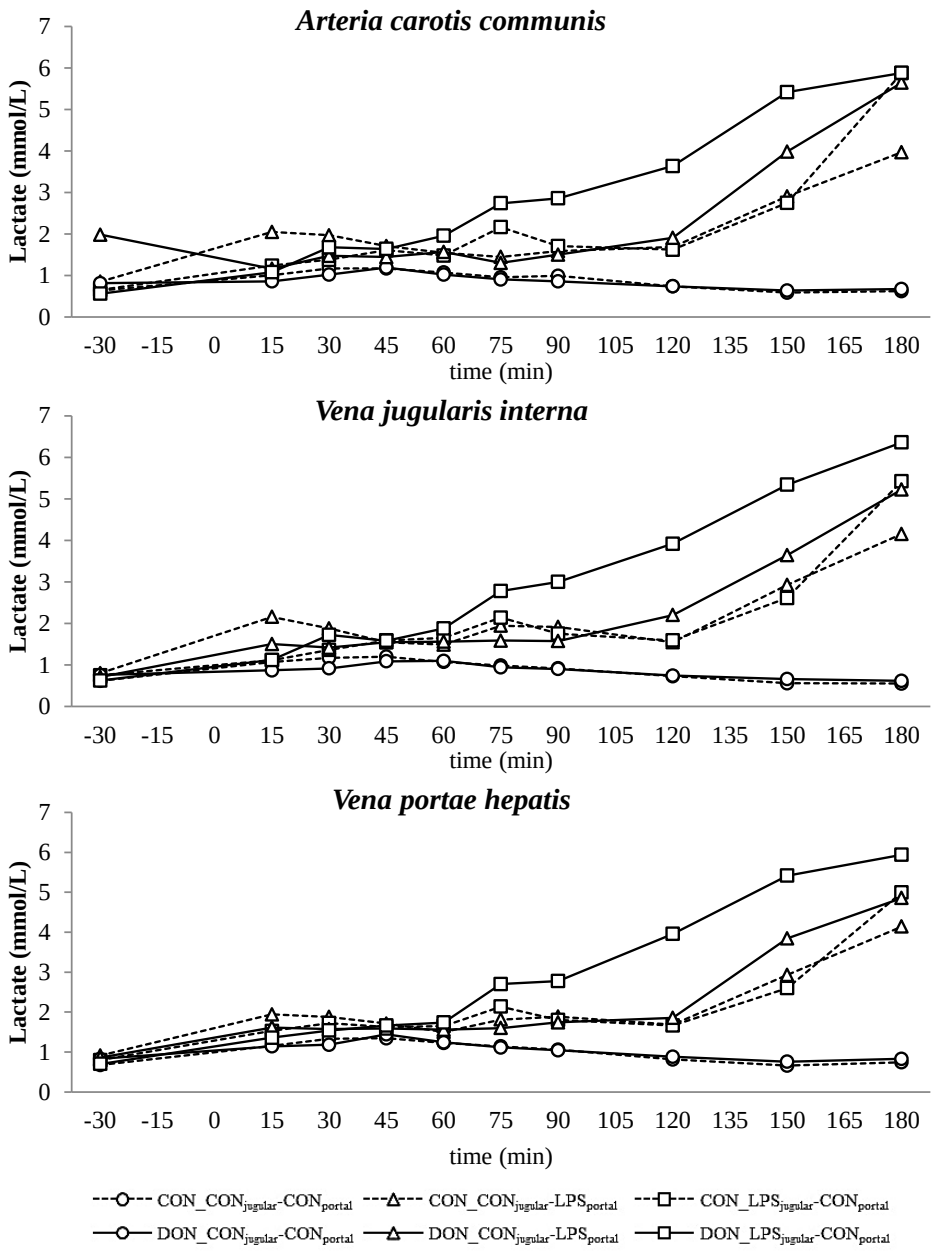
Fig. 3: Effect of chronic enteral *Fusarium* toxin deoxynivalenol (DON) exposure and pre- or post-hepatic *E. coli* lipopolysaccharide (LPS) infusion on arterial, jugular or portal blood glucose in pigs. Reference value: 70-115 mg/dl in venous blood [26]. Barrows were either fed a DON contaminated ration (4.59 mg/kg feed) or control feed during 29 days. Infusion groups were divided as follows: pre-hepatic LPS infusion (CON_CON$_{jugular}$-LPS$_{portal}$, n = 7 and DON_CON$_{jugular}$-LPS$_{portal}$, n = 6), post-hepatic LPS infusion (CON_LPS$_{jugular}$-CON$_{portal}$, n = 8 and DON_LPS$_{jugular}$-CON$_{portal}$, n = 6), and control infusion (CON_CON$_{jugular}$-CON$_{portal}$, n = 7 and DON_CON$_{jugular}$-CON$_{portal}$, n = 7). Infusion from time 0 until 60 min with 7.5 µg LPS/kg LW in 0.9 % saline. Feed was offered during 15 min prior to infusion start. LSMeans. PSEM = 1.75. Significance: Group (G): $p = 0.28$; Catheter (C): $p \leq 0.001$; Time (T): $p \leq 0.001$; G*C*T: $p \leq 0.001$. Table illustrates differences between DON- and CON-fed control infused groups at different times. ^{T} = trend ($P \leq 0.10$)



***post hoc* test (p-value) CON_LPS-CON vs. DON_LPS-CON**

time (min)	-30	15	30	45	60	75	90	120	150	180
A.carotis	0.499	0.611	0.327	0.236	0.105	0.105	**0.038**	0.061^{T}	**0.006**	0.623
V.jugularis	0.636	0.585	0.548	0.630	0.268	0.051^{T}	**0.015**	**0.035**	**0.047**	0.176
V.portae	0.975	0.736	0.637	0.495	**0.039**	0.132	**0.024**	**0.007**	**0.002**	0.129

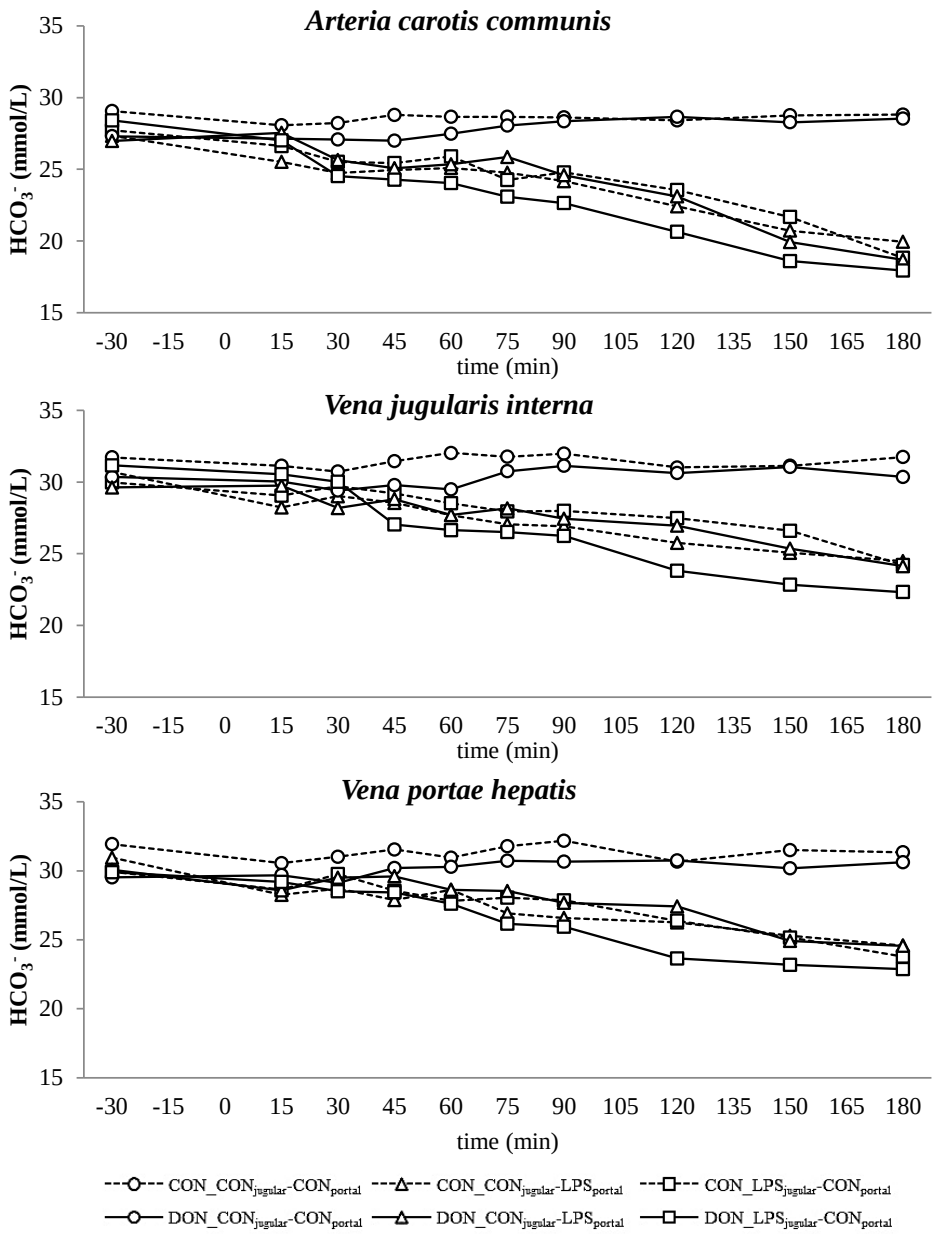
Fig. 4: Effect of chronic enteral Fusarium toxin deoxynivalenol (DON) exposure and pre- or post-hepatic *E. coli* lipopolysaccharide (LPS) infusion on arterial, jugular or portal blood pH in pigs. Reference value: 7.42 in arterial blood [26]. Barrows were either fed a DON contaminated ration (4.59 mg/kg feed) or control feed during 29 days. Infusion groups were divided as follows: pre-hepatic LPS infusion (CON_CON$_{jugular}$-LPS$_{portal}$, n = 7 and DON_CON$_{jugular}$-LPS$_{portal}$, n = 6), post-hepatic LPS infusion (CON_LPS$_{jugular}$-CON$_{portal}$, n = 8 and DON_LPS$_{jugular}$-CON$_{portal}$, n = 6), and control infusion (CON_CON$_{jugular}$-CON$_{portal}$, n = 7 and DON_CON$_{jugular}$-CON$_{portal}$, n = 7). Infusion from time 0 until 60 min with 7.5 µg LPS/kg LW in 0.9 % saline. Feed was offered during 15 min prior to infusion start. LSMeans. PSEM = 0.02. Significance: Group (G): $p \leq 0.001$; Catheter (C): $P \leq 0.001$; Time (T): $P \leq 0.001$; G*C*T: $P \leq 0.001$. Table illustrates differences between DON and CON fed post-hepatic LPS infused groups at different times. ^{T} = trend ($P \leq 0.10$)



***post hoc* test (p-value) CON_LPS-CON vs. DON_LPS-CON**

time (min)	-30	15	30	45	60	75	90	120	150	180
A.carotis	0.912	0.845	0.714	0.969	0.558	0.484	0.161	**0.014**	**0.001**	0.999
V.jugularis	0.871	0.993	0.656	0.993	0.775	0.424	0.120	**0.004**	**0.001**	0.245
V.portae	0.904	0.838	0.824	0.965	0.911	0.484	0.223	**0.004**	**<0.001**	0.252

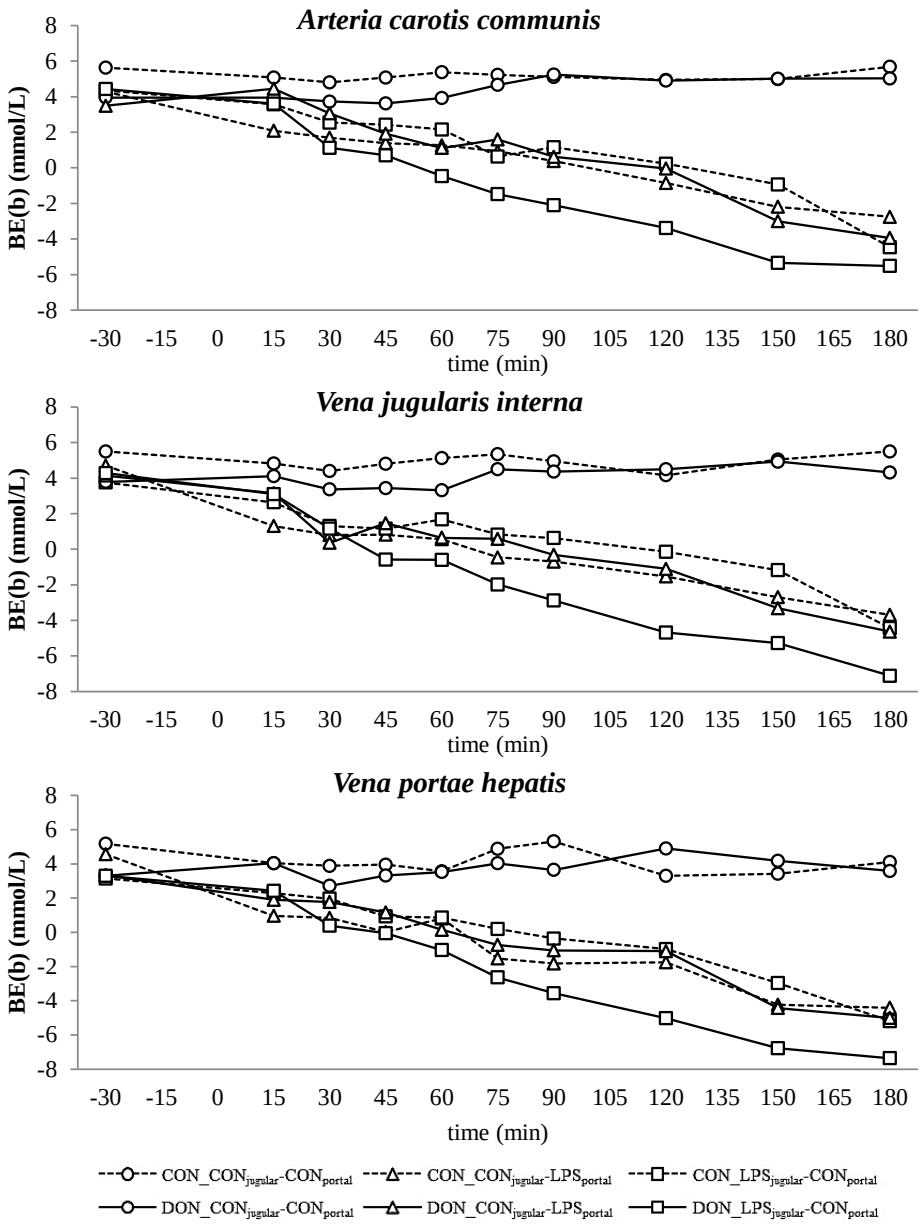
Fig. 5: Effect of chronic enteral *Fusarium* toxin deoxynivalenol (DON) exposure and pre- or post-hepatic *E. coli* lipopolysaccharide (LPS) infusion on arterial, jugular or portal blood lactate in pigs. Reference: 0,84±0,24 mmol/l in venous blood [28]. Barrows were either fed a DON contaminated ration (4.59 mg/kg feed) or control feed during 29 days. Infusion groups were divided as follows: pre-hepatic LPS infusion (CON_CON$_{jugular}$-LPS$_{portal}$, n = 7 and DON_CON$_{jugular}$-LPS$_{portal}$, n = 6), post-hepatic LPS infusion (CON_LPS$_{jugular}$-CON$_{portal}$, n = 8 and DON_LPS$_{jugular}$-CON$_{portal}$, n = 6), and control infusion (CON_CON$_{jugular}$-CON$_{portal}$, n = 7 and DON_CON$_{jugular}$-CON$_{portal}$, n = 7). Infusion from time 0 until 60 min with 7.5 µg LPS/kg LW in 0.9 % saline. Feed was offered during 15 min prior to infusion start. LSMeans. PSEM = 0.56. Significance: Group (G): : $p \leq 0.001$; Catheter (C): $p = 0.78$; Time (T): $p \leq 0.001$; G*C*T: $p \leq 0.001$. Table illustrates differences between DON and CON fed post-hepatic LPS infused groups at different times. ^{T} = trend ($P \leq 0.10$)



***post hoc* test (p-value) CON_LPS-CON vs. DON_LPS-CON**

time (min)	-30	15	30	45	60	75	90	120	150	180
A.carotis	0.639	0.789	0.506	0.443	0.215	0.431	0.153	0.054^{T}	**0.042**	0.558
V.jugularis	0.423	0.314	0.835	0.145	0.206	0.327	0.238	**0.014**	**0.012**	0.205
V.portae	0.980	0.714	0.408	0.934	0.896	0.202	0.195	0.066^{T}	0.181	0.539

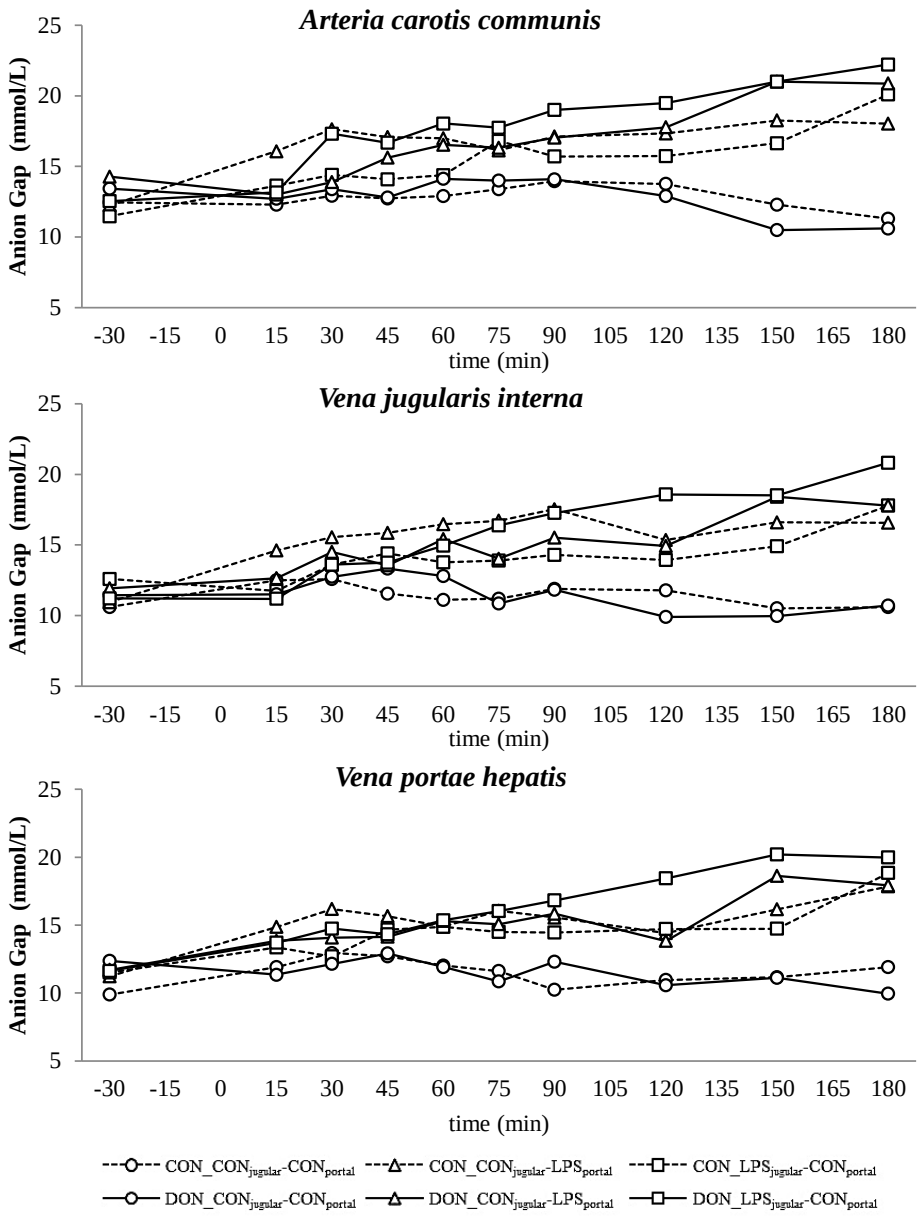
Fig. 6 Effect of chronic enteral *Fusarium* toxin deoxynivalenol (DON) exposure and pre- or post-hepatic *E. coli* lipopolysaccharide (LPS) infusion on arterial, jugular or portal blood bicarbonate (HCO_3^-) in pigs. Reference range: 20 – 30 mmol/L in arterial blood [26]. Barrows were either fed a DON contaminated ration (4.59 mg/kg feed) or control feed during 29 days. Infusion groups were divided as follows: pre-hepatic LPS infusion (CON_CON$_{jugular}$-LPS$_{portal}$, n = 7 and DON_CON$_{jugular}$-LPS$_{portal}$, n = 6), post-hepatic LPS infusion (CON_LPS$_{jugular}$-CON$_{portal}$, n = 8 and DON_LPS$_{jugular}$-CON$_{portal}$, n = 6), and control infusion (CON_CON$_{jugular}$-CON$_{portal}$, n = 7 and DON_CON$_{jugular}$-CON$_{portal}$, n = 7). Infusion from time 0 until 60 min with 7.5 µg LPS/kg LW in 0.9 % saline. Feed was offered during 15 min prior to infusion start. LSMeans. PSEM = 1.18. Significance: Group (G): : $p \leq 0.001$; Catheter (C): $p \leq 0.001$; Time (T): $p \leq 0.001$; G*C*T: $p \leq 0.001$. Table illustrates differences between DON and CON fed post-hepatic LPS infused groups at different times. ^{T} = trend ($P \leq 0.10$)



***post hoc* test (p-value) CON_LPS-CON vs. DON_LPS-CON**

time (min)	-30	15	30	45	60	75	90	120	150	180
A.carotis	0.957	0.988	0.413	0.324	0.133	0.226	0.062^{T}	**0.039**	**0.012**	0.545
V.jugularis	0.757	0.787	0.935	0.310	0.186	0.103	**0.042**	**0.009**	**0.018**	0.119
V.portae	0.931	0.941	0.364	0.566	0.268	0.099^{T}	0.064^{T}	**0.020**	**0.027**	0.214

Fig. 7: Effect of chronic enteral *Fusarium* toxin deoxynivalenol (DON) exposure and pre- or post-hepatic *E. coli* lipopolysaccharide (LPS) infusion on arterial, jugular or portal blood base-excess (BE(b)) in pigs. Reference range: -3.5 - 3.5 mmol/L in arterial blood [26]. Barrows were either fed a DON contaminated ration (4.59 mg/kg feed) or control feed during 29 days. Infusion groups were divided as follows: pre-hepatic LPS infusion (CON_CON$_{jugular}$-LPS$_{portal}$, n = 7 and DON_CON$_{jugular}$-LPS$_{portal}$, n = 6), post-hepatic LPS infusion (CON_LPS$_{jugular}$-CON$_{portal}$, n = 8 and DON_LPS$_{jugular}$-CON$_{portal}$, n = 6), and control infusion (CON_CON$_{jugular}$-CON$_{portal}$, n = 7 and DON_CON$_{jugular}$-CON$_{portal}$, n = 7). Infusion from time 0 until 60 min with 7.5 µg LPS/kg LW in 0.9 % saline. Feed was offered during 15 min prior to infusion start. LSMeans. PSEM = 1.18. Significance: Group (G): : $p \leq 0.001$; Catheter (C): $p \leq 0.001$; Time (T): $p \leq 0.001$; G*C*T: $p \leq 0.001$. Table illustrates differences between DON and CON fed post-hepatic LPS infused groups at different times. ^{T} = trend ($P \leq 0.10$)



***post hoc* test (p-value) CON_LPS-CON vs. DON_LPS-CON**

time (min)	-30	15	30	45	60	75	90	120	150	180
A.carotis	0.564	0.792	0.115	0.162	**0.048**	0.612	0.075^{T}	**0.043**	**0.019**	0.253
V.jugularis	0.451	0.759	1.000	0.716	0.516	0.170	0.102	**0.011**	**0.048**	0.095^{T}
V.portae	0.951	0.855	0.268	0.850	0.784	0.392	0.192	**0.041**	**0.003**	0.535

Fig. 8: Effect of chronic enteral *Fusarium* toxin deoxynivalenol (DON) exposure and pre- or post-hepatic *E. coli* lipopolysaccharide (LPS) infusion on arterial, jugular or portal blood anion-gap Gap(K^+) in pigs. Reference range: 10 – 25 mmol/L in venous blood [27]. Barrows were either fed a DON contaminated ration (4.59 mg/kg feed) or control feed during 29 days. Infusion groups were divided as follows: pre-hepatic LPS infusion (CON_CON$_{jugular}$-LPS$_{portal}$, n = 7 and DON_CON$_{jugular}$-LPS$_{portal}$, n = 6), post-hepatic LPS infusion (CON_LPS$_{jugular}$-CON$_{portal}$, n = 8 and DON_LPS$_{jugular}$-CON$_{portal}$, n = 6), and control infusion (CON_CON$_{jugular}$-CON$_{portal}$, n = 7 and DON_CON$_{jugular}$-CON$_{portal}$, n = 7). Infusion from time 0 until 60 min with 7.5 µg LPS/kg LW in 0.9 % saline. Feed was offered during 15 min prior to infusion start. LSMeans. PSEM = 1.22. Significance: Group (G): : $p \leq 0.001$; Catheter (C): $p \leq 0.001$; Time (T): $p \leq 0.001$; G*C*T: $p \leq 0.001$. Table illustrates differences between DON and CON fed post-hepatic LPS infused groups at different times. ^{T} = trend ($P \leq 0.10$)

3.4. Discussion

In this study, LPS was used to induce an inflammatory response. All LPS-treated pigs exhibited typical clinical symptoms of an acute phase response [16,29,30], such as an increased respiratory rate, fever, tremor, cyanosis, followed by hyperemic conjunctivae, injected episcleral vessels or leukopenia (Tesch et al., 2015, submitted, [31]). In all groups at time −30 min and within the control group over the course of the trial, no significant alterations of metabolic and hematological variables were observed, and all parameters were within their physiological range. It therefore can be stated that the performed manipulations, such as the surgery and the sampling procedure, did not constitute confounding factors. All LPS-treated animals exhibited a lactic acidosis as a consequence of LPS infusion [13]. Taking into consideration the observed pO2, pCO2 and lactate concentrations, we deduced that at first, acidosis was caused by a decreased pO2 (respiratory acidosis) and later on originated from an increase in systemic lactate concentration (metabolic acidosis) [32]. This is also mirrored in the variables BE and AG, which are a reflection of the different variables that affect the acid-base balance [33]. The alterations observed in AG can solely be ascribed to changes in $HCO3^-$ concentrations, since other electrolytes (Na^+, K^+, Cl^-, $Ca2^+$) were not influenced by the LPS challenge.

Since erythrocytes occupy a central role in oxygen transport, metabolism, as well as the acid-base balance, we also assessed red hemogram variables. No biologically relevant influence of DON or LPS was observed on red hemogram variables at any time during the trial. This is in line with a study of Grenier et al. [34] in which the effects of the mycotoxins DON and fumonisin, alone or in combination with a subcutaneous ovalbumin injection on different hematology variables, were investigated.

These results are further confirmed by two recent studies [35,36] investigating the influence of low-dose (≤2 mg/kg feed) chronic oral DON exposure on red hemogram variables and electrolytes in piglets and pre-puberal gilts, respectively. However, in both studies, neither biologically relevant alterations in red hemogram nor blood electrolytes were observed after four weeks of chronic oral DON exposure. Few studies have investigated the influence of an acute inflammatory response on sodium-potassium transport in red blood cells and skeletal muscle. Suri and colleagues [37] and Illner et al. [38] observed hyponatremia and hyperpotassemia and attributed this to alteration in the transport capacity of the RBC Na^+/K^+ pump. This change in ion transport across RBC and skeletal muscles has also been observed in other studies [39,40], but this hypothesis was challenged later

on [41,42]. In our study, we did not detect any changes in blood electrolytes and, based on these previous articles, might speculate that the sodium-potassium transport in red blood cells and skeletal muscle after an LPS-challenge was not changed.

A decrease in systemic pO2 during the initial state of an acute inflammatory response has been documented before and is probably caused by a decreased cardiac output, as well as alterations in the respiratory rate and depth [43]. This assumption is supported by the concurrent observed increase in respiratory rate in LPS-infused animals during this trial (Tesch et al., 2015, submitted, [31]). After an initial decrease of arterial pO2, a continuous increase during the rest of the trial, accompanied by a decrease in jugular pO2 and no alterations in jugular pCO2, was observed. This observation is indicative of an increase in tissue oxygen consumption, rather than a decrease in overall oxygen availability. These findings are in line with previous studies showing that the hyperlactatemia observed during sepsis is most likely caused by alterations in the glycolytic pathway, rather than hypoxia, along with increased tissue oxygen consumption [44]. In human studies, Revelly and co-workers [45] described an increased glucose and lactate rate of appearance in the blood of septic patients compared to healthy subjects. Furthermore, the hyperlactatemia resulted from an increased endogenous lactate production in sepsis, whereas lactate clearance was not altered compared to healthy patients, confirming the impact of altered glycolytic pathways in the development of lactic acidosis. It is further assumed that during a state of acute inflammatory response, the rate of pyruvate formation exceeds the oxidative capacity of mitochondria, causing an accumulation of pyruvate, and thereby, an increase in lactate formation [46]. This is further potentiated by a decrease in lactate utilization [47]. Besides an alteration in the glycolytic pathway, tissue perfusion heterogeneity is being put forward as a possible reason for hyperlactatemia accompanied by physiological systemic pO2 during sepsis by Gutierrez and colleagues [18]. Under shock conditions, the blood circulation is centralized to vital organs, and non-vital tissues are characterized by a compromised peripheral vascular perfusion [48]. In our study, we clinically observed cyanosis of the extremities and dermographism in five out of 28 LPS-treated pigs (Tesch et al., 2015, submitted, [31]), as well as liver hemorrhage in LPS-treated animals (Renner et al., 2015, [49]). In contrast to our observations, other studies with a similar setup reported dermographism in most of the LPS-treated animals, as well as macroscopic and microscopic intrahepatic hyperperfusion [8]. These symptoms can be attributed, amongst others, to tissue perfusion heterogeneity and, thus, would fit the hypothesis voiced earlier [18]. Further, an increased lactic acid output of leukocytes due to an increased glycolysis has been suggested as a

contributing factor to the lactic acidosis observed in septic animals [50]. In our study, leukocytes in particular were severely affected by LPS and an enhanced glycolysis, and thus, the output of lactic acid into the blood might have contributed to the present lactic acidosis (Tesch et al., 2015, submitted, [31]).

There was no difference between pre- or post-hepatic LPS infusion in CON-fed animals concerning the acid-base balance. An influence of DON was only observed in post-hepatic LPS-infused animals, whereas no dietary impact in pre-hepatic LPS-infused animals was observed regarding different acid-base balance variables in our trial. These results suggest a DON-related priming of post-hepatic cells involved in the exacerbation of metabolic disorders caused by LPS stimulation. Moreover, the lack of an assumed partial hepatic LPS clearance in post-hepatic LPS-infused DON-fed pigs compared to their pre-hepatic LPS-infused DON-fed counterparts might have triggered latent interactions between LPS and DON. Previously, it has been shown that an exposure to DON causes an altered immune response due to an upregulation of the transcriptional and post-transcriptional expression of cytokines, chemokines and inflammatory genes in porcine *in vitro* and *in vivo* studies [6-8]. In rodent studies, LPS priming of animals prior to DON exposure resulted in a stronger cytokine response compared to vehicle-treated animals [51], as well as simultaneous LPS and DON treatment of RAW264.7 macrophages [52]. However, we observed a uniform increase of TNF-α after 30 min with peak values at 60 min in all LPS-infused pigs, irrespective of the site of infusion or dietary treatment. Therefore, in our study, we could not confirm a superinduction of TNF-α in LPS-treated animals fed with a DON-contaminated diet (Tesch et al., 2015, submitted, [31]).

Similar to other studies that have investigated the influence of a systemic inflammatory response on blood glucose levels, we observed an initial hyperglycemia followed by eu- and hypoglycemia [21,53]. However, this kinetic was not significantly distinguishable from the post-prandial increase in blood glucose, which was observed in all groups as LPS infusion superimposed with the post-prandial effects. The DON fed animals exhibited a markedly lower portal glucose level, which can most likely be attributed to the negative effects of DON on glucose transport across the intestinal barrier, as described previously by Halawa et al. [54]. This effect was also observed in several other studies using different animal and *in vitro* models. In chickens, DON inhibited the jejunal SGLT-1 activity (sodium-linked glucose transporter 1), responsible for active glucose uptake into enterocytes from lumen [55]. Furthermore, in human cell line HT-29, SGLT-1, GLUT-5 (glucose transporter 5, D-fructose associated) and GLUT-1 (passive D-glucose transporter) were inhibited

by DON in a dose-dependent manner [56]. However, other studies in swine did not confirm this effect on SGLT-1 activity in brush border vesicles derived from the jejunum [57]. In addition to this DON effect, an additive effect of LPS infusion was detected. All LPS-infused groups had lower portal glucose concentrations 45 min after infusion start compared to the total control group. Generally, under shock conditions, hypoperfusion of the intestinal tract can be observed [58,59], and therefore, it can be hypothesized that the transport capacity of the intestinal mucosa is impaired. Furthermore, in several LPS studies, an inhibitory effect on intestinal glucose transport was observed, for instance decreased GLUT-5 [60] and SGLT-1 [61] levels in LPS-treated rabbits. Furthermore, Amador and co-authors [62] observed an inhibitory effect of TNF-α on SGLT-1 ex vivo in rabbit's intestine. We could thus hypothesize that in our study, the impaired portal glucose uptake in LPS-treated animals (45 min-180 min) might be, at least partially, attributed to an impairment in SGLT-1 transport capacity due to the increase in TNF-α (Tesch et al., 2015, submitted, [31]).

Our data suggest that chronic oral exposure to DON exacerbates lactic acidosis by a post-hepatic LPS-induced systemic inflammation, while a pre-hepatic LPS stimulation did not result in such amplification. This different responsiveness between pre- and post-hepatic-infused animals was not observed within the control-fed groups.

3.5. Experimental Section

Animal experiments were conducted according to the EC regulations concerning the protection of experimental animals and the guidelines of the German Animal Welfare Act approved by the Lower Saxony State Office for Consumer Protection and Food Safety (Lower Saxony State Office for Consumer Protection and Food Safety; File Number 33.4-42502-04-13/1274).

3.5.1. Experimental Design and Procedures

A total of 41 barrows (German landrace, Mariensee, Germany) were randomly assigned to either a group receiving natural DON-contaminated feed (DON; 4.59 mg DON/kg feed; n = 19) or a control group (CON; n = 22) control diet (Table 3). Experimental groups, their treatment and the number of animals are illustrated in Figure 9. The pigs had an average initial weight of 25.8 ± 3.7 kg (means ± SD) and were fed restrictively with 2 single portions of 700 g per day, mixed with water and provided as mash. All barrows were housed in individual floor pens during the first 21

days of the trial and subsequently transferred into individual metabolism crates (described in [63]) until Day 29.

Table 3: Diet composition, based on air dry matter (ADM) = 88.37%

Ingredients	CON	DON
	%	%
barley	53.30	53.30
maize (non contaminated)	15.00	7.50
maize (contaminated)	-	7.50
soybean meal	20.00	20.00
rapeseed	5.00	5.00
soybean oil	2.00	2.00
Premix[1]	3.00	3.00
Lysine-HCl	0.40	0.40
L-Threonine	0.12	0.12
DL-Methionine	0.15	0.15
HCl-insoluble ash[2]	1.00	1.00
analysed composition	**g/kg ADM**	**g/kg ADM**
crude protein	196.85	194.83
crude fat	47.48	46.51
crude ash	69.70	69.51
crude fiber	51.26	49.32
deoxynivalenol mg/kg	0.12	4.59

[1] Provided per kilogram of premix: Ca 25 g, P 6 g, Na 5.5 g, Mg 1 g, Fe 4000 mg, Cu 500 mg, Mn 2670 mg, Zn 3340 mg, I 67 mg, Se 13.5 mg, Co 8.3 mg, bas. Co-II-carb-monohydrat 8.3 mg, vitamin A 400000 I.U., vitamin D3 40000 I.U., vitamin E 1200 mg, vitamin B_1 37.5 mg, vitamin B_2 100 mg, vitamin B_6 100 mg, vitamin B_{12} 750 µg, vitamin K3 52.5 mg, nicotinic acid 500 mg, pantothenic acid 337.5 mg, choline chloride 5000 mg.

[2] > 97% SiO_2 (Sipernat® 22S, Evonic Industries, Hanau-Wolfgang, Germany)

In order to facilitate pre- or post-hepatic blood-sampling and infusion, pigs were surgically fitted with 5 differently-located, permanent indwelling catheters under general inhalation anesthesia (Isoflurane®, CP-Pharma, Burgdorf, Germany) at Day 27 of the trial when the animals had an average body weight of 40.5 ± 3.0 kg. Permanent Silastic® catheters were manufactured from Dow Corning (Midland, TX, USA) medical-grade tubing material (1.57 mm ID and 3.18 mm OD), autoclaved and placed in the *Vena jugularis interna*, *Vena jugularis externa*, *Vena lienalis*, *Vena*

portae hepatis and *Arteria carotis communis*. Catheters were tunneled to the neck and left flank, respectively, and fixed with catheter mounts/clamps (Arrow, Teleflex Medical GmbH, Kernen, Germany) on the skin. Catheters were fitted with three-way valves (Walther-CMP, Kiel, Germany) for sampling and flushed with heparinized physiological saline (1 mL sodium heparin (25.000 IE/5 mL) (Ratiopharm, Ulm, Germany); dissolved in 500 mL sterile 0.9% NaCl (B. Braun Melsungen AG, Melsungen, Germany) every 4 h and after each sampling to prevent blood coagulation. Two days between surgery and sampling day were allowed for recovery. Throughout this recovery period, half of the daily ration was fed (2 times 350 g/day). At Day 29 of the trial, animals were further divided into 3 infusion groups: LPS pre-hepatic (LPSportal-CONjugular), LPS post-hepatic (CONportal-LPSjugular) or control (CONportal-CONjugular), illustrated in Figure 9.

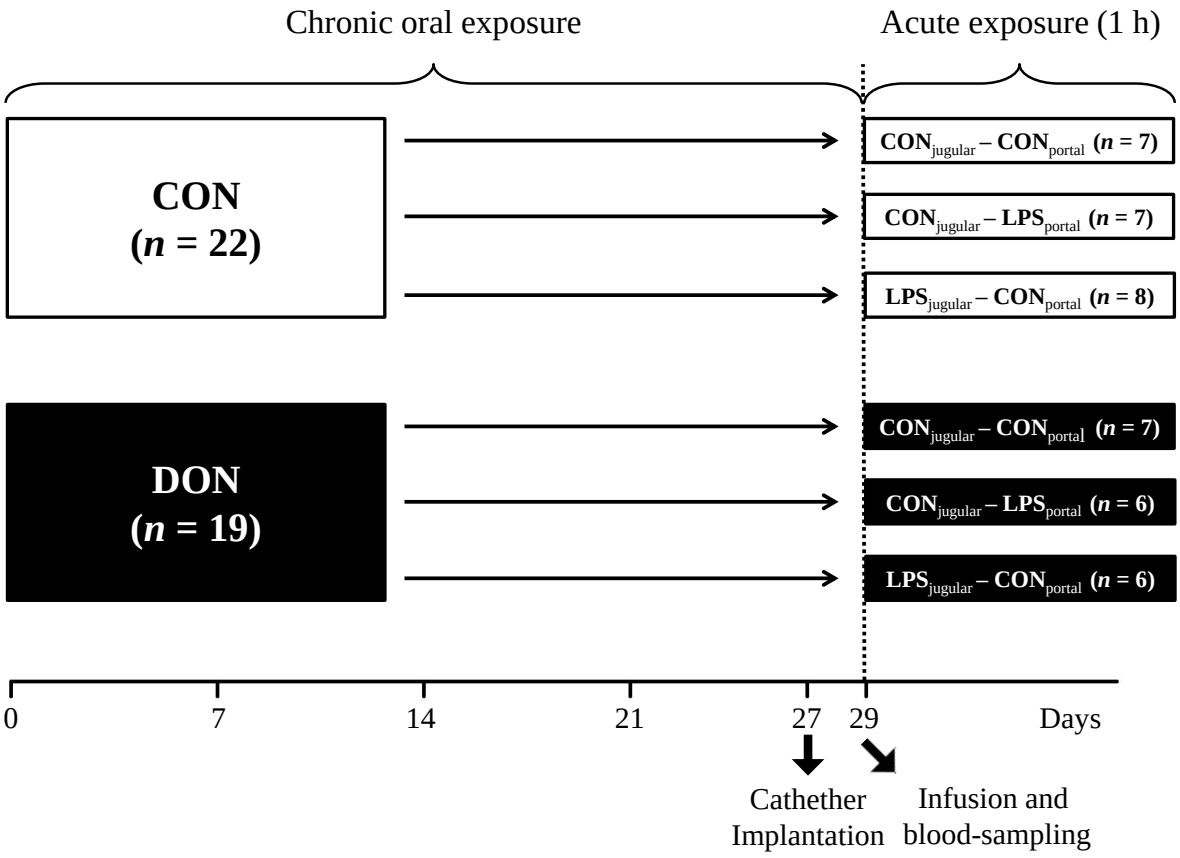


Figure 9: Experimental design.

On the sampling day, the time of infusion start was set as zero, and blood samples were taken at -30, 15, 30, 45, 60, 75, 90, 120, 150 and 180 min from *Vena jugularis interna*, *Vena portae hepatis* and *Arteria carotis communis*. Fifteen minutes prior to infusion, pigs received 700 g of feed each. LPS was infused at 7.5 µg LPS/kg BW for 1 h (Escherichia coli-LPS, O111:B4, Sigma-

Aldrich, Taufkirchen, Germany), and 0.9% NaCl was used as the control substance. Infusion was implemented using an infusion pump (IPC-N-4, ISMATEC Laboratoriumstechnik GmbH, Wertheim, Germany) and infusion tubes with a 2.06 mm inner diameter (PharMed® Ismaprene, Wertheim, Germany, ISMATEC), administered into either *Vena jugularis externa* or *Vena lienalis*. The pigs were slaughtered 195 min after infusion start.

3.5.2. Sample Analysis

For a red hemogram assessment, 1-mL blood samples were collected in EDTA tubes and analyzed immediately with an automated hematology analyzer (Celltac alpha MEK-6450, Nihon Kohden Corporation, Tokyo, Japan). Furthermore, blood samples (0.5 mL) for blood gases, electrolytes, pH, glucose and lactate were collected into blood sample syringes (SC-Sanguis Counting GmbH, Nümbrecht, Germany), and variables were assessed immediately using an automated blood gas and electrolyte analyzer (GEM Premier 4000, Werfen, Kirchheim, Germany). Anion gap and base excess were calculated using the equations detailed below.

3.5.3. Calculations

Equation used to calculate anion gap (AG) (Emmett and Narins 1977):

$$AG = (Na^+ + K^+) - (Cl^- + HCO_3^-)$$

Na^+ = sodium; K^+ = potassium; Cl^- = chloride; HCO_3^- = bicarbonate.

Equation used to calculate base excess (BE) (Lang and Zander 2002):

$$BE = (1 - 0.0143 \times cHb) \times [(0.0304 \times pCO_2 \times 10^{pH-6.1} - 24.26) + (9.5 + 1.63 \times cHb) \times (pH - 7.4)] - 0.2 \times cHb \times (1 - sO_2)$$

cHb = total hemoglobin concentration; pCO_2 = carbon dioxide partial pressure; sO_2 = oxygen saturation.

3.5.4. Statistical Analysis

Data were evaluated by using PROC MIXED in SAS Enterprise Guide 6.1 (SAS Institute 2013, Cary, NC, USA) using a restricted maximum likelihood model (REML). Group, catheter, time and their interaction were defined as fixed factors. A “REPEATED” statement was included to account

for the individual similarity at repeated measurements. The “compound symmetry” was found to be the most appropriate co-variance structure according to the corrected Akaike’s information criterion (AICC), and significant effects at different time points were further evaluated by multiple t-tests (“pairwise differences” (PDIFF)). Results are presented as least square means (LSMeans) and pooled standard error of means (PSEM).

3.6. Conclusions

Concluding, we could demonstrate chronic enteral DON-exposure had a definite priming effect on the pig’s organism, specifically the acid-base balance, thus aggravating the sole impact of LPS depending on the site of its entry. The involvement of the liver in this scenario was apparent as post-hepatic LPS exposure elicited a much stronger impact compared to pre-hepatic exposure in combination with mycotoxin feeding. Portal glucose uptake was significantly diminished in DON-fed animals compared to control, thus giving in vivo evidence to the previously reported impairment of glucose transporter activity in ex vivo studies.

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Author Contributions

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Conflicts of Interest

The authors declare no conflict of interest.

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4. Plasma Kinetics and Matrix Residues of Deoxynivalenol (DON) and Zearalenone (ZEN) are Altered in Endotoxaemic Pigs Independent of LPS Entry Site.

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4.1. Abstract

This study aimed to investigate a potential modulatory effect of E. coli lipopolysaccharide (LPS) on the kinetics of deoxynivalenol (DON) and zearalenone (ZEN) after pre- or post-hepatic LPS administration to unravel the putative role of the liver. Fifteen barrows were fed a diet containing mycotoxin contaminated maize (4.59 mg DON/kg feed, 0.22 mg ZEN/kg feed) for 29 days and equipped with pre-hepatic catheters (portal vein, "po") and post-hepatic catheters (jugular vein, "ju"), facilitating simultaneous infusion of LPS ("LPS group", 7.5 µg/kg body weight) or 0.9% sterile NaCl solution (control, "CON group", equivolumar to LPS group) and blood sampling. This resulted in three infusion groups, depending on infusion site: CONju-CONpo, CONju-LPSpo, and LPSju-CONpo. On day 29, pigs were fed their morning ration (700 g/pig) at -15 min, and blood samples were collected at regular intervals relative to infusion start. At 195 min, pigs were sacrificed and bile, urine, liquor, and liver samples collected. DON concentrations in jugular and portal blood decreased in both LPS-infused groups, whereas the ZEN concentrations increased, regardless of the treatment site. In liver tissue, a decrease of both toxin concentrations was observed in endotoxaemic pigs as well as a drop in hepatic conjugation, regardless of LPS-entry site. In contrast to our hypothesis, DON and ZEN were not differently altered depending on the LPS-entry site. Neither the absorption nor the accumulation of DON and ZEN in different tissues differed significantly between animals which were infused with LPS via either the jugular or portal vein.

Keywords: Deoxynivalenol, Zearalenone, Lipopolysaccharides, Kinetics, Blood plasma, Liver

4.2. Introduction

The Fusarium mycotoxins deoxynivalenol (DON) and zearalenone (ZEN) are frequently co-occurring in cereals and have a high practical relevance in animal production (Dänicke et al. 2005; Döll and Dänicke 2011; Pestka 2007). Especially pigs respond very sensitive to DON and ZEN and economic losses mainly occur due to a decreased feed intake and fertility, caused by DON and ZEN, respectively (Wu and Munkvold 2008).

Besides a possible co-contamination of feed with DON and ZEN, pigs can be exposed to different systemic and enteric infections, such as for example post-weaning colibacillosis (Escherichia coli), Salmonellosis, and E rysipelas (Erysipelothrix rhusiopathiae) (Pluske et al. 2002; Zimmerman et al. 2012) that might be associated with an acute phase reaction (APR). A common trigger of an APR, are lipopolysaccharides (LPS), for example due to an infection with gramnegative bacteria (Wyns et al. 2015). Depending on the site of infection, LPS could enter the systemic circulation either directly (e.g., after a peripheral infection) or via the porto-hepatic route (e.g., due to an enteric infection). Thus, the magnitude of APR might depend on the proportion of extrahepatic and hepatic macrophages inducing an APR and LPS clearing. The LPS-induced release of pro-inflammatory cytokines results in inflammation, apoptosis, microcirculatory failure, and perfusion heterogeneity in various tissues (Angus and van der Poll 2013). In pigs, labored respiration, increased respiratory rate, hyperthermia, tremor, cyanosis, injected episcleral vessels, hyperaemic conjunctivae, dermographism, retching and vomiting, lactic acidosis, and leukopenia can be observed clinically upon the systemic infusion of LPS (Dänicke et al. 2014; Wyns et al. 2015). A more pronounced lactic acidosis and an altered body temperature profile in DON-fed pigs was observed when LPS was systemically infused compared to their pre-hepatically infused counterparts (Bannert et al. 2015; Tesch et al. 2016). These findings hint at a role of the liver in clearing a portal LPS-entry and the consequences on the magnitude of the APR in the first-pass and further on suggest effects on mycotoxin kinetics, including metabolism and excretion.

The kinetics of DON, ZEN, and their respective metabolites in pigs have been described in several studies (Biehl et al. 1993; Burdon and Zabel 2002; Dänicke et al. 2004; Dänicke and Winkler 2015; Shin et al. 2009; Zinedine et al. 2007), albeit mostly in non-endotoxaemic pigs. For example, an LPS-induced APR in DON-exposed pigs decreased the clearance of DON and concomitantly increased plasma DON concentration (Dänicke and Brezina 2013). These results were discussed as consequences of a hemodynamically associated longer persistence of DON glucuronides, or of

an altered hepatic glucuronidation activity. However, the putative role of the liver could not be evaluated in that study since LPS was only administered post-hepatically. In the view of our observation that a post-hepatically induced APR differs from a pre-hepatic provoked one (Bannert et al. 2015; Tesch et al. 2016), we hypothesized that kinetics of DON and ZEN might also be different. In order to clarify this hypothesis, we used specimens collected from the cited experiment (Bannert et al. 2015; Tesch et al. 2016) and analyzed them for residues of DON, ZEN, and their metabolites.

4.3. Material and Methods

Animal experiments were conducted according to the EC regulations concerning the protection of experimental animals and the guidelines of the German Animal Welfare Act approved by the Lower Saxony State Office for Consumer Protection and Food Safety (file number 33.4-42,502-04-13/1274).

4.3.1. Experimental design, treatments, and procedure

This manuscript is part of a larger project, investigating the interactions between a chronic oral mycotoxin exposure and a subsequent intravenous LPS stimulus, either administered pre- or post-hepatically. Experimental design, treatments, and procedures have been previously described in detail in Bannert et al. (2015) and Tesch et al. (2016). A simplified overview of the experimental setup is given in Figure 1. Briefly, a total of 15 barrows (German Landrace, Mariensee, Germany) were chronically exposed to a diet containing mycotoxin-contaminated maize with 4.59 mg DON and 0.22 mg ZEN per kg diet for 29 days. The diet consisted of 53% barley, 20% soybean meal, 15% maize (7.5% Fusarium graminearum inoculated and 7.5% non-inoculated), 5% rapeseed, 2% soybean oil, 3% mineral and vitamin premix, and 1% insoluble ash (Sipernat®22S, Evonik Industries AG, Germany). More details about the diets are published in Bannert et al. (2015). Sizes of feed portions were increased continuously according to the daily voluntary intake in order to reach the final amount of 1400 g per animal and day (2 × 700 g).

All animals (mean body weight 40.5 ± 3.0 kg, mean ± SD) were equipped with indwelling catheters in *Vena jugularis externa* and *V. lienalis* for infusion, and *V. jugularis interna*, *V. portae hepatis*, and *Arteria carotis communis* for blood sampling. The arterial catheter was used for blood gas analysis already published elsewhere (Bannert et al. 2015). On sampling day (day 29), animals were exposed to either a pre-hepatic (CONju-LPSpo, *V. lienalis*, n = 5) or post-hepatic (LPSju-

CONpo, *V. jugularis externa*, n = 5) LPS challenge (7.5 µg LPS/kg BW for 1 h) or received a 0.9% NaCl solution (CONju-CONpo, n = 5). Fifteen minutes prior to infusion start (0 min = infusion start), animals were fed 700 g of the DON- and ZEN-containing diet. Blood samples were collected at -30, 15, 30, 45, 60, 75, 90, 120, 150, and 180 min relative to the start of the infusion from *V. jugularis interna* and *V. portae hepatis*. At 195 min, animals were sacrificed and samples of liver (Lobus hepatis dexter medialis), urine, bile, and cerebrospinal liquor (lumbar puncture) were collected. All samples were stored at -20 °C until further processing. Liver tissue was freeze dried and ground to pass a 1 mm screen before analysis.

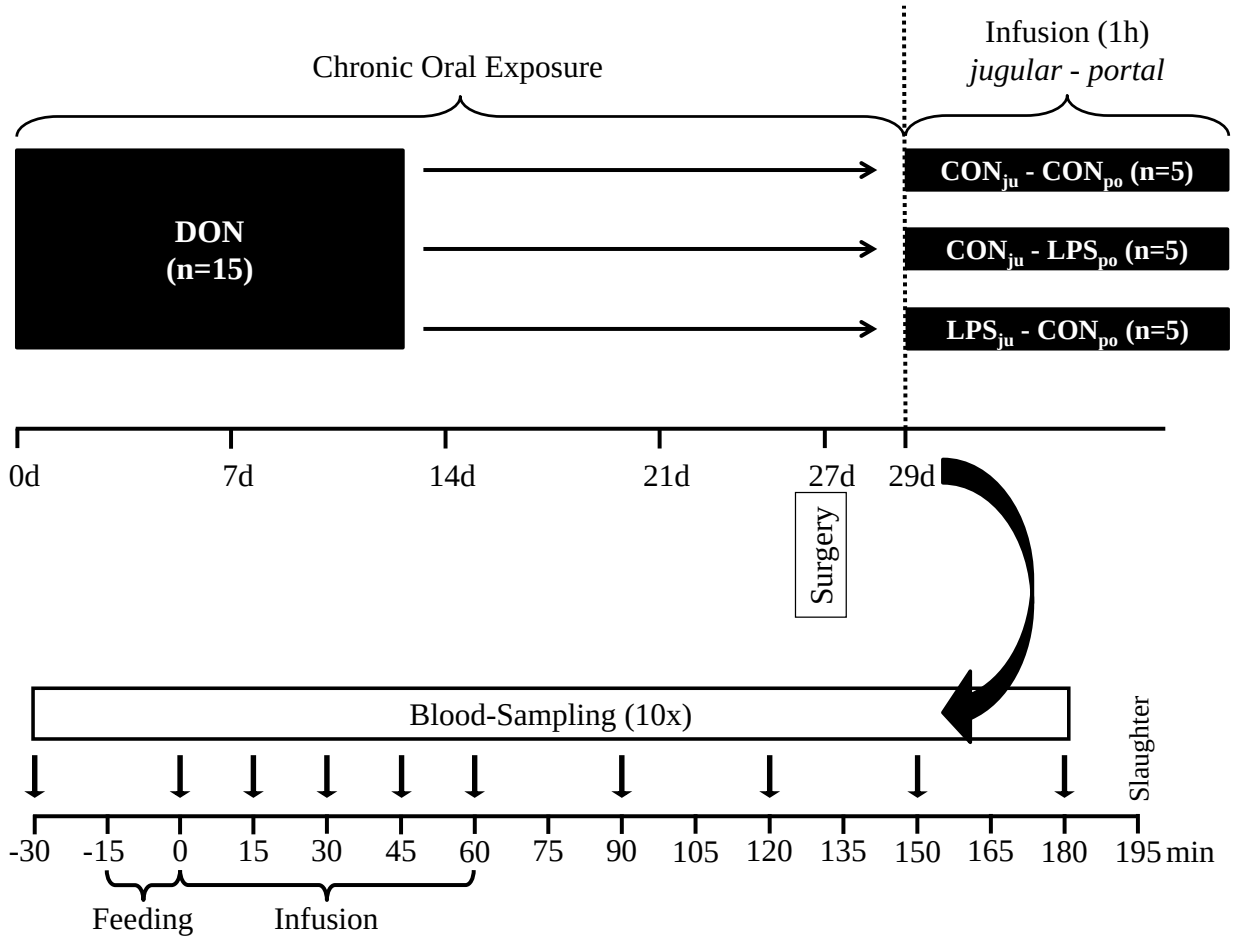

Fig. 1: Experimental Design

4.3.2. Sample preparation and mycotoxin analysis

Feed analysis

After cleanup step using immune affinity columns (IAC) (for DON: DONprep, R-Biopharm, Darmstadt, Germany; for ZEN: ZearalaTest, Vicam, Klaus Ruttmann, Hamburg, Germany), high-

performance liquid chromatography (Shimadzu, Duisburg, Germany) with diode array detection (HPLC-DAD, for DON) and fluorescence detection (HPLC-FLD, for ZEN) was performed for mycotoxin analysis in feed (Oldenburg et al. 2007). The sample preparation and analysis for ZEN was performed according to VDLUFA (2006) method with slight modifications: For preparation, a 10 g sample and for HPLC measurement acetonitrile/bidistilled water (65 + 35 v/v) as eluent and an Aqua® 5 µm C18 column, 250 × 4.6 mm (Phenomenex, Aschaffenburg, Germany), were used. The limit of detection (LOD, S/N = 3) was 15 and 1.2 µg/kg, the limit of quantification (LOQ, S/N = 10) was 50 and 4.0 µg/kg, and the mean recovery was approximately 93 % for DON and 92 % for ZEN.

Besides the analysis of DON and ZEN in compound feed, a quantitative analysis of various other mycotoxins in the contaminated maize batch was performed with a high-performance liquid chromatography-electrospray ionization tandem mass spectrometry (HPLC-ESI-MS/MS) based multi mycotoxin method using an Agilent 1290 HPLC coupled to an Applied Biosystems 5500 QTrap mass spectrometer by Biomin Holding GmbH and IFA-Tulln (Tulln, Austria). Analytical values are detailed in the supplemental material (S1).

Analysis of biological samples

The mycotoxins DON, ZEN, and their metabolites de-epoxy-deoxynivalenol (DOM-1), zearalenol (α-ZEL), β-zearalenol (β-ZEL), zearalanone (ZAN), α-zearalanol (α-ZAL), and β-zearalanol (β-ZAL) were analyzed with liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) in negative mode using a 4000 QTrap (Applied Biosystems, CA, USA) coupled with a 1200 series HPLC (Agilent Technologies, Waldbronn, Germany) as described earlier (Brezina et al. 2014b). The sample preparation methods are described in detail for plasma, liquor, bile (Brezina et al. 2014a, b, 2016), and urine (Winkler et al. 2015). Briefly, all samples were incubated at 37 °C over night with or without β-glucuronidase solution (Sigma G 0876, 100,000 U/mL/type H-2 from Helix pomatia by Sigma-Aldrich, Steinheim, Germany) at pH 5.5 using sodium acetate buffer (VWR, Darmstadt, Germany). After incubation, the plasma, liquor, and urine samples were cleaned up by solid phase extraction by Oasis™ HLB SPE (Waters, Milford, MA, USA) and bile samples using the IAC DZT-MS Prep (R-Biopharm AG, Darmstadt, Germany).

The liver sample preparation was performed by a modification (Lippelt et al. 2014) of the method for bile (Brezina et al. 2016) as follows: to 0.5 g of the freeze dried liver, a mix of the internal

standards, 3.5 mL bidistilled water, and 2.5 mL sodium acetate buffer (pH 5.5; VWR, Darmstadt, Germany) were added; then, the mixture was incubated as described a bove for p lasma, either with 50 µL β-glucuronidase or without. After incubation, 30 mL acetonitrile (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) were added, the mixture was mixed for 1 h and centrifuged, and the liquid phase was concentrated in a rotary evaporator. As a next step, 10 mL PBS (Merck KGaA, Darmstadt, Germany) was added and the pH was adjusted to 7.0-7.3 with 1 M NaOH (Merck KGaA, Darmstadt, Germany). Thereafter, the solutions were cleaned up by DZT-MS Prep columns as described for bile (Brezina et al. 2016). The LOD, LOQ, and mean recovery of plasma, urine, bile, liver, and liquor samples are listed in Table 1. While values lower than LOD were set to zero, values between LOD and LOQ were used for further statistical evaluation. Creatinine measurements in urine were performed according to an inhouse HPLC-method (Winkler et al. 2015).

4.3.3. Calculations and statistics

The samples were either incubated with (total mycotoxin, i.e., free plus conjugated) or without β-glucuronidase (free mycotoxin). The degree of conjugation was calculated as follows:

$$\text{conjugated mycotoxin [\%]} = \frac{\text{mycotoxin } [total] - \text{ mycotoxin } [free]}{\text{mycotoxin } [total]} \times 100$$

The degree of metabolite formation (including their conjugated forms) was expressed by dividing one single metabolite or the sum of the metabolites by the sum of mycotoxin metabolites (DOM-1 or α-ZEL, β-ZEL, ZAN, α-ZAL, and β-ZAL) and nonmetabolized mycotoxin (DON or ZEN).

$$\text{metabolized mycotoxin [\%]} = \frac{[\text{metabolite or sum of metabolites}]}{([\text{DON or ZEN}] + [\text{sum of metabolites}])} \times 100$$

The carry over factor (edible animal products: liver) or transfer factor (non-edible animal products: urine, bile and liquor) was calculated as follows:

$$\text{carry over or transfer factor} = \frac{[\text{DON or ZEN}] + [\text{sum of metabolites}] \text{ tissue}}{[\text{DON or ZEN}] + [\text{sum of metabolites}] \text{ diet}}$$

Plasma data were evaluated using PROC MIXED in SAS 9.4 (SAS Institute Inc., Cary, NC, USA) with group, (sampling) location and time as fixed factors using a restricted maximum likelihood model (REML). A REPEATED statement was included, and according to the corrected Akaike's information criterion (AICC) the compound symmetry was found to be the most appropriate covariance structure. Significant effects at different times were further evaluated by multiple t-tests (pairwise differences (PDIFF)), and results are presented as least square means (LSMeans) and pooled standard error of means (PSEM). Plasma curves of toxin concentrations were used to calculate an area under the curve (AUC) with the trapezoid method and statistically analyzed via ANOVA by using group and (sampling) location as main factors.

Normally distributed data of bile, urine, liquor, and liver were analyzed via one-way ANOVA and Spearman correlation in Statistica 13 (StatSoft Inc., Tulsa, USA). Values are reported as mean and standard deviation (SD) in the case of normally distributed data and medians and ranges (minimum - maximum) for non-Gaussian distributed data. The toxin concentrations of plasma at 180 min, bile, urine, liquor, liver, and the relative liver weight were correlated using Spearman correlations.

4.4. Results

4.4.1. Mycotoxin exposure

DON exposure amounted to 79.3 ± 5.6 µg/kg BW and ZEN exposure to 3.80 ± 0.27 µg/kg BW (mean ± SD) in a single 700 g feed portion during the final 11 days of trial. In total, the pigs received 133.1 ± 3.0 mg DON and 6.5 ± 0.1 mg ZEN (mean ± SD) over 29 days of trial.

4.4.2. Clinical signs and pathological findings

A detailed description and discussion of clinical signs and pathological findings are provided in Bannert et al. (2015) and Tesch et al. (2016). Briefly, the observed clinical signs in response to LPS included labored respiration, increased respiratory rate, hyperthermia, tremor, cyanosis, injected episcleral vessels, hyperaemic conjunctivae, dermographism, retching and vomiting, lactic acidosis and leukopenia. Since only 4 of the 15 animals vomited, and only small amounts of feed (2.67 ± 0.92% of morning feed portion; mean ± SD) were regurgitated, it was concluded that most likely, this would not have influenced the toxicokinetics of DON and ZEN.

Table 1: Mean recoveries (n = 4-9, limits of detection (LOD, S/N = 3) and quantification (LOQ, S/N = 10) of ZEN, DON and their metabolites from the analysis of plasma, urine, bile, liver (freeze-dried) and liquor samples of pigs.

toxin		**ZEN**	**α-ZEL**	**β-ZEL**	**ZAN**	**α-ZAL**	**β-ZAL**	**DON**	**de-DON**
	recovery [%]	107	99	105	105	83	111	105	90
plasma	**LOD [ng/ml]**	0.03	0.4	0.5	0.3	0.1	0.2	0.7	0.5
	LOQ [ng/ml]	0.10	1.3	1.7	0.9	0.5	0.7	2.3	1.7
	recovery [%]	89	93	90	88	132	79	93	82
urine	**LOD [ng/ml]**	0.20	2.0	5.0	2.0	1.6	5.0	15.0	10.0
	LOQ [ng/ml]	0.50	5.0	12.0	7.0	5.7	15.0	50.0	33.0
	recovery [%]	94	91	94	90	107	100	90	92
bile	**LOD [ng/ml]**	0.03	0.2	0.4	0.5	0.3	0.2	1.5	1.0
	LOQ [ng/ml]	0.08	0.4	1.3	1.6	1.0	0.5	5.1	3.3
	recovery [%]	94	106	109	78	75	85	100	84
liver	**LOD [ng/ml]**	0.05	0.1	0.3	0.2	0.3	0.2	2.5	2.5
	LOQ [ng/ml]	0.15	0.3	0.6	0.6	1.0	0.5	8.3	8.3
	recovery [%]	104	96	98	102	84	106	94	71
liquor	**LOD [ng/ml]**	0.02	0.3	0.2	0.2	0.2	0.3	1.5	1.0
	LOQ [ng/ml]	0.07	0.9	0.7	0.7	0.5	0.7	4.5	3.0

Note: concentration ranges (ng/ml): plasma & liquor: ZEN: 0.67-6.67, metabolites of ZEN: 2-20, DON & de-DON: 8-80; urine: ZEN: 6.65-266.5, metabolites of ZEN: 20-800, DON & de-DON: 80-3200; bile: ZEN: 5.34-133.5, metabolites of ZEN: 16-400, DON & de-DON: 64-1600; liver: ZEN: 1.3-26.7, metabolites of ZEN: 4-80, DON & de-DON: 16-320).

4.4.3. DON and DOM-1 in plasma

Fifteen minutes prior to feeding (at time -30 min), all groups exhibited similar plasma DON concentrations 4.0 ± 1.0 ng/mL (mean ± SD, Fig. 2). Fifteen minutes after feeding, an increase in plasma DON concentrations was observed in all groups ($p < 0.01$). In the control group, plasma DON concentrations increased at the portal sampling location from time -30 min until 30 min ($p < 0.01$) and exhibited a plateau of 25.0 ± 1.1 ng/mL (mean ± SD) from 45 to 180 min. At the jugular sampling location, a similar pattern was observed ($p < 0.05$ between -30 and 30 min); however, a plateau was only reached at 75 min (75 to 180 min: 23.8 ± 0.7 ng/mL). Also, in the LPS-infused animals, a lower increase in DON concentrations was observed at the jugular compared to the

portal sampling location in the first minutes after feeding ($p < 0.01$ at 15 min). Furthermore, both LPS-infused groups exhibited a decrease in plasma DON concentrations during the infusion (between time 30 and 60 min, only significant at portal sampling location, $p < 0.05$). Thereafter, until 180 min, a continuous lower concentration compared to the control group was observed (statistically significant at different points in time). This was also reflected by 26% lower AUC of LPS-infused animals in comparison to control (significant group effect, Table 2), irrespective of infusion site. The degree of conjugation amounted to 32% on average and was not influenced by treatments ($p_{Group} = 0.57$, $p_{Location} = 0.48$, $p_{Group*Location*Time} = 0.33$).

Table 2: Area under the curve (AUC) of plasma mycotoxin concentrations. AUC of DON, de-DON, ZEN and α-ZEL concentrations of jugular and portal blood plasma (Fig. 2 & 3) of pigs chronically exposed to a naturally DON and ZEN contaminated feed (4.59 mg DON/kg feed and 0.22 mg ZEN/kg feed for 29 d). The pigs were either infused with 7.5 µg LPS/kg BW in the *V. lienalis* (LPS_{portal}), the *V. jugularis externa* ($LPS_{jugular}$) or as control with 0.9% saline infusion (n = 5 each group). Blood samples were collected at -30, 15, 30, 45, 60, 75, 90, 120, 150 and 180 min relative to infusion-start. Data is presented as means (SD).

	toxin	**control**	**LPS_{portal}**	**$LPS_{jugular}$**
AUC - jugular plasma	**DON**	4180 (697)	2893 (653)	3037 (959)
	de-DON	479(67)	426 (32)	342 (115)
	ZEN	19 (13)	59 (13)	54 (15)
	α-ZEL	61 (42)	62 (51)	76 (52)
AUC - portal plasma	**DON**	4565 (675)	3424 (1353)	3647 (1156)
	de-DON	566(258)	459 (86)	451 (204)
	ZEN	55 (26)	124 (67)	147 (54)
	α-ZEL	87 (38)	153 (77)	134 (58)
		group	**location**	**group*location**
ANOVA p-values	**DON**	**0.019**	0.157	0.965
	de-DON	0.184	0.177	0.844
	ZEN	**0.002**	**< 0.001**	0.267
	α-ZEL	0.312	**0.007**	0.422

No significant alterations in plasma DOM-1 concentrations were observed during the entire observation period in all groups (Figure 2). However, on average, a significant sampling location effect due to higher DOM-1 concentrations in the portal compared to jugular sampling location (2.3 versus 2.0 ng/mL) was observed. This could not be confirmed by the AUC due to time-dependent inconstant DOM-1 fluctuations (Table 2).

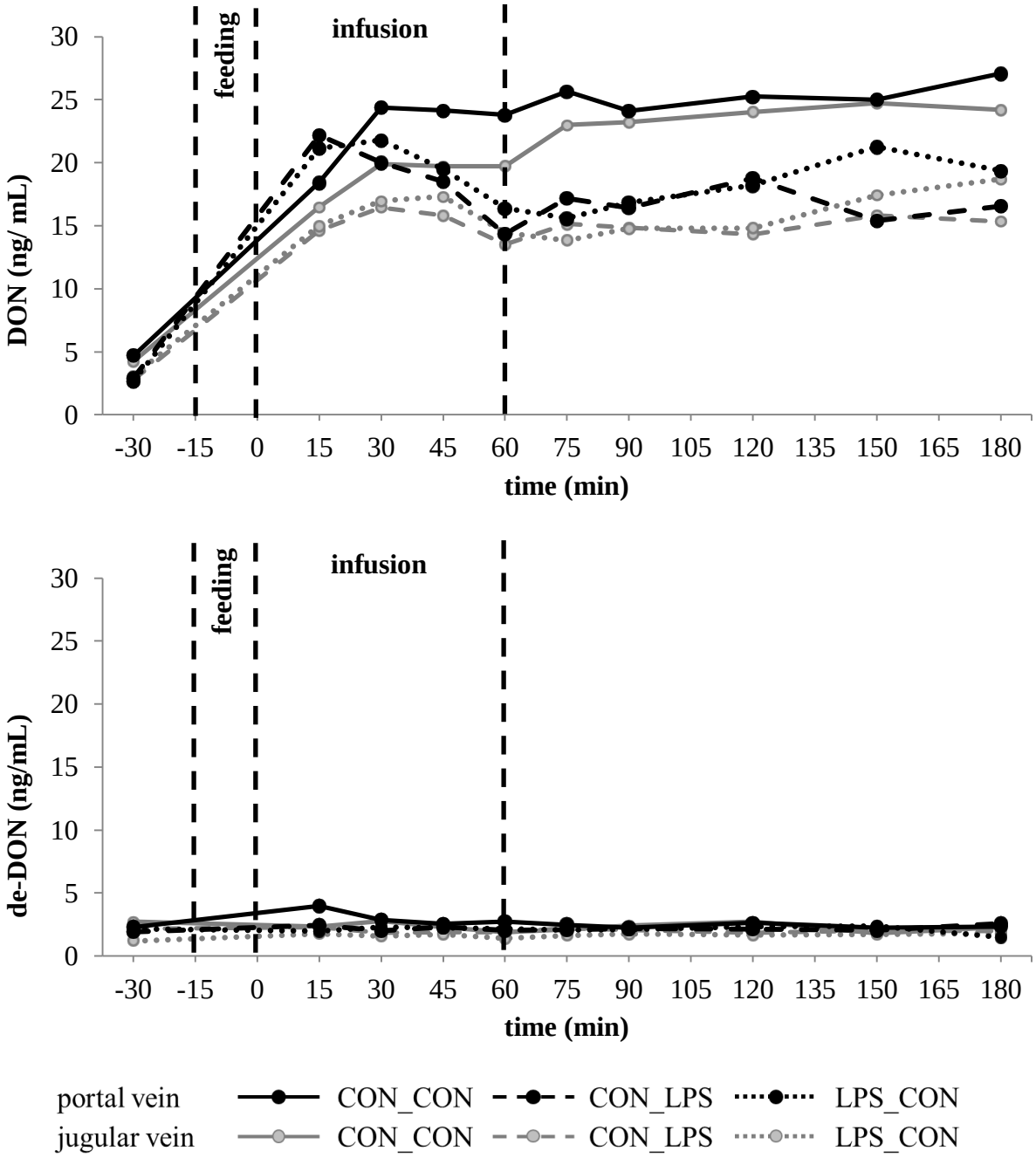

Figure 2: Concentrations of DON & de-DON in jugular and portal plasma of pigs chronically exposed to a naturally DON and ZEN contaminated feed (4.59 mg DON/kg feed and 0.22 mg ZEN/kg feed for 29 d). The pigs were either infused with 7.5 µg LPS/kg BW in the *V. lienalis* (CON_{ju}_LPS_{po}), the *V. jugularis externa* (LPS_{ju}_CON_{po}) or as control with 0.9% saline infusion (CON_{ju}_CON_{po}, n = 5) from time 0 until 60 min. Feed (700 g) was offered during 15 min prior to infusion start. **DON:** LSMeans. PSEM = 2.57. Main effects (F-test): Group (G): $p = 0.1$; Location (L): $p \leq 0.001$; Time (T): $p \leq 0.001$; G*L*T: $p \leq 0.026$. **de-DON:** LSMeans. PSEM = 0.42. Main effects (F-test): Group (G): $p = 0.33$; Location (L): $p \leq 0.001$; Time (T): $p \leq 0.398$; G*L*T: $p \leq 0.602$.

For the degree of conjugation of DOM-1, a significant group*location*time interaction was observed due to significant differences between groups at several points in time, however, without an obvious pattern within groups. The jugular sampling location exhibited a significantly higher degree of conjugation with 80.6 %, compared to 72.3 % at the portal sampling location (p_{Group} = 0.4, $p_{Location}$ < 0.001, p_{Time} = 0.93, $p_{Group*Location*Time}$ = 0.04). For the degree of metabolization of DON in plasma only, a time effect was observed, due to a decrease from 40.0 to 13.8 % between time -30 and 15 min (p_{Time} < 0.01) and continuous low levels thereafter (average of 10.5 ± 0.6 %, mean ± S D) in all groups and sampling locations (p_{Group} = 0.58, $p_{Location}$ = 0.29, $p_{Group*Location*Time}$ = 0.94).

4.4.4. ZEN and its metabolites in plasma

Blood plasma was analyzed for ZEN, α-ZEL, β-ZEL, ZAN, α-ZAL, and β-ZAL. Only ZEN and α-ZEL were detectable (Table 1 and Fig. 3).

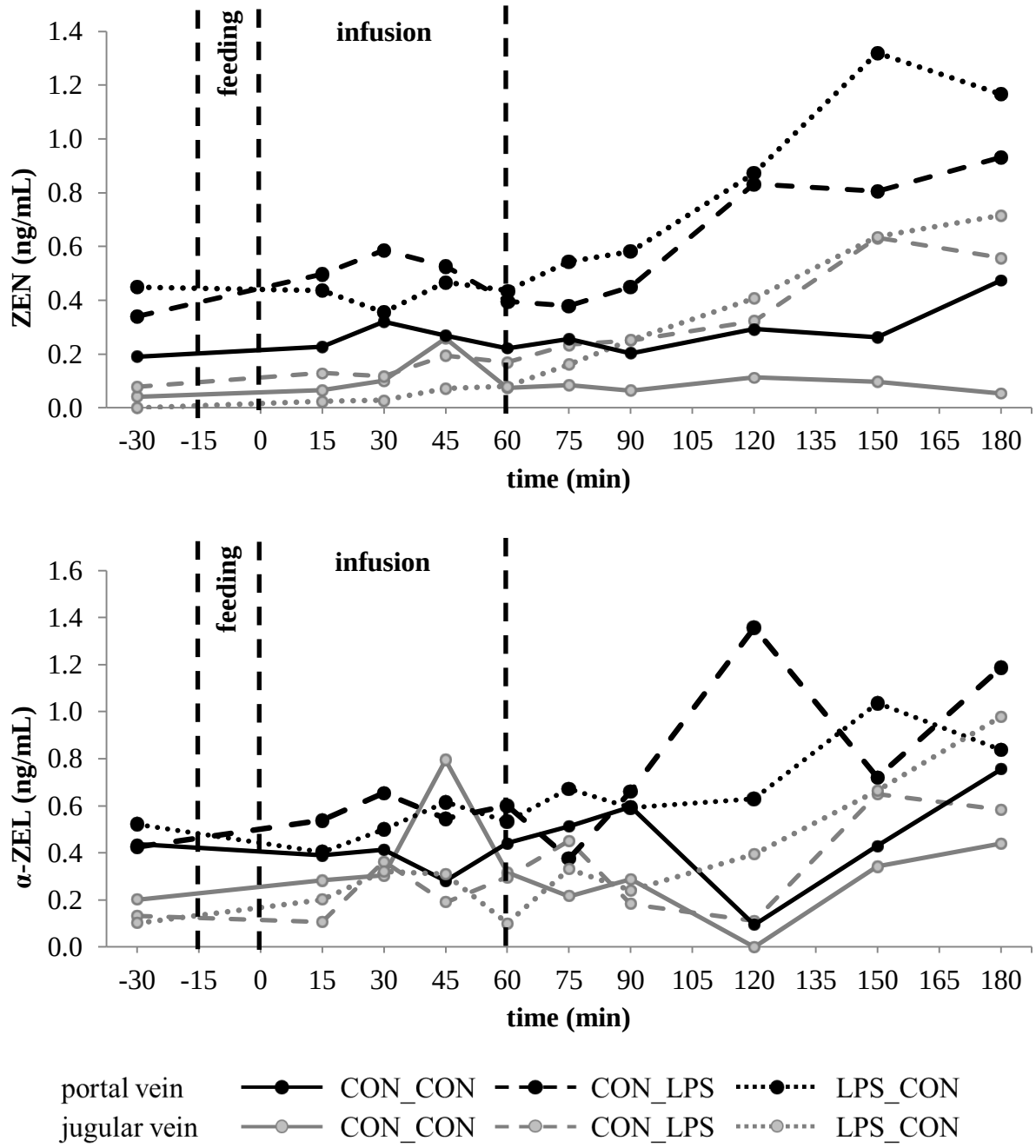

Figure 3: Concentrations of ZEN & α-ZEL in jugular and portal plasma of pigs chronically exposed to a naturally DON and ZEN contaminated feed (4.59 mg DON/kg feed and 0.22 mg ZEN/kg feed for 29 d). The pigs were either infused with 7.5 µg LPS/kg BW in the *V. linealis* (CON_{ju}_LPS_{po}), the *V. jugularis externa* (LPS_{ju}_CON_{po}) or as control with 0.9% saline infusion (CON_{ju}_CON_{po}, n = 5) from time 0 until 60 min. Feed (700 g) was offered during 15 min prior to infusion start. **ZEN:** LSMeans. PSEM = 0.11. Main effects (F-test): Group (G): $p = 0.022$; Location (L): $p \leq 0.001$; Time (T): $p \leq 0.001$; G*L*T: $p \leq 0.001$. **α-ZEL:** LSMeans. PSEM = 0.18. Main effects (F-test): Group (G): $p = 0.594$; Location (L): $p \leq 0.001$; Time (T): $p \leq 0.001$; G*L*T: $p \leq 0.019$.

In the control group, no significant alterations in plasma ZEN concentrations were observed throughout the observation period (Figure 3). In the LPS-infused animals, plasma ZEN

concentrations remained unaltered until 90 min and exhibited an increase thereafter until 180 min (on average from 0.45 to 0.93 ng/mL at the portal and from 0.25 to 0.56 ng/mL at the jugular sampling location, $p < 0.01$), with no significant difference between LPS-infusion sites. This was also reflected in a 37.3 % higher AUC in LPS-infused compared to control-infused animals (Table 2). Generally, higher ZEN concentrations were observed at the portal compared to the jugular sampling location (on average 0.20 ng/mL jugular vs 0.50 ng/mL portal and prior to feeding (at time -30 min) 0.04 ng/mL jugular vs 0.33 ng/mL portal, $p < 0.05$, Figure 3), which was also reflected in a 39.6% higher AUC (Table 2). Similar to ZEN, α-ZEL concentrations remained unaltered in the control groups over the course of the observational period and increased in the LPS-infused animals towards the end of the trial, with significant differences between time -30 and 150/180 min within both groups (portal location: $p < 0.05$, jugular location: CONju-LPSpo $p_{150min} = 0.19$, $p_{180min} < 0.001$; LPSju-CONpo $p_{150min} < 0.01$, $p_{180min} = 0.16$). This alteration, however, was not visible in the general AUC due to time dependent variations of α-ZEL concentrations. Generally, plasma α-ZEL concentrations were lower at the jugular (0.33 ng/mL, Fig. 3) compared to the portal sampling location (0.59 ng/mL), which was also reflected in a 55.8 % lower AUC (significant sampling location effect, Table 2). Overall, higher variations were observed in α-ZEL compared to ZEN concentrations (e.g., 45 min, 120 min). The conjugation degree of ZEN and its metabolites amounted to 100 % in all plasma samples. The degree of ZEN metabolization in plasma only showed a time effect with an average of 47.6 % ($p < 0.01$; $p_{Group} = 0.68$; $p_{Location} = 0.93$; $p_{Time} < 0.002$; $p_{Group*Location*Time} = 0.12$). It was not appreciably altered from -30 to 180 min. Only at 120 min in jugular plasma, the degree of metabolization was significantly decreased ($p < 0.01$).

4.4.5. DON and DOM-1 in urine, bile, liver, and liquor

While DON was detected in all matrices, DOM-1 was detected in urine, bile and liver, but not liquor (Table 3). The highest DON concentrations were found in urine followed by bile, liver and liquor. Overall, DON concentrations appeared to be lower in response to the LPS treatment in all matrices. This effect was only statistically significant in liver tissue in portal LPS-infused animals. Like in plasma, also in all other matrices, DOM-1 concentrations remained unaffected by LPS treatment, alongside with a numerical decrease in overall DON concentration. This resulted in an increase in the degree of metabolization in the LPS-infused animals (significant in bile: CONju-LPSpo and liver: LPSju-CONpo and CONju-LPSpo). The total amounts of DON in the complete liver accounted for 31.9 ± 5.2 µg (mean ± SD) in CONju-CONpo, 27.1 ± 5.6 µg in CONju-LPSpo, and 31.0 ± 8.0 µg in LPSju-CONpo group and were not influenced by treatments (p = 0.475).

Furthermore, a decrease of the degree of conjugation of DON in the liver was observed in the LPS-infused animals. Similarly, the carry over factor of DON in liver tissue decreased in the LPS-infused animals (only significant for CONju-LPSpo). In liquor, no DOM-1 was detected. In order to examine the relationships between DON and DOM-1 concentrations in jugular plasma and liquor (at 180 min), the corresponding transfer of toxin from plasma to liquor was calculated: at on average 0.30 for CONju-CONpo, 0.37 in CONju-LPSpo, and 0.49 in LPSju-CONpo with no significant effects ($p = 0.419$).

Table 3: DON residues in urine, bile, liver and liquor. Comparison of DON & de-DON concentrations in different matrices of pigs chronically exposed to a naturally DON and ZEN contaminated feed (4.59 mg DON/kg feed and 0.22 mg ZEN/kg feed for 29 d). The pigs were either infused with 7.5 µg LPS/kg BW in the *V. lienalis* (LPS_{portal}), the *V. jugularis externa* ($LPS_{jugular}$) or as control with 0.9% saline infusion (n = 5 each group) during 60 min, beginning 195 min prior to slaughtering. All samples were collected at slaughtering. Measurements in liver tissue were conducted in freeze dried samples and concentrations extrapolated to fresh matter.

	control	**LPS_{portal}**	**$LPS_{jugular}$**	**p-values**
urine	*n=4*	*n=5*	*n=5*	
DON total (ng/mL)	3063.7 (984.5)	1770.0 (1509.6)	1840.0 (549.3)	0.197
DON conjugation (%)	43.2 (11.9)	43.9 (13.5)	55.6 (8.9)	0.223
de-DON total (ng/mL)	145.0 (83.0)	161.1 (132.1)	191.4 (69.0)	0.779
de-DON conjugation (%)	87.7 (15.1)	84.6 (13.3)	93.1 (4.0)	0.513
metabolization (%)	4.5 (1.9)	8.8 (2.8)	10.4 (5.7)	0.122
transfer factor	0.699 (0.225)	0.421 (0.357)	0.443 (0.106)	0.245
bile	*n=5*	*n=4*	*n=4*	
DON total (ng/mL)	234.3 (95.8)	212.5 (153.1)	167.7 (78.6)	0.682
DON conjugation (%)	85.2 (6.5)	84.1 (5.1)	94.4 (6.9)	0.078
de-DON total (ng/mL)	48.9 (41.5)	111.2 (90.3)	54.6 (36.2)	0.288
de-DON conjugation (%)	96.7 (2.6)	96.1 (1.3)	98.1 (2.6)	0.496
metabolization (%)	14.6 (7.3) [a]	33.3 (4.6) [b]	22.0 (6.7) [a]	**0.005**
transfer factor	0.062 (0.030)	0.070 (0.053)	0.048 (0.025)	0.707
liver	*n=5*	*n=5*	*n=5*	
DON total (ng/g)	38.3 (3.9) [a]	26.2 (6.7) [b]	28.4 (7.7) [ab]	**0.025**
DON conjugation (%)	58.4 (14.4) [a]	22.6 (16.4) [b]	35.4 (12.2) [b]	**0.007**
de-DON total (ng/g)	1.97 (0.23)	2.27 (0.73)	2.82 (0.96)	0.203
de-DON conjugation (%)*	100 (100-100)	100 (100-100)	100 (26.6-100)	n.c.
metabolization (%)	4.9 (0.7) [a]	8.0 (1.7) [b]	9.1 (1.8) [b]	**0.003**
carry over factor	0.009 (0.001) [a]	0.006 (0.002) [b]	0.007 (0.002) [a]	**0.047**
liquor	*n=5*	*n=5*	*n=5*	
DON total (ng/mL)	8.0 (2.0)	5.9 (1.9)	8.8 (3.0)	0.188
DON conjugation (%)*	100 (100-100)	100 (100-100)	100 (100-100)	n.c.
de-DON total (ng/mL)*	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	n.c.
transfer factor	0.0017 (0.0004)	0.0013 (0.0004)	0.0019 (0.0007)	0.188

For normally distributed data a one-way ANOVA was performed. Data is presented as means (SD) or median (min-max) in case of non-normal distribution (*). Different letters indicate a significant difference ($p<0.05$) between groups.

For DON concentrations in urine, a positive correlation was observed with DON concentrations in bile and portal plasma (Table 4). Furthermore, also DON concentrations in the liver and portal and jugular plasma correlated positively. However, a closer correlation was observed between DON concentrations in liver and portal plasma compared to jugular plasma (0.796 versus 0.631). No correlation between DON liquor concentrations and DON concentrations in other matrices was observed. The relative liver weight (on average 20.1 g/kg in CONju-CONpo, 25.9 g/kg in CONju-LPSpo, and 26.9 g/kg in LPSju-CONpo (Renner et al. 2017) correlated negatively with DON liver, and jugular and portal plasma (Table 4).

Table 4: Spearman correlation matrix of DON concentrations in urine, bile, liver, liquor, and jugular and portal plasma. Significant coefficients ($p < 0.05$) are highlighted in bold type. The pigs were chronically exposed to a naturally DON and ZEN contaminated feed (4.59 mg DON/kg feed and 0.22 mg ZEN/kg feed for 29 d) and either infused with 7.5 µg LPS/kg BW in the *V. lienalis*, the *V. jugularis externa* or as control with 0.9% saline infusion (n = 15) during 60 min, 180 min prior to slaughtering. All samples were collected at slaughtering, except for plasma samples (15 min prior).

	DON urine	DON bile	DON liver	DON liquor	DON plasma jugular	DON plasma portal	relative liverweight
DON urine	1	**0.636**	**0.568**	0.051	0.525	**0.596**	-0.059
DON bile		1	0.352	-0.016	0.335	0.379	0.170
DON liver			1	0.200	**0.631**	**0.796**	**-0.608**
DON liquor				1	0.218	0.354	-0.361
DON plasma jugular					1	**0.875**	**-0.621**
DON plasma portal						1	**-0.711**
relative liverweight							1

4.4.6. ZEN and metabolites in urine, bile, liver, and liquor

The highest ZEN concentrations were measured in bile, followed by urine and liver (Table 5). In bile and urine, LPS treatment caused a numerical increase in ZEN, α-ZEL, β-ZEL, and ZAN concentrations as well as of the transfer factor. Only in urine, the ZAN concentrations were significantly higher in jugular LPS-infused group. The total amount of ZEN in the complete liver was 2.4 ± 0.7 µg (mean ± SD) in CONju-CONpo, 1.4 ± 0.5 µg in CONju-LPSpo, and 1.3 ± 0.1 µg in LPSju-CONpo group, with a significant effect ($p < 0.01$). In the liver, in contrast to bile and urine, the concentrations of ZEN, α-ZEL, β-ZEL, the sum of metabolites, and the carry over factor significantly decreased in both LPS-infused groups by 32-44 % with no difference between LPS infusion site. No α-ZAL or β-ZAL was found in any matrices. The degree of conjugation of ZEN (significant in CONju-LPSpo) and α-ZEL (numerical) in the liver decreased in LPS-infused animals, whereas the degree of conjugation of ZEN and α-ZEL in bile was only slightly altered under the influence of an LPS infusion. The degree of conjugation of ZEN, α-ZEL, β-ZEL, and ZAN in urine of β-ZEL and ZAN in bile was 100 % and not affected due to LPS.

Table 5: ZEN residues in urine, bile, liver and liquor. Comparison of ZEN & metabolites concentrations in different matrices of pigs chronically exposed to a naturally DON and ZEN contaminated feed (4.59 mg DON/kg feed and 0.22 mg ZEN/kg feed for 29 d). The pigs were either infused with 7.5 µg LPS/kg BW in the *V. lienalis* (LPS_{portal}), the *V. jugularis externa* ($LPS_{jugular}$) or as control with 0.9% saline infusion (n = 5 each group) during 60 min, beginning 195 min prior to slaughtering. All samples were collected at slaughtering. Measurements in liver tissue were conducted in freeze dried samples and concentrations extrapolated to fresh matter.

	control	**LPS_{portal}**	**$LPS_{jugular}$**	**p-values**
bile	*n=4*	*n=5*	*n=5*	
ZEN total (ng/mL)	239.0 (86.4)	477.3 (275.3)	395.6 (280.8)	0.303
ZEN conjugation (%)	99.6 (0.3) [a]	99.1 (0.1) [b]	99.7 (0.4) [a]	**0.032**
α-ZEL total (ng/mL)	261.9 (153.1)	458.9 (124.2)	476.7 (352.1)	0.320
α-ZEL conjugation (%)	99.9 (0.2)	99.6 (0.1)	99.9 (0.2)	0.057
β-ZEL total (ng/mL)	32.8 (11.7)	64.9 (37.8)	56.4 (42.7)	0.333
β-ZEL conjugation (%)*	100 (100-100)	100 (99.5-100)	100 (99.5-100)	n.c.
ZAN total (ng/mL)	5.5 (1.9)	11.3 (7.7)	8.5 (7.1)	0.380
ZAN conjugation (%)*	100 (100-100)	100 (100-100)	100 (100-100)	n.c.
sum metabolites (ng/mL)	303.4 (167.8)	535.0 (163.0)	541.7 (401.8)	0.326
metabolization (%)	54.1 (8.2)	54.8 (9.2)	57.2 (1.9)	0.810
transfer factor	2.5 (1.1)	4.6 (1.9)	4.3 (3.1)	0.305
urine	*n=5*	*n=4*	*n=4*	
ZEN total (ng/mL)	66.3 (23.2)	210.2 (185.5)	199.5 (89.6)	0.216
ZEN conjugation (%)*	100 (100-100)	100 (100-100)	100 (100-100)	n.c.
α-ZEL total (ng/mL)	57.6 (28.5)	131.0 (106.7)	149.7 (67.4)	0.235
α-ZEL conjugation (%)*	100 (100-100)	100 (100-100)	100 (100-100)	n.c.
β-ZEL total (ng/mL)	8.1 (3.9)	20.4(17.13)	21.3 (10.1)	0.251
β-ZEL conjugation (%)*	100 (100-100)	100 (100-100)	100 (100-100)	n.c.
ZAN total (ng/mL)	0.6 (1.2) [a]	8.0 (6.2) [ab]	11.1 (5.8) [b]	**0.032**
ZAN conjugation (%)*	100 (100-100)	100 (100-100)	100 (100-100)	n.c.
sum metabolites (ng/mL)	66.3 (32.9)	159.4 (131.6)	182.4 (82.6)	0.212
metabolization (%)	48.8 (5.7)	44.3 (3.7)	47.7 (5.8)	0.418
transfer factor	0.6 (0.2)	1.7 (1.4)	1.7 (0.8)	0.212
liver	*n=5*	*n=5*	*n=5*	
ZEN total (ng/g)	2.9 (1.1) [a]	1.3 (0.5) [b]	1.2 (0.1) [b]	**0.003**
ZEN conjugation (%)	96.6 (2.4) [a]	92.4 (5.5) [ab]	81.9 (12.8) [b]	**0.039**
α-ZEL total (ng/g)	4.8 (0.5) [a]	1.7 (0.7) [b]	2.3 (0.7) [b]	**≤ 0.001**
α-ZEL conjugation (%)	98.8 (1.7)	96.6 (7.7)	82.9 (20.1)	0.136
β-ZEL total (ng/g)	0.83 (0.25) [a]	0.23 (0.17) [b]	0.28 (0.22) [b]	**0.001**
β-ZEL conjugation (%)*	100 (100-100)	100 (n.c.-100)	100 (n.c.-100)	n.c.
ZAN total (ng/g)*	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	n.c.
sum metabolites (ng/g)	5.6 (0.7) [a]	2.0 (0.8) [b]	2.6 (0.9) [b]	**≤ 0.001**
metabolization (%)	66.4 (5.4)	59.7 (5.6)	67.2 (5.9)	0.109
carry over factor	0.039 (0.008) [a]	0.015 (0.005) [b]	0.017 (0.004) [b]	**≤ 0.001**
liquor	*n=5*	*n=5*	*n=5*	
ZEN total (ng/mL)*	0.00 (0.00-0.03)	0.00 (0.00-0.00)	0.00 (0.00-0.54)	n.c.
sum metabolites (ng/mL)*	0.0 (0.0-0.0)	0.0 (0.0-0.0)	0.0 (0.0-0.0)	n.c.

For normally distributed data a one-way ANOVA was performed. Data is presented as means (SD) or median (min-max) in case of non-normal distribution (*). Different letters indicate a significant difference ($p<0.05$) between groups.

In liquor, ZEN concentrations higher than LOD were found only in three pigs, while metabolites were non-detectable. A positive correlation was observed for urine ZEN concentrations and bile as well as for jugular and portal plasma ZEN concentrations. A closer correlation was detected for the portal sampling location than for the jugular site (0.789 versus 0.662, Table 6). Jugular and portal plasma ZEN concentrations also correlated positively. Furthermore, a negative correlation between liver and jugular plasma ZEN concentrations, but not portal plasma, was observed. ZEN concentrations in all matrices correlated positively with the relative liver weight, except for liver ZEN concentrations where a negative correlation was observed (Table 6).

Table 6: Spearman correlation matrix of ZEN concentrations in urine, bile, liver, and jugular and portal plasma. Significant coefficients ($p < 0.05$) are highlighted in bold type. The pigs were chronically exposed to a naturally DON and ZEN contaminated feed (4.59 mg DON/kg feed and 0.22 mg ZEN/kg feed for 29 d) and either infused with 7.5 µg LPS/kg BW in the *V. lienalis*, the *V. jugularis externa* or as control with 0.9% saline infusion (n = 5) during 60 min, 180 min prior to slaughtering. All samples were collected at slaughtering, except for plasma samples (15 min prior).

	ZEN urine	ZEN bile	ZEN liver	ZEN plasma jugular	ZEN plasma portal	relative liverweight
ZEN urine	1	**0.629**	-0.341	**0.662**	**0.789**	0.468
ZEN bile		1	-0.489	0.337	0.401	**0.593**
ZEN liver			1	**-0.566**	-0.414	**-0.611**
ZEN plasma jugular				1	**0.885**	0.470
ZEN plasma portal					1	0.429
relative liverweight						1

4.2. Discussion

In order to investigate the kinetics of DON and ZEN and the specific role of the liver in endotoxaemic pigs, we used a model for inducing an APR by infusion of LPS either pre- or post-hepatically. It had been shown that the route of LPS administration modified the outcome of APR as indicated by varying body temperature kinetics and differences in the degree of lactic acidosis (Bannert et al. 2015; Tesch et al. 2016). These results suggested that the liver plays a role in mediating LPS effects with possible consequences for the kinetics of DON and ZEN.

After feeding, a rapid increase in DON plasma concentrations was observed, which is in line with previous studies (Prelusky et al. 1988; Avantaggiato et al. 2004; Dänicke et al. 2010, 2004; Goyarts and Dänicke 2006). In the control group, a plateau was observed between time 30 and 180 min, similar to earlier data (Goyarts and Dänicke 2006), suggesting a similar absorption and plasma clearance rate during this time interval. The higher concentrations of DON in portal compared to jugular blood can most likely be attributed to the ongoing absorption of DON from the intestines and the distribution in the system after passing through the liver ("first-pass effect") and the continuous urinary elimination.

The plasma concentrations of DON in LPS-infused pigs were lower than in control-infused animals from 30 to 180 min, irrespective of infusion site. Thus, the LPS-entry route had no effect on the toxicokinetics of DON. One reason for the lower plasma concentration in LPS-infused animals could be a reduced gastrointestinal (GI) motility due to an APR. In endotoxaemic canines, the gastric, duodenal, and jejunal activity was altered and the migrating motor complex (MMC) was stopped for 48 h after single LPS injection (Cullen et al. 1995, 1996). Based on its polarity, DON is supposed to be transported with the liquid phase rather than with solid ingesta. In endotoxaemic rats, a decrease of GI emptying in the liquid phase was reported (Wirthlin et al. 1996). Circular smooth muscle activity in rats was decreased after single LPS injection (Eskandari et al. 1997), resulting in less propulsion of ingesta. Another possible cause could be a centralization of the blood circulation caused by the APR (Cinel and Dellinger 2007), leading to a decreased enteric absorption of DON.

No alterations in plasma DOM-1 concentrations were observed after feeding or LPS infusion, which can most likely be ascribed to the fact that the metabolization of DON mainly occurs in the lower intestines by microbiota (Eriksen et al. 2002; Dänicke et al. 2004). Since samples were collected only up to 180 min post infusion start, it is likely that DOM-1 formation and absorption

of the preceding feeding were not captured. The establishment of a steady state could be a result of the chronic exposure and the continuous fermentation of feed and DOM-1 absorption in the lower intestines (Eriksen et al. 2002), which was obviously not affected by LPS infusion until 180 min after infusion start. Since DOM-1 concentrations remained unaltered over the course of the sampling period, and DON absorption concurrently decreased, an increase in the degree of metabolization (ratio between the sum of metabolites and the sum of DON and its metabolites) was observed in all matrices, except for liquor.

DON is mainly eliminated via the urinary, and to a lesser degree also via the biliary route (Coppock et al. 1985; Prelusky et al. 1988; Döll et al. 2003). This was confirmed in our study with highest DON concentration observed in urine, and a higher transfer factor of 0.456 in urine compared to 0.052 in bile (mean across all animals, n = 15). LPS had no effect on DON and DOM-1 concentrations in bile and liquor. In creatinine-corrected urine, a slight decrease in DON concentration was observed in LPS-infused groups ($p \leq 0.055$) which corresponded to the observations in plasma and thus reflected the compromised DON absorption from the digestive tract. DON possesses a high capability to cross biological membranes which is reflected in the fact that DON was also found in liquor, which is in line with other studies (Prelusky et al. 1990; Brezina et al. 2014a). LPS are suspected to cause blood-brain-barrier dysfunction (Banks and Erickson 2010), leading to the assumption that the DON transfer from the circulation might increase due to LPS treatment. However, this was not confirmed by the present results.

LPS-infused pigs (Renner et al. 2017). In our experiment, the decreased ZEN concentration and carry over factor in liver after LPS infusion is supporting that point, indicating the disturbed capacity of liver function. Moreover, hepatic DON and ZEN conjugation, usually mediated by UDP-glucuronosyltransferase, was significantly decreased and peripheral total bilirubin concentration increased in these endotoxaemic pigs (Renner et al. 2017), reflecting again an impairment on hepatic function. An additional possible entry route for ZEN into plasma could be through the cell membrane due to its lipophilic character, followed by incorporation into chylomicrons in the lymphatic system (Jandacek 1982). In a study by Wilson et al. (1985), this route was investigated for aflatoxin B1, which is also lipophilic, but no aflatoxin was found in the lymphatic system.

These results show that the chronic oral exposure of the mycotoxin ZEN and the resulting enrichment in the enterohepatic cycle could possibly enhance the negative effects of an infection

due to a sudden increase of systemic ZEN concentrations upon an APR, representing an additional strain to an already stressed metabolism. Further research is needed to investigate the influence of this observation on the etiopathology of different diseases. In liquor samples, no or only small amounts of ZEN and its metabolites were detected, which is in line with a study with a similar setup by Brezina et al. (2014a). In this study, ZEN was found in some liquor samples 3-4 h after feeding with 0.17 and 0.29 mg ZEN/kg feed.

4.3. Conclusion

We observed macroscopic hemorrhage in the liver together with a marked edema in the gallbladder wall as well as pathohistological alterations due to LPS infusion. However, our hypothesis that the absorption and accumulation of DON and ZEN in various tissues would be altered in endotoxaemic pig's dependent on LPS-entry site was not confirmed. Our data nevertheless illustrate the central role of the liver as a detoxifying and regulating organ. This was reflected in a decreased degree of conjugation of DON and ZEN in liver tissue, which is in line with other studies illustrating a decreased conjugation of DON in endotoxaemic animals (Banhegyi et al. 1995; Dänicke et al. 2014; Sobrova et al. 2010), and the impairment of the accumulation of ZEN in the enterohepatic circle during an APR.

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Compliance with ethical standards

Conflict of interest: The authors declare no conflict of interest.

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5. On the Distribution and Metabolism of Fusarium-Toxins along the Gastrointestinal Tract of Endotoxaemic Pigs

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5.1. Abstract

The aim of this study was to investigate the potential modulatory effect of E. coli lipopolysaccharides (LPS) on residues of deoxynivalenol (DON), de-epoxy-deoxynivalenol (DOM-1), zearalenone (ZEN) and its metabolites α-zearalenol (α-ZEL), β-zearalenol (β-ZEL), zearalanone (ZAN), α-zearalanol (α-ZAL) and β-zearalanol (β-ZAL) after pre- or post-hepatic administration along the gastrointestinal axis. Fifteen barrows were exposed to a naturally mycotoxin contaminated diet (4.59 mg DON/kg feed and 0.22 mg ZEN/kg feed) and equipped with jugular (ju) and portal (po) catheters. On sampling day (day 29), the barrows were infused with LPS or a control fluid (LPS, 7.5 µg/kg body weight; control, 0.9% NaCl) either pre- or post-hepatically, resulting in three infusion groups: CONju-CONpo, CONju-LPSpo and LPSju-CONpo. At 195 min relative to infusion start (210 min post-feeding), pigs were sacrificed and content of stomach and small intestine (proximal, medial and distal part) as well as faeces were collected. In all LPS- infused animals, higher amounts of dry matter were recovered irrespective of LPS-entry site suggesting a reduced gastric emptying and a decreased gastrointestinal motility under endotoxaemic conditions. DON metabolism in the gastrointestinal tract (GIT) remained unaltered by treatments and included an increase in the proportion of DOM-1 along the GIT, particularly from distal small intestine to faeces. Variables describing ZEN metabolism suggest a stimulated biliary release of ZEN and its metabolites in LPS-infused groups, particularly in the LPSju-CONpo group. In conclusion, the GIT metabolism of ZEN was markedly influenced in endotoxaemic pigs whereby a jugular induction of an acute phase reaction was more effective than portal LPS infusion hinting at a strong hepatic first-pass effect.

Keywords: Deoxynivalenol, gastrointestinal tract, lipopolysaccharides, metabolites, pigs, zearalenone

5.2. Introduction

Pigs respond with reduced voluntary feed intake and fertility disorders upon the ingestion of the mycotoxins deoxynivalenol (DON) and zearalenone (ZEN), respectively (Wu and Munkvold 2008). Due to their wide occurrence and co-occurrence in animal feed, they are responsible for considerable economic losses particularly in pig production (Dänicke et al. 2005; Pestka 2007; Döll and Dänicke 2011; Dänicke and Winkler 2015).

The two mycotoxins differ substantially in their toxicokinetics (Burdon and Zabel 2002; Dänicke and Brezina 2013; Dänicke and Winkler 2015). DON is rapidly absorbed in the upper gut or metabolised in the lower intestines to de-epoxy-deoxynivalenol (DOM-1) by intestinal microbes and partially absorbed thereafter (Kollarczik et al. 1994; Eriksen et al. 2002; Dänicke and Brezina 2013). ZEN is also well absorbed in the small intestine but the biotransformation of ZEN into the different metabolites occurs in the intestinal lumen (pre-absorptive stage, microbial) and mucosa (absorptive stage), as well as in the liver (metabolic stage) (Olsen et al. 1987; Malekinejad et al. 2006; Zinedine et al. 2007; Dänicke and Winkler 2015). After ingestion, DON is rapidly distributed throughout the body and can be found in almost all matrices shortly thereafter. It is however also eliminated rapidly, mainly via the renal route (Coppock et al. 1985; Prelusky et al. 1988; Goyarts and Dänicke 2006). In contrast to DON, ZEN is not only eliminated via urine but also with bile due to a pronounced entero hepatic cycling (Biehl et al. 1993; Dänicke et al. 2005).

In pig production, different systemic and enteric infections are known to be associated with an acute phase reaction (APR) (Zimmerman et al. 2012). Lipopolysaccharides (LPS), constituting the outer membrane of gram-negative bacteria, are a common trigger of an APR. The infusion or injection of LPS is therefore a frequently used and well described animal model for the investigation of APR related pathomechanisms (Wyns et al. 2015). In pigs, the infusion of LPS results in an increased respiratory rate and laboured respiration, hyperthermia and lactic acidosis, tremor, cyanosis and dermographism, injected episcleral vessels and hyperaemic conjunctivae, as well as alterations in different immune variables such as a leukopenia (Dänicke et al. 2014; Bannert et al. 2015; Tesch et al. 2016). On the level of the gastro-intestinal tract (GIT), a decrease in passage rate is observed (Cullen et al. 1995, 1996; Wirthlin et al. 1996; Eskandari et al. 1997) which might also be due to the APR associated centralization of blood circulation. Under these conditions, the perfusion of the GIT is reduced leading to a compromised small intestinal barrier (Klunker et al. 2013) with possible consequences for mycotoxin absorption, to an altered intestinal milieu due to

a cessation of mucosal digestive processes and cellular functions such as protein synthesis (Kullik et al. 2013), the associated accumulation of nutrients, enzymes and enhanced mucosal shedding and to a shift of fluid spaces. All these processes might have consequences for the gastrointestinal passage and metabolism of DON and ZEN and consequently for absorption, hepatic clearance and inner exposure. Moreover, the LPS-entry route might modulate the magnitude of the APR given the central role of the liver in post-absorptive LPS and mycotoxin metabolism and first-pass elimination.

Therefore, it was hypothesised that a pre- or post-hepatically LPS-induced APR influences the recovery of dry matter, DON, ZEN and their metabolites along the GIT. Specimens, analysed in the present investigations, were generated in an experiment where general indicators of APR, portal and systemic blood kinetics and tissue residue levels of DON and ZEN were reported (Bannert et al. 2015, 2017; Tesch et al. 2016). As pre-absorptive GIT mycotoxin metabolism clearly influences both portal and systemic mycotoxin profiles, another objective of the present study was to relate gastrointestinal DON and ZEN metabolism with post-absorptive metabolism.

5.3. Material and Methods

Animal experiments were conducted according to the EC regulations concerning the protection of experimental animals and the guidelines of the German Animal Welfare Act approved by the Lower Saxony State Office for Consumer Protection and Food Safety (Lower Saxony State Office for Consumer Protection and Food Safety; File Number 33.4-42,502-04-13/1274).

5.3.1. Experimental design, treatments and procedures

Experimental design and respective procedures have been described previously (Bannert et al. 2015; Tesch et al. 2016), but they are illustrated in Figure 1 as an overview. Briefly, a total of 15 German Landrace barrows (Mariensee, Germany) were chronically exposed to a naturally Fusarium toxin contaminated diet containing 4.59 mg DON and 0.22 mg ZEN per kg feed. The total mean intake during 29 days of trial of DON and ZEN amounted 133.0 ± 3.0 mg and 6.5± 0.1 mg (mean ± SD), respectively. The daily feed allowance was increased continuously over the course of the trial to attain a voluntary intake of 1400 g of feed per day, provided as two equal rations daily (feeding times: 07:00 h, 14:00 h; fed as slurry). The diet consisted of 53 % barley, 20 % soybean meal, 15 % maize (7.5 % Fusarium graminearum inoculated and 7.5 % non-inoculated), 5 % rapeseed, 2 % soybean oil, 4 % mineral and vitamin premix, amino acids, and for digestibility

estimation 1 % of a marker (Sipernat®50S, Evonik Industries AG, Germany) was added (Bannert et al. 2015). During experimental days 24 and 29, barrows were housed in metabolism crates and on day 27 surgically equipped with indwelling catheters in *Vena jugularis externa* and *V. lienalis* for infusion, and *V. jugularis interna*, *V. portae hepatis* and *Arteria carotis communis* for blood sampling. On sampling day (day 29), the animals (mean body weight [BW]: 40.5 ± 3.0 kg, mean ± SD) received 700 g of the Fusariumtoxin contaminated diet 15 min prior to infusion start and were then exposed to either a pre-hepatic (CONju-LPSpo, n = 5, *V. lienalis*) or post-hepatic (LPSju-CONpo, n = 5, *V. jugularis externa*) LPS-challenge (7.5 µg LPS/kg BW and concomitant catheters infused with isotone NaCl solution), or received an isotone NaCl solution (CONju-CONpo, n = 5) for 60 min. The chosen LPS dose provided a defined and transient endotoxaemic response (clinical symptoms) and was established in a previous dose-response study (Dänicke et al. 2012). Blood samples were collected from either *V. jugularis interna* or *V. portae hepatis* at different times (-30, 15, 30, 45, 60, 75, 90, 120, 150, 180 min relative to start of infusion) and at 195 min after infusion start (= 210 min after feeding the morning ration), barrows were sacrificed via electro-stunning and exsanguination and the entire GIT was quickly dissected. Stomach, small and large intestine were separated from each other and the small intestine (SI) divided into three equal sections (upper section SI 1, middle section SI 2 and lower section SI 3). Thereafter, the entire contents of stomach and the three parts of the SI were collected separately.

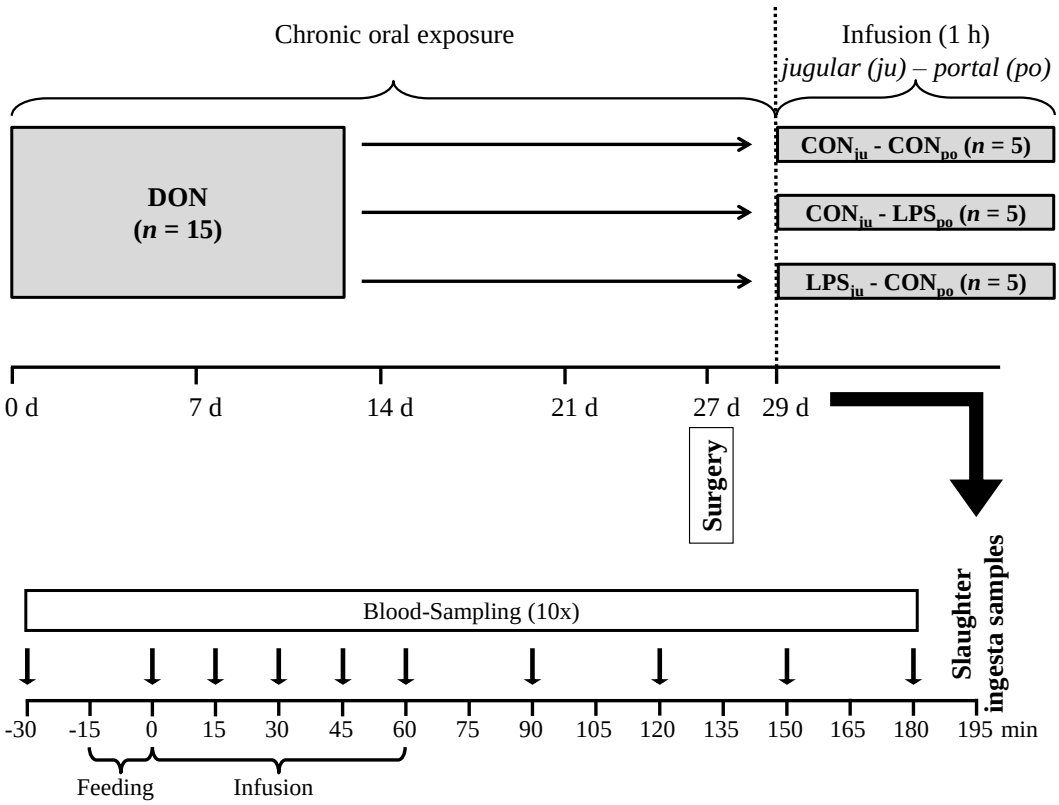

Figure 1: Experimental Design

Faecal samples were collected within one hour prior to (voluntary voided) and at slaughter (rectal sample) and pooled for each animal to obtain sufficient material for analyses. All GIT contents were weighed, stored at -20°C and subsequently freeze-dried and ground to 1 mm particle size for further analyses. HCl-insoluble ash was employed as indigestible marker, according to the official methods of the VDLUFA, (Naumann and Bassler 1993) and only analysed in stomach, SI 3 and faeces, due to insufficient sample material from SI 1 and SI 2.

5.3.2. Mycotoxin analysis in feed and GIT contents

Briefly, for the mycotoxin analysis in the feed, a high-performance liquid chromatography with diode array detection (HPLC-DAD, for DON) or a fluorescence detection (HPLC-FLD, for ZEN, Shimadzu, Duisburg, Germany) was performed as described in Oldenburg et al. (2007) with slight changes as described in Bannert et al. (2017).

The analyses of the GI contents for mycotoxin concentrations were performed as described for liver in Bannert et al. (2017) with slight modifications. The sample preparation was performed as follows: 2 g of freeze-dried sample were mixed with internal standards, 14 ml bidistilled water, 10

ml sodium acetate-buffer (pH 5.5; VWR, Darmstadt, Germany) and 200 µl β-glucuronidase (Sigma G 0876, 100 000 U/ml/type H-2 from Helix pomatia by Sigma-Aldrich, Steinheim, Germany) were added. The mixture was incubated at 37°C overnight. Thereafter, 120 ml acetonitrile (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) were added, and after mixing (1 h; 240 rpm), the sample was centrifuged and the supernatant was concentrated in a rotary evaporator. Then 10 ml PBS (pH 7.4, Merck KGaA, Darmstadt, Germany) was added and the pH was adjusted to 7.0-7.3 with 1 M NaOH (Merck KGaA, Darmstadt, Germany). Thereafter, the solutions were cleaned up by DZT-MS Prep columns as described for bile by Brezina et al. (2016).

The mycotoxins DON, ZEN and their metabolites de-epoxy-deoxynivalenol (DOM-1), α-zearalenol (α-ZEL), β-zearalenol (β-ZEL), zearalanone (ZAN), α-zearalanol (α-ZAL) and β-zearalanol (β-ZAL) were analysed with liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) in negative mode using a 4000 QTrap (Applied Biosystems, CA, USA) coupled with a 1200 series HPLC (Agilent Technologies, Waldbronn, Germany) as described by Brezina et al. (2014).

The limit of detection (LOD), limit of quantification (LOQ) and mean recovery are listed in Table 1. While values lower than LOD were set to zero, values between LOD and LOQ were used for further statistical evaluation.

Table 1: Mean recoveries (n = 4, limits of detection (LOD, S/N = 3) and quantification (LOQ, S/N = 9) of zearalenone (ZEN), deoxynivalenol (DON) and their metabolites from the analysis of samples from gastric content, small intestines (SI) contents and faeces of pigs.

		Toxin							
		ZEN	α-ZEL[¶]	β-ZEL[#]	ZAN[†]	α-ZAL[‡]	β-ZAL[◊]	DON	DOM-1[♦]
Gastric content	Recovery [%]	97.3	98.5	96.1	96.8	96.3	98.4	97.8	72.2
	LOD [ng/ml]	0.03	0.1	0.3	0.1	0.2	0.1	2	0.6
	LOQ [ng/ml]	0.1	0.4	0.6	0.4	0.6	0.3	6.7	1.2
SI content	Recovery [%]	83.9	92.4	91.6	78.7	163.6	127.3	109.8	82.4
	LOD [ng/ml]	0.08	0.6	0.5	0.4	0.3	0.1	1	0.4
	LOQ [ng/ml]	0.3	1.2	1	1.4	0.7	0.3	3.3	1
Faeces	Recovery [%]	96.1	114.6	103.1	107.4	111.8	110.0	117.4	88.6
	LOD [ng/ml]	0.04	0.4	0.3	0.4	0.3	0.4	0.3	0.3
	LOQ [ng/ml]	0.13	1.3	1.0	1.5	1.0	1.2	1.0	1.0

*Concentration ranges [ng/ml]: ZEN: 26.7–106.8, metabolites of ZEN: 80–320, DON and DOM-1: 320–1280; α-ZEL, α- zearalenol; [#]β-ZEL, β-zearalenol; [†]ZAN, zearalanone; [‡]α-ZAL, α-zearalanol; [◊]β-ZAL β-zearalanol; [♦]DOM-1, de-epoxy- deoxynivalenol.

5.3.3. Calculations and statistics

Total amounts of DM recovered from chyme of stomach, SI 1, SI 2 and SI 3 were expressed as percentage of DM intake as follows:

$$\text{Dry matter (DM) recovery [\%]} = \frac{(\text{total amount of ingesta } [g\ DM])}{(\text{total feed intake } [g\ DM])} \cdot 100$$

The apparent mycotoxin recovery in chyme was calculated based on HCl-insoluble ash as indigestible marker:

$$\text{Mycotoxin recovery [\%]} = \frac{100\ \cdot\ (\text{marker [\%] diet}\ \cdot\ \text{toxin } [\mu g/g\ DM] \text{ ingesta})}{(\text{marker [\%] ingesta}\ \cdot\ \text{toxin } [\mu g/g\ DM] \text{ diet})}$$

Metabolite profiles of DON and ZEN were calculated as relative proportion [%] of the sum of the main mycotoxin and their respective metabolites, demonstrating their relative distribution along the GIT.

Data were analysed by a two-way analysis of variance with group, location (GIT site) and their interaction as fixed factors and Fisher LSD test as post hoc procedure. Statistical significance was

declared at $p \leq 0.05$ and a trend at $p \leq 0.10$. All statistical analyses were performed using Statistica 13 (StatSoft Inc., Tulsa, USA).

5.4. Results

5.4.1. Apparent dry matter recovery in subsequent segments of the GIT

At the time of slaughter (210 min post-feeding), the highest amounts of dry matter contents were present in stomach and very low contents in small intestinal sections (Figure 2). Distribution pattern in control group CONju-CONpo turned out to be as follows (means ± SD): 75.3 ± 13.3% in stomach, 1.3 ± 0.5% in SI 1, 4.8 ± 2.3% in SI 2 and 6.2 ± 3.0% in SI 3. Dry matter recovery was influenced by LPS treatments. Compared to the control group, LPS infusion increased the amounts of DM in stomach while lower amounts were recovered from small intestinal segments S2 and S3 irre- spective of LPS-entry route ($p \leq 0.01$). No significant difference was observed for SI 1 between both LPS infusion groups.

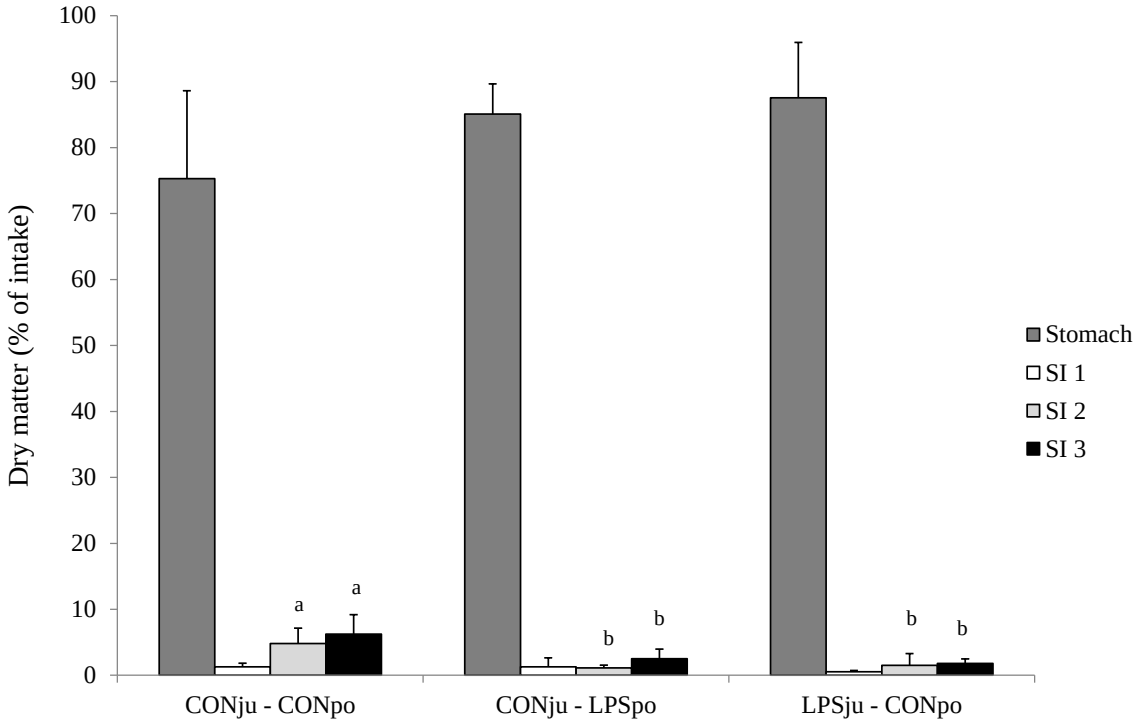

Figure 2: Dry matter (DM) recovery (% of DM intake with the morning ration) from stomach and the small intestine (SI; effect of location) 195 min after start of infusion (=210 min after morning feed) (bars are means with standard deviation, n = 5). Different lower case letters indicate significant differences ($p < 0.05$) between groups at the respective location (Fisher LSD test as post-hoc procedure). SI 1, SI 2 and SI 3, proximal, medium and distal section of SI, respectively; CON_{ju}-CON_{po}, post-hepatic and pre- hepatic infusion with 0.9 % NaCl (control) (n = 5); CON_{ju}-LPS_{po}, post-hepatic infusion with 0.9% NaCl; pre-hepatic infusion with LPS (n = 5); LPS_{ju}-CON_{po}, post-hepatic infusion with LPS, pre-hepatic infusion with 0.9% NaCl (n = 5). Main effects of ANOVA (F-test): $p_{group} = 0.839$, $p_{location} < 0.001$, $p_{group \times location} = 0.007$.

5.4.2. Recovery of ZEN and DON in GI contents

For the recovery of ZEN along the GI tract (Table 2), only a significant location effect was observed with the highest recovery in stomach (103.7 ± 14.1%) and SI 3 (97.6 ± 49.9%) and lowest in faeces (18.8 ± 7.4%). Further, for sum of ZEN and metabolites, a significant group × location interaction occurred. CONju-CONpo exhib- ited a decrease between stomach and SI 3, and faeces. In LPSju-CONpo however, the recovery of sum of ZEN and metabolites was significantly higher in SI 3 and lower in the faeces compared to the stomach, as well as compared to SI 3 and faeces of the CONju-CONpo- and CONju-LPSpo-infused group.

Table 2: Recovery [%] of zearalenone (ZEN), deoxynivalenol (DON) and the sum of their respective metabolites in stomach, distal part of small intestine (SI 3) and faeces (means ± standard deviation).

		Treatment groups		
Toxin	Location	CON_{po} - CON_{ju} ♦	LPS_{po} - CON_{ju} ◊	CON_{po} - LPS_{ju} ¶
ZEN	Stomach	103.59 ± 16.49	107.54 ± 16.77	100.06 ± 10.47
p_{group} = 0.209	SI 3	71.49 ± 39.19	92.56 ± 41.14	135.26 ± 56.14
$p_{location}$ < 0.001	Faeces	19.95 ± 8.59	21.39 ± 7.00	15.34 ± 7.02
$p_{group*location}$ = 0.060				
ZEN + metabolites†	Stomach	112.06 ± 17.84 ^{A}	117.57 ± 16.69 ^{A}	109.54 ± 12.53 ^{A}
p_{group} < 0.016	SI 3	117.25 ± 68.28 ^{Aa}	168.94 ± 92.74 ^{Aa}	287.59 ± 85.00 ^{Bb}
$p_{location}$ < 0.001	Faeces	44.92 ± 15.05 ^{B}	46.04 ± 13.07 ^{B}	37.49 ± 16.65 ^{C}
$p_{group*location}$ = 0.001				
DON	Stomach	47.21 ± 6.16	45.17 ± 12.47	43.15 ± 2.58
p_{group} = 0.969	SI 3	1.65 ± 0.67	3.74 ± 3.55	4.46 ± 2.71
$p_{location}$ < 0.001	Faeces	0.06 ± 0.01	0.05 ± 0.01	0.07 ± 0.01
$p_{group*location}$ = 0.696				
DON + metabolite‡	Stomach	47.37 ± 6.19	45.36 ± 12.51	43.34 ± 2.59
p_{group} = 0.985	SI 3	1.74 ± 0.71	3.92 ± 3.55	4.65 ± 2.74
$p_{location}$ < 0.001	Faeces	0.56 ± 0.33	0.49 ± 0.2	0.85 ± 0.68
$p_{group*location}$ = 0.684				

Notes: ♦CONju-CONpo, pre-hepatic and post-hepatic infusion with 0.9 % NaCl (control, n = 5); ◊CONju-LPSpo, post-hepatic infusion with 0.9% NaCl; pre-hepatic infusion with LPS (n = 5); ¶LPSju-CONpo, post-hepatic infusion with LPS, pre-hepatic infusion with 0.9% NaCl (n = 5); †Comprise α-zearalenol (α-ZEL), β-zearalenol (β-ZEL), zearalanone (ZAN), α-zearalanol (α-ZAL) and β-zearalanol (β-ZAL); ‡de-epoxy-deoxynivalenol (DOM-1). ^{ab}Means with different superscripts are significantly different between groups in respective matrix ($p < 0.05$); ^{AB}means with different superscripts are significantly different between matrices within respective group ($p < 0.05$).

For the recovery of DON and the sum of DON and DOM-1 again, a significant location effect was observed with the highest recovery in the stomach (45.2 ± 7.8 and 45.4 ± 7.8) and lowest in SI 3 (3.2 ± 2.6 and 3.3 ± 2.5) and faeces (0.06 ± 0.01 and 0.64 ± 0.46, mean ± SD).

5.4.3. Metabolite profiles of ZEN and DON in GI contents

The relative distribution of ZEN and its metabolites (Figure 3) in the entire metabolite profile showed a distinct pattern along the proximodistal GI axis (reflected in a significant effect of location, $p < 0.001$): parental ZEN represented the main portion in stomach and declined in small intestine with lowest levels in faeces. Concomitantly, α-ZEL as major metabolite increased along the digestive tract to faeces as did β-ZEL, but the latter to a considerably lower degree. The sum of metabolites ZAN, α-ZAL and β-ZAL also decreased from stomach to faeces, but represented a rather negligible proportion in the metabolite profile. Besides the impact of location, the main factor "group" demonstrated also a significant effect on mycotoxin profiles: in group CONju- CONpo, parental ZEN amounted to 64.8% and was decreased in LPS-challenged groups (CONju-LPSpo 61.5%, LPSju-CONpo 55.2%), whereas this picture was reversed for main metabolite α-ZEL, effectively increasing in challenged groups (29.7% in CONju-CONpo, 32.8% in CONju-LPSpo and 39.3% in LPSju-CONpo). Metabolites β-ZEL and the sum of ZAN, α-ZAL and β-ZAL showed no significant alterations due to LPS infusion.

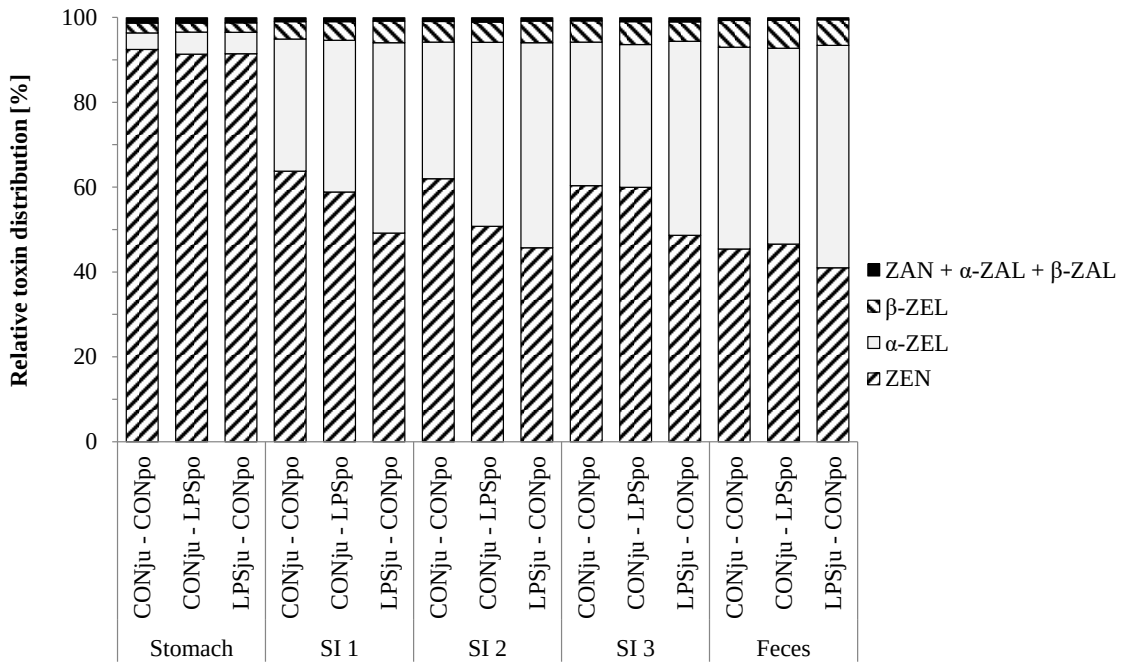

Figure 3: Relative distribution of zearalenone (ZEN) and its metabolites (α-zearalenol [α-ZEL], β- zearalenol [β-ZEL], zearalanone [ZAN], α-zearalanol [α-ZAL], β-zearalanol [β-ZAL]) along the gastro- intestinal axis (displayed as relative proportions of their sum). SI 1, SI 2 and SI 3, proximal, medium and distal section of the small intestine, respectively; CON_{ju}-CON_{po}, pre-hepatic and post-hepatic infusion with 0.9 % NaCl (control, n = 5); CON_{ju}-LPS_{po}, post-hepatic infusion with 0.9% NaCl; pre-hepatic infusion with LPS (n = 5); LPS_{ju}-CON_{po}, post-hepatic infusion with LPS, pre-hepatic infusion with 0.9% NaCl (n = 5). Main effects of ANOVA (F-test): ZEN: $p_{group} < 0.001$, $p_{location} < 0.001$, $p_{group \times location} = 0.415$; α-ZEL: $p_{group} = 0.001$, $p_{location} < 0.001$, $p_{group \times location} = 0.487$; β-ZEL: $p_{group} = 0.955$, $p_{location} < 0.001$, $p_{group \times location} = 0.983$; ZAN+α-ZAL+β-ZAL: $p_{group} = 0.228$, $p_{location} < 0.001$, $p_{group \times location}$ 0.664.

Parental DON (Figure 4) had its highest proportion in the stomach and SI 1 (99.6 ± 0.1% and 98.9 ± 0.6%), which decreased slightly, albeit significantly, towards the distal end (96.3 ± 2.0% in SI 2, 94.1 ± 2.7% in SI 3) and the largest decline in faeces (11.5 ± 3.8%). Concurrently, an increase in DOM-1 proportions was observed. LPS infusion did not influence DON and DOM-1 proportions throughout the GI tract.

5.4.4. Correlation of DON and ZEN content in SI 3 with plasma levels

The time-dependent appearance of parental toxins DON and ZEN in portal and jugular plasma and their corresponding areas under the curve (AUC) were reported recently in the frame of this study (Bannert et al. 2017). In order to examine possible absorption/ reabsorption effects in more detail,

these AUC's were correlated with the corresponding total toxin residues recovered from the intestinal segment SI 3. No significant relationship was observed for DON in plasma and the toxin content in SI 3 digesta (plasma AUCportal r = 0.228, p = 0.413; plasma AUCjugular r = 0.476, p = 0.073). In contrast, a negative correlation was evident for plasma ZEN and its corresponding content in SI 3 digesta (Figure 5), mainly attributed to the marked correlation for groups LPSju-CONpo and CONju-LPSpo.

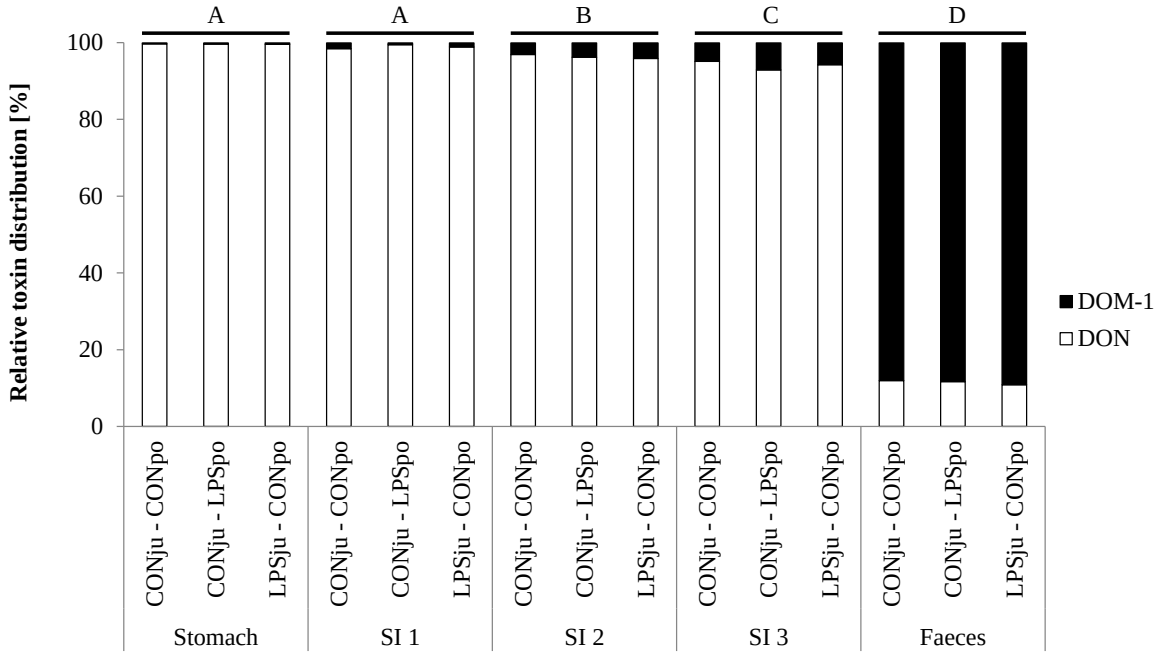

Figure 4: Relative distribution of deoxynivalenol (DON) and its metabolite de-epoxy-deoxynivalenol (DOM-1) along the gastrointestinal tract axis (displayed as individual proportions of their sum). Unlike capital letters indicate significant differences between matrices ($p < 0.05$). SI 1, SI 2 and SI 3, proximal, medium and distal section of the small intestine, respectively; CON_{ju}-CON_{po}, pre-hepatic and post-hepatic infusion with 0.9 % NaCl (control, n = 5); CON_{ju}-LPS_{po}, post-hepatic infusion with 0.9% NaCl; pre-hepatic infusion with LPS (n = 5), LPS_{ju}-CON_{po}, post-hepatic infusion with LPS, pre-hepatic infusion with 0.9% NaCl (n = 5). Main effects of ANOVA (F-test): DON: $p_{group} = 0.725$, $p_{location} < 0.001$, $p_{group \times location} = 0.936$; DOM-1: $p_{group} = 0.725$, $p_{location} < 0.001$, $p_{group \times location} = 0.936$.

5.5. Discussion

In order to investigate whether metabolism and recovery of DON and ZEN and their metabolites along the GIT would be altered due to an endotoxaemic state, a model was used to induce an APR by infusion of LPS, either pre- or post-hepatically. We could show a more pronounced APR in post-hepatically infused pigs, compared to their pre-hepatically infused counterparts, as expressed

by an altered body temperature profile as well as a more pronounced lactic acidosis post-hepatically hinting at a specific role of the liver in LPS clearance (Bannert et al. 2015; Tesch et al. 2016).

The present data on dry matter recovery as percentage of feed intake indicate that the solid phase of the morning ration was still largely lodged in the stomach (>80%) 3 h post-feeding and that ingesta propulsion towards the small intestine appeared to be diminished by LPS as indicated by the significantly lower dry matter recovery in the second and third SI sections. These findings support the view that LPS adversely influences the GI motility (Cullen et al. 1995, 1996; Wirthlin et al. 1996; Eskandari et al. 1997). However, the more pronounced APR in pre-systemically LPS-infused pigs in this study apparently did not exacerbate consequences for GI-motility as LPS-entry route did not affect dry matter passage differently.

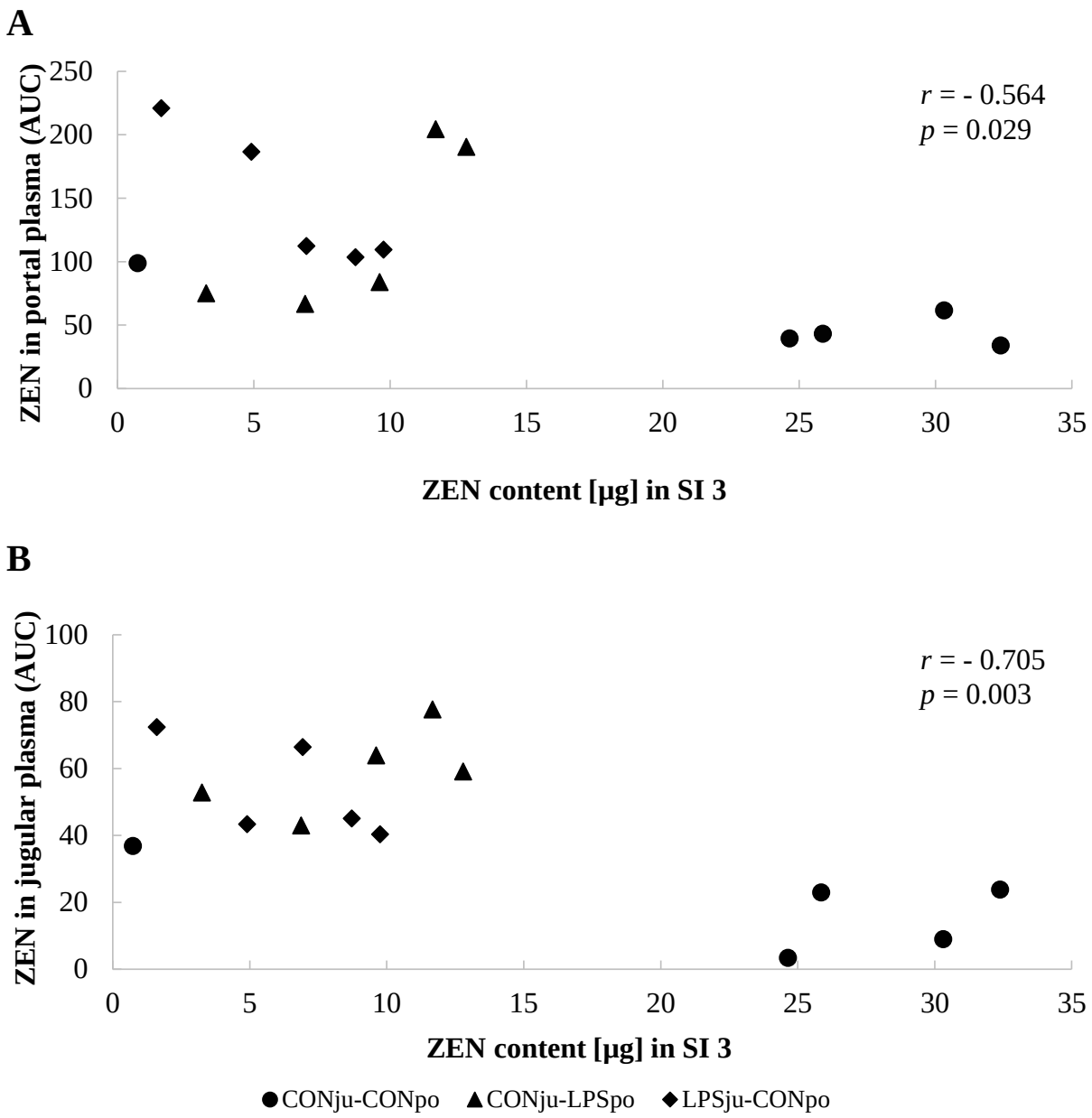

Figure 5: Scatterplot of ZEN content in portal and jugular plasma (panel A and B, respectively) and in the distal section of the small intestine (SI 3). AUC, area under the curve, calculated from toxin concentration measured at different time points (-30, 15, 30, 45, 60, 75, 90, 120, 150 and 180 min relative to feeding time) as described in Bannert et al. (2017). CON_{ju}-CON_{po}, post-hepatic and pre-hepatic infusion with 0.9 % NaCl (control, n=5); CON_{ju}-LPS_{po}, post-hepatic infusion with 0.9% NaCl; pre-hepatic infusion with LPS (n=5), LPS_{ju}-CON_{po}, post-hepatic infusion with LPS, pre-hepatic infusion with 0.9% NaCl (n=5).

Our data confirm the previously described rapid absorption of DON (Dänicke et al. 2004) in the upper GIT after ingestion as indicated by the fast-rising plasma absorption already within 15 min post feeding (Bannert et al. 2017) and the 50 % recovery of the parental toxin

3 h post feeding in the stomach. This appearance in plasma takes place despite the fact that most of the pig's morning meal (DM basis) is still located in the stomach and raises the question on the nature of DON absorption. Due to its hydrophilic nature, DON could be predominantly transported in the liquid ingesta phase assuming that there's a difference in the propulsion of solid and liquid ingesta. Studies on gastric emptying in pigs fed different fibre sources could indeed demonstrate that in the first 3 h post feeding the liquid phase leaves the stomach faster compared to the solid phase, but after 3 h, this difference is ameliorated (Johansen et al. 1996). This supports the notion that the majority of DON leaves the stomach in the liquid phase of ingesta and thus can be absorbed in the small intestine in a very short time as evidenced in plasma concentrations. In contrast to this, another study reports no differences in mean retention time between solid and liquid phase of ingesta (Wilfa by the gastric mucosa. In our current experimental setup, we are not able to discern between these two potential mechanisms and further research on this is expedient.

Furthermore, we observed a lower DON, but no DOM-1 absorption in LPS-infused pigs compared to their CON-infused counterparts as verified by their significantly lower DON- AUC values, representing an indicator for the amount of DON transferred from the intestinal lumen to the plasma during the observation period of 3 h post feeding (Bannert et al. 2017). Taking the data presented here into account, the reason for a diminished DON appearance in the blood circulation of LPS-challenged pigs might not be entirely attributed to a compromised DON absorption from the small intestine, as similar amounts of DON were recovered from the lumen of SI 3 (1.74 - 4.65 % of DON intake) and not higher levels in LPS-groups as one might expect. Moreover, DON residue concentrations in urine and bile were not significantly different among the treatment groups (Bannert et al. 2017). Therefore, the lower portal and systemic DON availability in LPS-exposed groups might be caused by distributing of DON in extravasal spaces or by forced renal elimination rates which are not reflected by urine concentration at the end of the observation period. Given the fact that an LPS-induced APR is associated with anuria or oliguria and inconspicuous urine DON and DOM-1 concentration at the end of the observation period, forced renal elimination rates might be rather unlikely. In addition, the biliary route of DON elimination appeared not to be influenced either as indicated by unaltered bile concentrations. Earlier studies have shown that DOM-1 formation mainly occurs in the hindgut (Kollarczik et al. 1994; Eriksen et al. 2002), which is confirmed in our data, showing an increase of DOM-1 proportion along the GIT, with slightly increasing percentage until SI 3, but representing the major portion (~80%) in faeces. LPS challenge did not affect the formation of DOM-1 irrespective of its non-detection in blood, further

confirming the notion that this is primarily produced by microorganisms present in the large intestine (Worrell et al. 1989).

In contrast to DON, ZEN recovery in the stomach still reflected pigs' feed intake, i.e. amounting to 100% of toxin intake and thus hinting at its passage with the solid ingesta phase. Data on dry matter recovery and portal plasma concentrations (Bannert et al. 2017) corroborate this. Furthermore, ZEN and metabolites are present in very high proportions, well above 100% at SI 3 level, indicating that ZEN recovery distal from the stomach reflects toxin exposure from previous meals rather than being representative for the morning meal before the onset of infusion. This also strongly supports the concept of enterohepatic recycling and thus accumulation in the GIT in chronic ZEN exposure. Although recovery in stomach and faeces appeared to be not affected by LPS, ZEN and its metabolites in SI 3 ingesta showed a strong increase in LPS-challenged pigs. This was particularly due to a stronger formation and/or a selective enrichment of metabolites, as this impact was less evident in parental ZEN recovery. Concurrently, plasma data show very little absorption during the observation period, only in LPS-infused pigs we noted a significant increase in ZEN and α-ZEL plasma levels starting 120 min post challenge, particularly evident in portal blood (Bannert et al. 2017). These coinciding events might have their origin in a decreased peristalsis and a stimulated bile release into the small intestine. It was suggested that LPS causes a release of gall bladder content into the gut and a subsequent decrease in bile formation, thereby interrupting the enterohepatic cycle, leading to an accumulation of ZEN and metabolites in the gut as well as an increased uptake in the distal part of the small intestines (Bannert et al. 2017). This was supported by the decreased gall bladder filling inrt et al. 2007) and thus could support the hypothesis of a substantial DON absorption all LPS infused animals at slaughtering (Renner et al. 2017). The involvement of a disrupted bile cycling is supported by the data of the present manuscript, showing that the recovery of ZEN and its metabolites at the terminal small intestine (SI 3) well exceeded dietary ZEN intake up to approximately 3-fold. Moreover, the impaired peristalsis due to LPS-challenge - as indicated by dry matter recovery data - most likely resulted in a prolonged retention of chyme in the GIT, enhancing contact time between chyme (incl. ZEN), the resident microbiota and the mucosal lining, additionally accounting for the increased pre-absorptive ZEN metabolite formation in SI 3. Interestingly, the highest toxin recovery was detected for the group infused systemically with LPS (LPSju-CONpo) supporting the view that the more pronounced APR mounted by this group also influenced bile release, metabolism and peristalsis more prominently than their portal-infused counterparts.

Further evidence of APR-driven modification of cycling of bile and its constituents, including ZEN and its metabolites, can be deduced from the relationships between the total amounts of ZEN and its metabolites recovered from the distal small intestine (SI 3) and the portal and jugular $AUC_{time(0->180min)} \times ZEN_{residue\ concentration}$ (Figure 5). This was most evident for the LPS-challenged groups whereas for placebo-infused pigs this relationship was hardly detectable. Higher ZEN residues were recovered in LPS infused groups at the distal small intestine, probably caused by the delayed stomach emptying, but the corresponding portal and jugular $AUC_{time(0->180min)} \times ZEN_{residue\ concentration}$ were also highest in these groups strongly hinting at a reabsorption of ZEN from bile. ZEN appearance in the portal vein, reflecting the drainage from the GIT, only occurred 120-180 min after start of LPS infusion, allowing the time for expulsed bile (including ZEN and metabolites) to travel from the duodenum to the terminal ileum. This accounts for the higher toxin recovery at the end of the small intestine,

The present data further show that ZEN is metabolised as soon as it enters the small intestine. The proportions of ZEN and its metabolites to each other might be the combined result of pre-absorptive metabolism of ZEN and of metabolites entering the gut via bile excretion. Again, the group characterised by the most pronounced APR (LPSju-CONpo) showed the highest α-ZEL proportions compared both to the non-stimulated and to the portal LPS-infused groups. The higher ZEN metabolism degree in this group might indicate a higher proportion of bile contribution to this metabolite pattern in the small intestine.

5.6. Conclusion

In conclusion, DON metabolism in the GIT remained unaltered by treatments and included an increase in the proportion of DOM-1 along the GIT, particularly from distal small intestine to faeces. ZEN metabolism was characterised by a stimulated biliary release of ZEN and its metabolites in LPS-infused groups, particularly in the LPSju-CONpo group, supporting the concept of enterohepatic recycling of ZEN. It can be concluded that GIT metabolism of ZEN was markedly influenced in endotoxaemic pigs whereby a jugular induction of APR was more effective than portal LPS infusion hinting at a strong hepatic first-pass effect.

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6. General Discussion and Conclusions

In pig production, a chronic exposure with DON and ZEN and a concurrent bacterial infection is a very possible scenario (Pluske et al., 2002; Zimmerman et al., 2012). To investigate the possible effects of a simultaneous chronic DON and ZEN exposure and systemic inflammation on clinical, metabolic and immunological traits as well as the toxicokinetics of DON and ZEN we conducted a study by using LPS as a well-established model substance to trigger an APR (Salomao et al., 2012; Wyns et al., 2015) in pigs chronically exposed to a natural DON and ZEN contaminated diet. To investigate the role of the liver as central detoxifying organ, LPS was infused via either the pre- or post-hepatic route. With attention to a possible effect of DON and ZEN to the LPS- induced immune response itself the experimental design arranged six animal groups: CON_CONju-CONpo, CON_CONju-LPSpo, CON_LPSju-CONpo, DON_CONju-CONpo, DON_CONju-LPSpo and DON_LPSju-CONpo (detailed description in Chapter 2-5).

Due to the relative low concentrations of DON and ZEN in the feedstuff we did not observe acute toxic effects. The measurements described in Chapter 4 and 5, however, show that we were able to successfully reproduce a chronic exposure with both mycotoxins comparable to practical production conditions and with the toxicokinetics expected (Chapter 1.2.1).

6.1. Influence of a Chronic DON and ZEN Feeding on Clinical, Metabolic and Immunological Traits during an LPS-Induced APR

Clinically, the initial innate immune response caused by the LPS infusion was characterized by an increase in respiratory rate, reddened conjunctivae, injected episcleral vessels, shivering, retching, and vomiting, as well as a marked fever response, without any impact of the DON-feeding or infusion site on the severity of the clinical symptoms, except for the body temperature. A marked diet effect was found in post-hepatic LPS-infused animals with the DON-fed animals exhibiting consistently lower temperature (~0.5 °C) as compared to their LPS-infused, CON-fed counterparts (Tesch et al., 2015). Furthermore, in the CON-fed pre-hepatic infused animals a more rapid return to physiological clinical levels was observed (Fig. 6; Tesch et al., 2015).

On the systemic level, the animals further exhibited substantial disturbance of their acid-base balance, characterized by a lactic acidosis. This effect was more pronounced in the mycotoxin exposed post-hepatic infused animals (Chapter 3). An initial hyperglycemia followed by eu- and hypoglycemia, which is commonly observed during an APR, was recorded in all animals. Also, in this context, the chronic

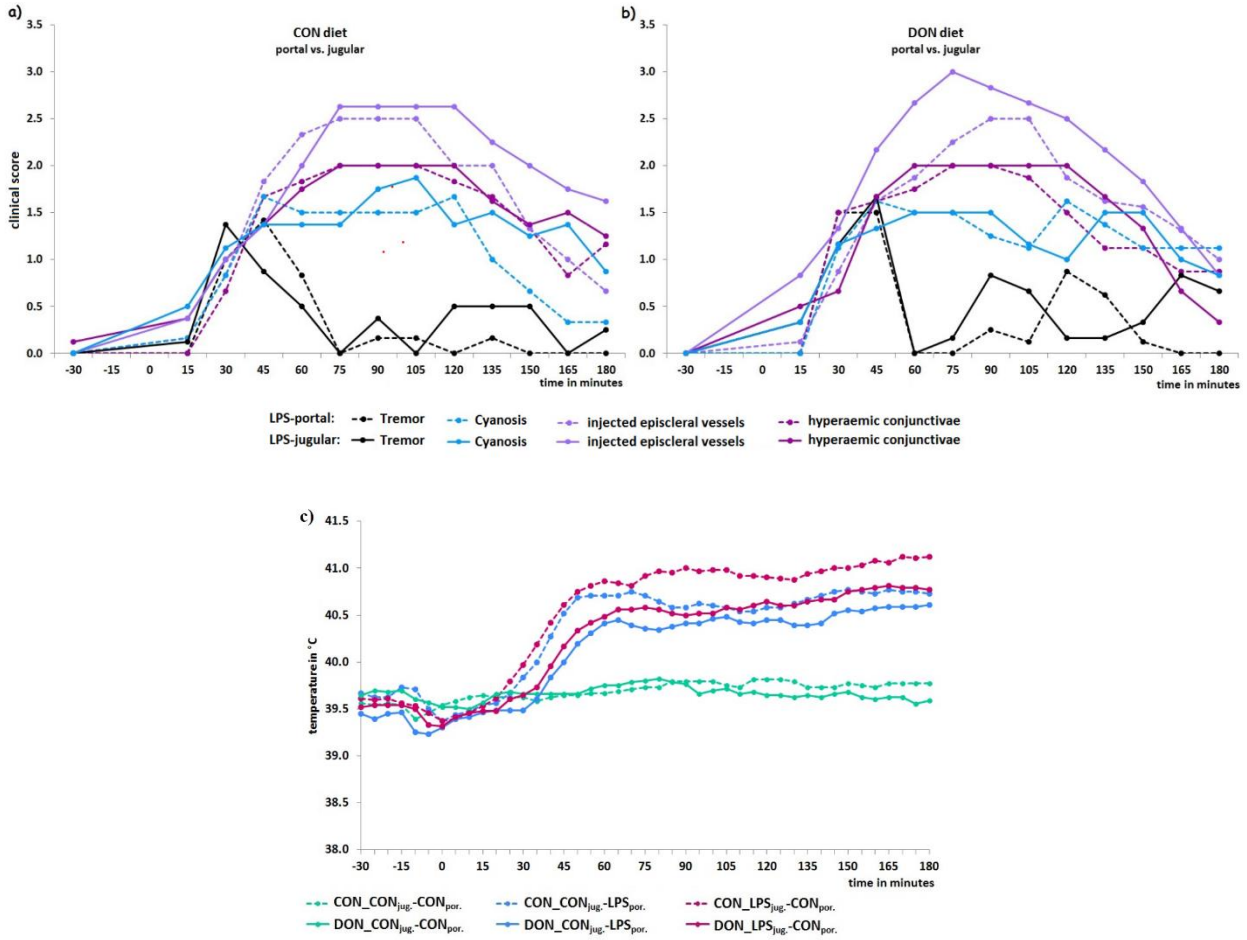


Fig. 6: LPS caused a sequential series of four key symptoms and the kinetics of those individual clinical signs is depicted in the two upper graphs for each dietary treatment. (**a**) showing CON-fed and (**b**) DON-fed groups. Tremor reached its highest level already between 30 and 45 min while the other symptoms showed their maximum degree between 75 and 105 min. Thereafter, from 120 min onwards, symptoms declined slowly to the base level. Data represent LSmeans ($PSEM_{Tremor}$ ± 0.07, $PSEM_{injected\ episcleral\ vessels/cyanosis}$ ± 0.15, $PSEM_{hyperaemic\ conjunctivae}$ ± 0.11) and statistical main effects were distributed as follows: cyanosis $p_{group} < 0.001$, $p_{time} < 0.001$, $p_{group \times time} < 0.01$, tremor/hyperaemic conjunctivae/injected episcleral vessels $p_{group} < 0.001$, $p_{time} < 0.001$, $p_{group \times time} < 0.001$. Body core temperature measurement (in 5 min intervals) for all experimental groups (depicted in **c**). All LPS-infused groups increased significantly and showed a hyperthermia in contrast to control-infused groups. DON-feeding had also a marked effect in LPS-infused animals and showed consistently lower temperature (~0.5 °C) as compared to their control-fed counterparts. Data represent LSmeans (PSEM ± 0.02) and statistical main effects were distributed as follows: $p_{group} < 0.01$, $p_{time} < 0.001$, $p_{group \times time} < 0.001$. Copied and adapted from Tesch et al. (2015). *Permission granted by MDPI publishing under the Open Access Creative Common CC BY license.* *https://doi.org/10.3390/toxins8010003*

feeding of mycotoxin contaminated feed and the post-hepatic infusion of LPS lead to an aggravation of the metabolic situation (Chapter 3). Immediately after the onset of the LPS infusion an increase of TNF-α, and a leukopenia based on neutropenia and lymphopenia was observed; characteristic for an APR (Tesch et al., 2015). In this respect no impact of the mycotoxin exposure or infusion site was detected. This was also the case for an important variable for the activation of the immune system, the kynurenine/tryptophan ratio (**Kyn/Trp;** Moffett and Namboodiri, 2003;

Schröcksnadel et al., 2006), which exhibited an increase at 180 min *post infusionem*, but no effect of the dietary treatment or infusion site (Tesch, 2017). This is in line with previous studies, showing no influence of an i.v. DON administration during an LPS challenge on these variables (Dänicke et al., 2013).

While the systemic imbalance caused by the immune response to LPS was clearly enhanced in the post-hepatic infused animals, this effect was not recorded on the clinical site. This leads to the assumption that the observed clinical variables may not be sensible enough to detect such small differences in the manifestation of an immune response; or *vice-versa* that the (minimal) differences in those variables are only measurable with laboratory instruments and have no influence on the intensity of the clinical signs. This is in line with clinical observations indicating certain metabolic and endocrinologic variables, such as serum lactate and calcitonin, constituting more reliable prognostic markers than the stand-alone clinical symptoms (Lee and An, 2016; Muller et al., 2000). The general interrelations between the clinical symptoms and metabolic and immunological variables are elaborately discussed in Tesch (2017).

As mentioned above, only in the CON treated pre-hepatic infused pigs a more rapid return to physiologically clinical levels was observed. This is in line with the severity of the disturbance of the metabolic traits, being more pronounced in the post-hepatic infused individuals. The greater decrease in systemic glucose availability in these animals lead more quickly to a state of exhaustion or fatigue, commonly observed in the later stage of an APR (Eikermann et al., 2006; Koluder, 2006). In the mycotoxin exposed animals this effect was however not obvious. A possible explanation for the different clinical course in these subjects could lie in the decreased systemic availability of glucose. The serum glucose decreasing effect observed in the DON-fed pigs is in line with previous studies that observed a decrease in intestinal glucose absorption after exposure to DON in different *in vitro* and *in vivo* models (Chapter 3; Awad et al., 2007; Halawa et al., 2012; Maresca et al., 2002). Kvidera et al. (2017a) have further illustrated that the decrease in systemic glucose concentration during an APR can be attributed not only to a decreased intestinal absorption of glucose, but also to a massive increase in glucose utilization by the immune system. By maintaining euglycemia through a continuous dextrose infusion in endotoxaemic pigs, the glucose demand during an APR was estimated to 1.1 g/kg $BW^{0.75}$/h (Kvidera et al., 2017b). This value has been further confirmed in other species (e.g. cattle) by this research group (Kvidera et al., 2016). Furthermore, a recent study illustrates the role of the iron-sequestering ferritin H chain (**FTH**) during sepsis in sustaining endogenous glucose production via liver gluconeogenesis (Weis et al.,

2017). An FTH overexpression or ferritin administration exerts a protective effect against the development of hypoglycemia during a sepsis event, thereby rendering disease tolerance. A possible interplay of DON in this context gives reason for further investigation.

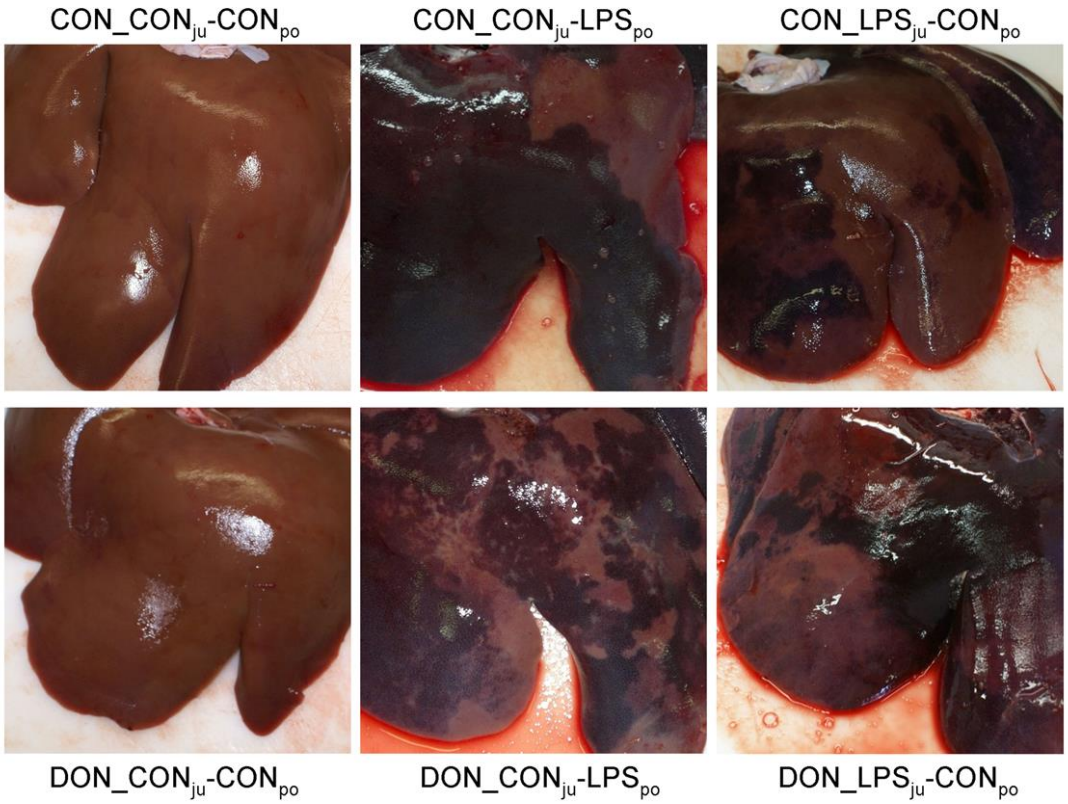
Hemorrhages in the liver together with a marked edema in the gallbladder were observed on a macroscopic level, with no differences between the entry site or mycotoxin exposure (Fig. 7; Renner et al., 2017). Furthermore, the degree of conjugation of DON and ZEN was decreased in the liver tissue of LPS infused animals, regardless of LPS-entry site (Chapter 4). This is in line with other studies illustrating a decreased conjugation of DON in endotoxaemic animals (Banhegyi et al., 1995; Dänicke et al., 2014; Sobrova et al., 2010) and our own measurements showing a decrease in uridine 5'-diphospho glucuronosyltransferase 1 A1 (**UDP-GT1-A1**) mRNA expression, independent of LPS-infusion site and dietary treatment (Renner et al., 2017). A shift of neutrophils from the blood stream to the liver further suggests an activation of the innate immune system to eliminate bacterial constituents such as LPS. Also, different variables for the evaluation of liver lesions like AST, γGT, ALP, and total bilirubin were increased upon the LPS-infusion (Renner et al., 2017). These massive macroscopic and functional alterations of liver and gallbladder highlight the role of the liver as detoxifying organ and main target for LPS and the LPS-induced local inflammation. The more pronounced systemic effect of an LPS infusion, when administered post-hepatically, on the metabolic level as well as on that of mycotoxin kinetics (see next chapter), illustrates the attenuating effect of the liver through the “first-pass” effect. These results are further complemented by the finding of a decreased capability of the liver to scavenge LPS in mycotoxin fed animals during our trial (Kahlert et al., 2019).

Although an immune-modulating effect of the chronic mycotoxin exposure was observed in the form of a leucopenia and additionally an influence on ALP and bilirubin levels was found, a mitigating effect during an APR, as observed in Stanek et al. (2012), was not demonstrated. These contradictory findings between the two studies need further investigation. Relevant aspects which warrant a distinct differentiation are the influence of other co-occurring mycotoxins in naturally contaminated diets which are often used in this kind of trials, and their possible interplay, as well as the exposure periods and levels (Döll and Dänicke, 2011; see chapter 1.2.3).

In our study setup we observed the initial immune response to a PAMP. The effects of a chronic mycotoxin feeding on the different sequential stages of an immune response (e.g. antibody built

up; Janeway et al. (2001) and recovery were, however, not captured. Future studies should include this aspect, as well as different disease scenarios to thrive practical applicability of the results.

a



b Relative liver weight (g/kg BW)

CON_CON$_{ju}$-CON$_{po}$	CON_CON$_{ju}$-LPS$_{po}$	CON_LPS$_{ju}$-CON$_{po}$	DON_CON$_{ju}$-CON$_{po}$	DON_CON$_{ju}$-LPS$_{po}$	DON_LPS$_{ju}$-CON$_{po}$
21.2 (± 1.6)^{a}	26.6 (± 2.0)^{b}	25.0 (± 3.1)^{b}	21.0 (± 1.8)^{a}	25.6 (± 3.3)^{b}	26.1 (± 1.7)^{b}

Fig. 7: (**a**) Representative photographs of livers, reflecting the common hepatic macroscopy in each experimental group. Hemorrhages are visible in all LPS-treated groups, but not in groups with saline infusion, irrespective of dietary treatment. (**b**) Relative liver weight (g/kg BW) in LPS-treated groups was significantly higher compared to saline-infused counterparts, irrespective of diet. Values are presented as LSMeans (±SEM), and those with uncommon superscripts are significantly different from each other ($p < 0.05$). Copied from Renner et al. (2017). *Permission granted by Springer Nature: Mycotoxin Research publishing under the Open Access Creative Common CC BY license. https://doi.org/10.1007/s12550-017-0279-9*

6.2. Influence of an LPS-Induced APR on Toxicokinetics of DON and ZEN

The LPS challenge also had, as hypothesized, a significant influence on the toxicokinetics of DON and ZEN and complemented the effects observed on systemic level (elaborately described and discussed in Chapter 4 and 5). In contrast to ZEN, the influx of DON with the liquid phase of the stomach contents into the blood circulatory system was very quick after feeding. Due to its hydrophilic nature, it was further rapidly distributed throughout the assessed body tissues. This was, however, influenced by the LPS challenge. The DON concentrations increased in the blood within 15 min in all infusion groups, but the area under the plasma DON concentration x time curve (AUC) was lower for DON, but not DOM-1, following LPS-infusions which indicates that the uptake from the GIT was decreased during the challenge until slaughtering 195 min after feeding. This can most likely be ascribed to a decreased perfusion of the GIT during the LPS challenge. Whereas DON exhibits short term kinetics due to its hydrophilicity, our results indicate that the kinetics of ZEN was defined by an accumulation in the enterohepatic pathway. We suggest that the content of the gallbladder was released into the GIT upon the LPS challenge (Renner et al., 2017), leading to alterations in the toxicokinetics of ZEN in this period. This was mirrored in an increase in α-ZEL in the lower GIT and also in higher portal blood plasma ZEN concentrations compared to the jugular plasma. At the intestinal site, a significant influence of the LPS-infusion site was observed with the proportion of α-ZEL being highest in the post-hepatic LPS-infused animals compared to their pre-hepatic and control infused counterparts, lining up with the above-named metabolic observations and emphasizing the role of the liver as main detoxifying organ and the "first-pass" effect in toxin metabolism.

6.3. Conclusions

To summarize our observations, the symptoms of an LPS induced APR were altered in animals chronically exposed to DON and ZEN, as well as aggravated when LPS was infused post-hepatically. These results confirm our hypothesis, that the systemic response to an endotoxin challenge is pronounced differently according to the entry route of LPS (pre- or post-hepatic) and that a chronic mycotoxin exposure has an influence on the magnitude of the APR.

On a practical level, these results imply, that a chronic exposure to DON and ZEN alters the initial inflammatory response, though depending on the LPS entry route for APR induction (pre- or post-hepatic). The enrichment of ZEN and metabolites in the enterohepatic cycle could furthermore

possibly enhance the negative effects of an inflammatory response due to a triggered release of ZEN in the systemic circulation upon an APR.

The guidance values for critical dietary concentrations of 0.9 mg DON and 0.1 mg (piglets, gilts), respectively 0.25 mg (sows, fattening pigs) ZEN per kg feed, set by the European Commission (2006), assume optimal housing and management situation, as well as an undisturbed health status as a prerequisite. In practice these ideal circumstances are, however, often not met/encountered. Therefore, the biological and practical relevance of our results need to be further investigated in, for example, study setups investigating these aspects in different stages of an immune response and/or course of infectious diseases or epidemiological studies, correlating mycotoxin exposure to the occurrence and severity of different infectious diseases in different housing and management settings.

7. References

(account for chapters 1. Background, 2. Aims of Study, and 6. General Discussion and Conclusion)

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8. Curriculum Vitae

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9. Eidesstattliche Erklärung / Declaration under Oath

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

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

Leipzig, 16.05.2022 Erik Bannert

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