PREDISPOSING EFFECT OF THE MYCOTOXINS DEOXYNIVALENOL AND FUMONISINS ON NECROTIC ENTERITIS IN BROILER CHICKENS

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“Learn how to see. Realize that everything connects to everything else.”

Leonardo da Vinci

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LIST OF ABBREVIATIONS

3ADON	3-acetyldeoxynivalenol
15ADON	15-acetyldeoxynivalenol
5-HIAA	5-hydroxyindoleacetic acid
5-HT	5-hydroxytryptamine
1-P	1-phosphate
αHF	alpha-hemolysin
ABA	aminobenzoic agent
ACN	acetonitrile
ADME	absorption, distribution, metabolism and excretion
AFs	aflatoxins
AFB_1	aflatoxin B_1
AGP	antibiotic growth promoters
AIMD	apparent ileal methionine digestibility
AMP	aerobic mesophilic bacteria
APEC	avian pathogenic *Escherichia coli*
AUC_{0-t}	area under the plasma concentration-time curve from time 0 to t
AXOS	arabinoxylan oligosaccharides
BLASTN	nucleotide basic local alignment search tool
β-PFT	β-pore forming toxin
BW	body weight
BWG	body weight gain
CaCo	human colon adenocarcinoma cell line
CKK	cholecystokinin
CCLi1	chemokines C-C motif ligand 1 inflammatory
Cl	total body clearance
CLDN3	claudin 3
CLDN4	claudin 4
CLDN5	claudin 5
Cl/F	clearance divided by the absolute oral bioavailability
C_{max}	maximal plasma concentration
Ct	treshold cycle values
CXCLi1	chemokine C-X-C motif ligand 1 inflammatory

CXCLi2	chemokine C-X-C motif ligand 2 inflammatory
CYP450	cytochrome P450
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DOM-1	de-epoxy-DON
DON	deoxynivalenol
DP	declustering potential
dsDNA	double-stranded DNA
ECMM	extracellular matrix molecules
ELEM	equine leukoencephalomalacia
$ERK_{1/2}$	extracellular signal regulated protein kinase 1 and 2
ETEC	enterotoxigenic *Escherichia coli*
EU	European Union
EUR	euro
F	oral bioavailability
FBs	fumonisins
FB_1	fumonisin B_1
FB_2	fumonisin B_2
FB_3	fumonisin B_3
FCR	feed conversion ratio
FHB	*Fusarium* Head Blight Disease
FOS	fructo-oligosaccharides
FRC	feed conversion rate
Fuc	fucose
Gal	galactose
GalNac	N-acetylgalactosamine
GlcNac	N-acetylglucosamine
GLUT	glucose transporter protein
GSH	glutathione
Hck	hematopoietic cell kinase
HFB_1	hydrolyzed FB_1
HIF-1α	hypoxia inducible factor 1 subunit alpha
HMOX	heme-oxygenase

HPLC-FL	high performance liquid chromatography with fluorescence detection
Hsp	heat shock protein
HT-29	human colonic and rectal adenocarcinoma cell line
HT-29 16E	human colonic goblet cell line
IARC	International Agency for Research on Cancer
IEC	intestinal epithelial cell
IFN-ɣ	interferon-γ
Ig	immunoglobulin
IgA	immunoglobulin A
IL	interleukin
IPEC	porcine IEC
IS	internal standard
Isc	current
IV	intravenous
$JNK_{1/2}$	c-Jun N-terminal kinase 1 and 2
k_{el}	elimination rate constant
LAB	lactic acid bacteria
LC-MS/MS	liquid chromatography-tandem mass spectrometry
LD_{50}	lethal dose for 50% of the subjects
LOAEL	lowest-observed-adverse-effect level
LOD	limit of detection
LOQ	limit of quantification
Man	mannose
MAPKs	mitogen-activated protein kinases
MCP-1	monocyte chemo-attractant protein-1
MDA	malondialdehyde
MDR1	multi-drug resistance protein 1 – P-glycoprotein
MOS	mannan-oligosaccharides
MRP2	multi-drug resistance-associated protein 2
mRNA	messenger ribonucleic acid
MRT	mean residence time
MS	mass spectrometry
MUC1	intestinal membrane-associated mucin
MUC2	secretory mucin

MUC3	secretory mucin
NE	necrotic enteritis
NELoc1	major pathogenicity locus
NetB	NE β-like toxin
NeuAc	N-acetyl-neuraminic acid
NIV	nivalenol
NSPs	non-starch polysaccharides
OPA	ortho-phthalaldehyde
OTA	ochratoxin A
OTU	operational taxonomic unit
PAM	porcine alveolar macrophage cell line
pBD1	porcine β-defensin 1
pBD2	porcine β-defensin 2
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PD	potential difference
$pHFB_1$	partially hydrolyzed FB_1
PKR	RNA-activated protein kinase
PO	*per os*
PPE	porcine pulmonary edema
PRRSV	porcine reproductive and respiratory syndrome virus
PYY	peptide YY
qRT-PCR	quantitative real-time polymerase chain reaction
R	microbial richness
RelF	relative oral bioavailability
RELMβ	resistin-like molecule beta
RFOS	raffinose family oligosaccharides
RNA	ribonucleic acid
RNS	reactive nitrogen species
ROS	reactive oxygen species
rRNA	ribosomal RNA
RSR	ribotoxic stress response
Rt-TEER	transepithelial electrical resistance
Sa	sphinganine

$SAPK_{1/2}$	stress-activated protein kinases
SD	standard deviation
SE	standard error
SEM	standard error of the mean
SEPEC	septicemic *Escherichia coli*
SFB	segmented filamentous bacterium
SGLT1	Na^+-dependent glucose transporter 1
SLC	solute carrier family
SLC3A1	rBAT – dimerizes with brushborder amino acid transporter $b^{0,+}AT$
SLC6A19	brushborder amino acid transporter B^0AT
SLC7A6	basolateral -amino acid transporters y^+LAT2
SLC7A7	basolateral -amino acid transporters y^+LAT1
SLC7A9	brushborder amino acid transporter $b^{0,+}AT$
SLC15A1	brushborder peptide transporter PepT1
SLC30A1	basolateral zinc transporter 1
So	sphingosine
SOCs	supressors of cytokin signaling
STEC	shigatoxin-producing *Escherichia coli*
$T_{1/2el}$	elimination half-life
$T_{1/2elIV}$	elimination half-life after IV administration
$T_{1/2elPO}$	elimination half-life after PO administration
TBARS	thiobarbituric acid reactive substances
TCA	propane-1,2,3,tricarboxylic acid
TEER	transepithelial electrical resistance
TGY	tryptone glucose yeast
Th1	type 1 T helper cell
Th2	type 2 T helper cell
TiO_2	titanium dioxide
TNF-α	tumor necrosis factor α
T_{max}	time to maximal plasma concentration
UPGMA	unweighted pair group method with arithmic means
US(A)	United States (of America)
V:C	villus to crypt ratio
Vd	volume of distribution

Vd/F	volume of distribution divided by the absolute oral bioavailability
VH	villus height
XDH	xanthine dehydrogenase
XO	xanthine oxidase
XOR	xanthine oxidoreductase
ZEA	zearalenone
ZEN	zearalenone
ZO-1	zona occludens 1

GENERAL INTRODUCTION

Partially based on:

Antonissen G., Martel A., Pasmans F., Ducatelle R., Verbrugghe E., Vandenbroucke V., Li S.-J., Haesebrouck F., Van Immerseel F., Croubels S. (2014) The impact of *Fusarium* mycotoxins on human and animal host susceptibility to infectious diseases. Toxins 6 (2): 430-452.

1 Broiler chickens

Broiler chickens (*Gallus gallus domesticus*) are a gallinaceous domesticated fowl, bred and raised specifically for meat production. Broiler strains used in intensive industrial farming systems are bred to be very fast growing. In general broilers reach their slaughter weight of 2.4 to 2.5 kg at between five to seven weeks of age, although slower growing strains reach slaughter weight at approximately 14 weeks of age. The specific broiler live weights farmers produce depend on the market in a specific country, region or the market segment that has to be supplied [129]. The estimated world broiler meat production in 2014 was 85.3 million tonnes [65]. Belgium accounts for 2.4% of the broiler production in the European Union (EU)[416]. Industrial poultry production is the fastest growing and most quickly changing segment of highly globalized livestock industry. Improvements in animal genetics, incubation and hatching, dietary formulations, health programs, bird management, and processing have made poultry meat the cheapest meat to produce [105]. By 2020, 124 million tonnes of poultry meat will be produced globally, being an increase of 25% in just a decade. The largest increase in production of chicken meat is expected in India, China, and Brazil. Growth of the broiler industry in the United States of America (USA) and the EU is forecast to be under the global average [64,65,357].

The extraordinary performance of the broiler meat producing industry over the past decades has coincided with increasing usage of concentrate feed, partially cereals and soybean meal. Since feed costs make up about 70% of the total cost of intensive poultry production systems, the availability of cheap feed is one of the most important factors affecting industry development [64]. Animal health and nutrition are interdependent. The interaction between the two is driven by feed-microbiota-host interactions in the gastrointestinal tract.

1.1 The gastrointestinal tract of broiler chickens

Gut health, an intricate and complex area combining nutrition, microbiology, immunology and physiology, has a key role to play in animal health [94,153,451]. The avian digestive tract starts at the beak and ends in the cloaca. The primary function of the gut is the conversion and digestion of feed into its basic components for absorption and utilization by the animal. In sequential order it is composed of an esophagus, crop, proventriculus, gizzard (ventriculus), small intestine (duodenum, jejunum, and ileum), and large intestine (caeca, colon and rectum) (Figure 1). The crop is an expansion of the cervical esophagus that functions as a feed storage organ. Absorption of nutrients and xenobiotics in esophagus or crop is minimal since these

segments are lined with incompletely keratinized stratified squamous epithelia. The avian stomach consists of two different parts: a proventriculus or glandular stomach and a ventriculcus or gizzard. The proventriculus secretes mucus, hydrochloric acid, and pepsinogen, whereas the gizzard functions in mechanical digestion and gastric proteolysis. The small intestine, which is subdivided in duodenum, jejunum and ileum, is the major site for enzymatic digestion and nutrient absorption in the avian gut. The duodenum starts from the pylorus and forms a loop that encircles the pancreas. Subsequently, the rest of the small intestine is subdivided into the jejunum and ileum with the Meckel's diverticulum as marker. The intestinal epithelial surface is characterized by the presence of villi, microvilli and crypts, which increase the surface area for absorption of nutrients and xenobiotics. The exocrine function of the pancreas and liver assists in nutrient digestion by secreting digestive enzymes, bile acids and salts in the duodenum. Both caeca are important in fermentation of vegetal matter and in water balance. The colon and rectum is found between the ileocecal junction and the cloacal coprodeum [60,94].

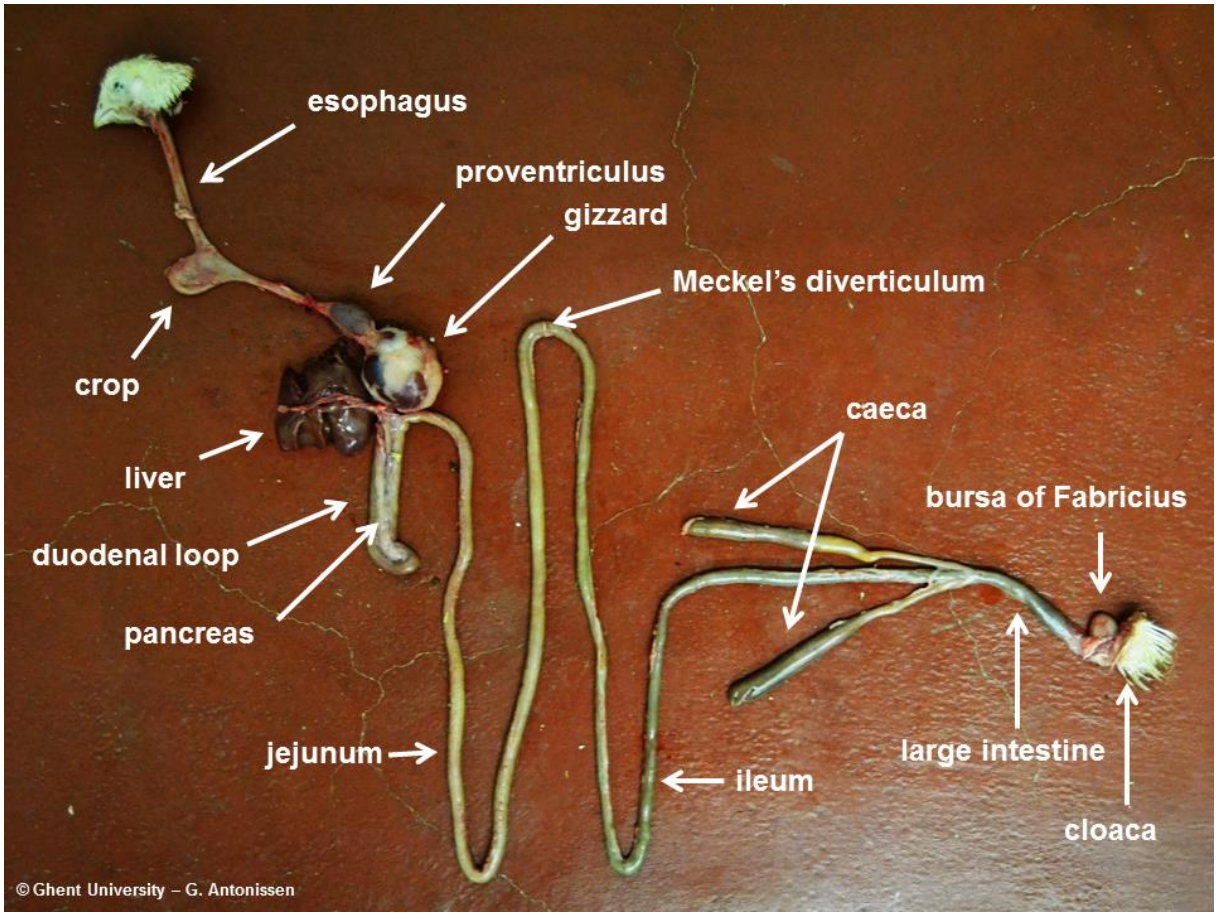

Figure 1. *Gastrointestinal tract of a three-week-old broiler chicken.*

2 Mycotoxins

2.1 The mycotoxin problem

Primary fungal metabolites, such as proteins, carbohydrates, nucleic acids, and lipids are essential for fungal growth. In contrast, secondary metabolites are not involved directly in the normal growth, development, or reproduction of the fungus. However, they may have an important role in ecological interactions with other organisms. Principally, these secondary metabolites are directed against substrate competitors, such as bacteria (antibiotics) or other fungal species (antimycotics), but may also protect the host plant from insect damage (insecticides). Therefore, many fungal secondary metabolites are of interest to the pharmaceutical, food, and agrochemical industries. On the other hand, other secondary fungal metabolites, mostly produced by saprophytic moulds, exhibit an intrinsic toxicity even at low concentrations, resulting in their collective classification as mycotoxins. The name mycotoxin is a combination of the Greek word for fungus ‘mykes’ and the Latin word ‘toxicum’ meaning poison. These low molecular weight compounds pose a potential health threat to humans and animals through ingestion of contaminated food or feed [139,383].

Conjecturally, there is historical evidence of mycotoxins affecting human and animal health back as far as the time reported in the Dead Sea Scrolls, noting destruction of “houses of mildew”. Although they have been included as the cause of the last of the Ten Plagues of Egypt, their historic occurrence and impact were not obvious until the Middle Ages, when ergot alkaloids poisoning outbreaks in Europe were responsible for the death of thousands of people. The onset of modern mycotoxicology was the discovery of aflatoxins in peanut meal incorporated in poultry feed in England in 1961, leading to Turkey X disease [342].

Mycotoxin producing fungi can be divided in two classes, namely field and storage associated fungi. Field fungi, such as *Fusarium* species, produce mycotoxins during growth in the field, whereas storage fungi, such as *Aspergillus* and *Penicillium* species, mainly produce mycotoxins after crop harvesting [138,327]. Many factors are involved in mycotoxin contamination. Climate represents the most important element in fungal colonization and mycotoxin production [316]. Temperature and relative humidity are considered as the main influencing climatological factors (Figure 2) [138,190].

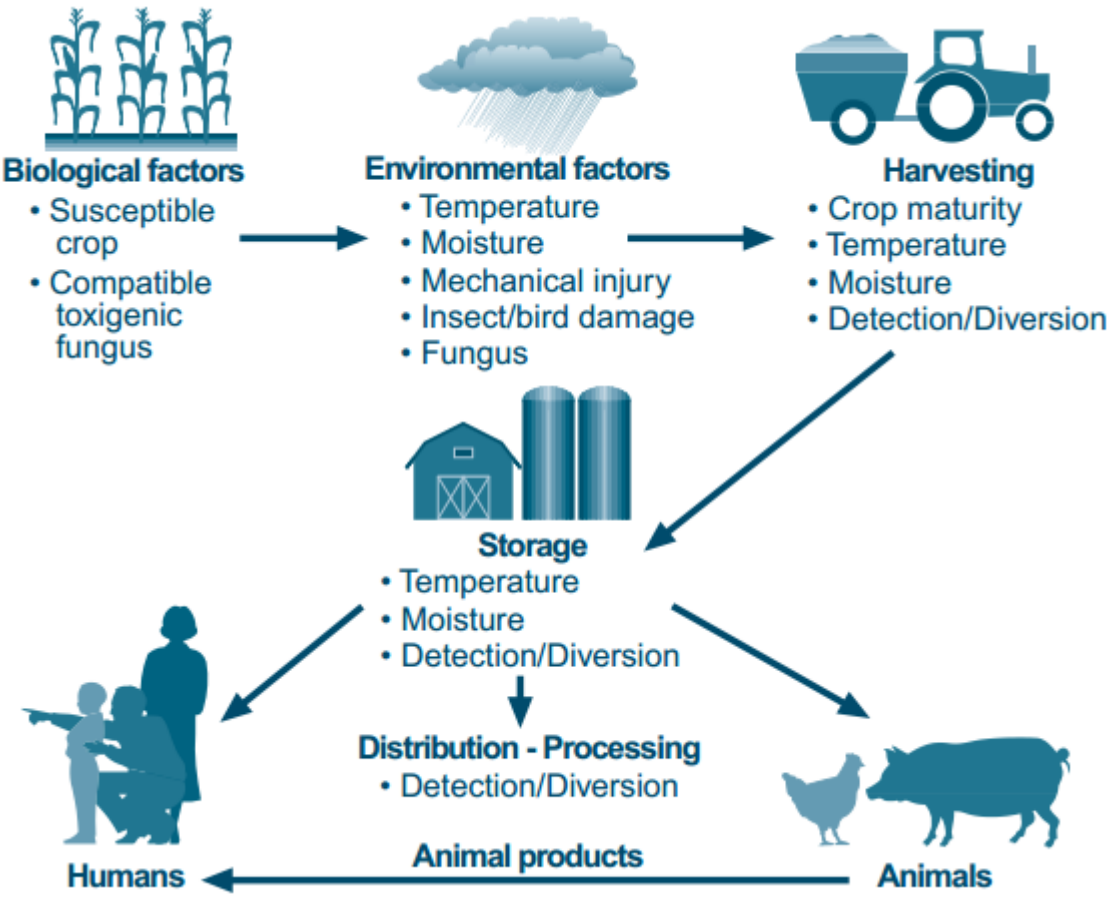

Figure 2. Factors affecting mycotoxin occurrence in the food and feed chain *(adapted from [62]).*

Since not all fungal growth results in mycotoxin contamination, detection of fungi is not necessarily associated with the presence of mycotoxins. Thanks to modern laboratory methods and growing interest in the research field of mycotoxicology, hitherto, more than 300 different mycotoxins have been identified [35,39,195]. Based upon their frequency of occurrence and detection, and/or severity of the disease they produce, some of these mycotoxins are considered as more important than others [39]. Mycotoxins of importance for the poultry industry are mainly produced by fungi of the genera *Aspergillus*, *Fusarium* or *Penicillium* [97]. From a global perspective, the most important mycotoxins or mycotoxin groups are aflatoxins (AFs), ochratoxin A (OTA), and three groups of *Fusarium* mycotoxins, i.e. trichothecenes, fumonisins (FBs) and zearalenone (ZEN or ZEA) [39,327]. The prevalence of different fungal species, and associated mycotoxins, is region dependent. *Fusarium* mycotoxins are the most frequently occurring mycotoxins on a worldwide basis. Schatzmayr and Streit [358] demonstrated in a long-term survey program from 2004 to 2012 that ZEN was present in 37% of all samples, with the highest prevalence in North Asia (56% positives, average contamination of 386 µg/kg feed). Deoxynivalenol (DON), a type B trichothecene, and FBs

were present in more than half of all samples worldwide, 56% and 54%, respectively. As shown in Figure 3, the prevalence of DON and FBs in European feed samples varied between 33-64% and 33-70%, respectively. The global average DON concentration of positive samples was 1,009 µg/kg feed, with a maximum of 50,289 µg/kg. Similarly, the global average FBs concentration of positives was 1,647 µg/kg, with a maximum of 77,502 µg/kg [358]. Based on the results of a long-term global mycotoxins survey program in feed and feed raw materials (Figure 3), it has been shown that OTA and AFs were most often detected in South Asia (55% and 78% positives; average contamination of 20 and 128 µg/kg feed, respectively). Notwithstanding high levels of mycotoxins were frequently detected, the majority of samples was compliant with the EU regulations and recommendations [131,132,358]. Furthermore, most fungal species are able to simultaneously produce different mycotoxins, feed materials can be contaminated by several fungi, and complete feed is made from various commodities [327,387].

Since mycotoxin production is highly connected to environmental factors such as temperature and humidity (Figure 2), mycotoxicology is likely to be affected by climate change [316]. Elsgaard *et al.* [122] predict a shift in the cropping patterns of corn and wheat in Europe due to climate changes. The production of corn is suggested to be characterized by generally increased cropping shares and a northwards expansion. Wheat is defined by divergent changes in cropping patterns, with increase in the northern parts and decrease in the southern parts of Europe. Climate change will induce a shift in composition of *Fusarium* species affecting cereal crops. Among *Fusarium* species associated with *Fusarium* Head Blight disease (FHB) in wheat, the prevalence of *F. graminearum*, the main DON producer, has already increased in Central Europe and is likely to increase in the North due to expected future weather conditions [251,273,313]. *F. verticillioides*, a producer of FBs, is currently the most common species on corn in Southern Europe. Since FBs have been associated with both dry weather during grain fill and late season rains, the production of these toxins will probably increase in Central and Northern Europe due to climate change [273,313].

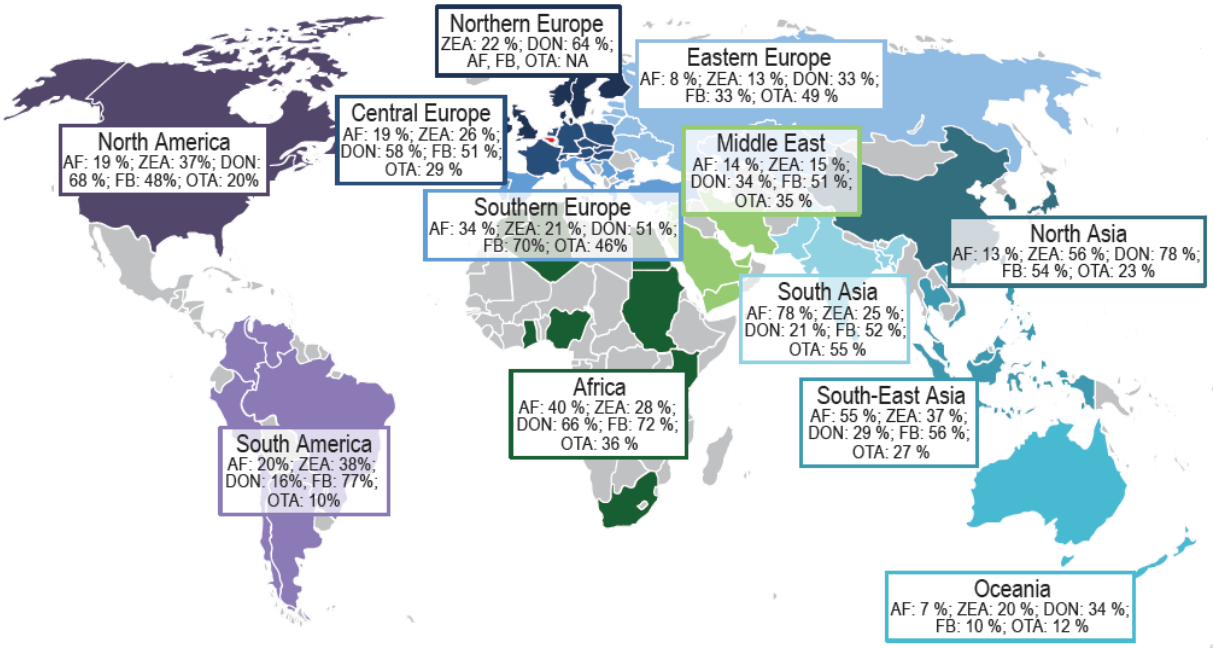

Figure 3. Global occurrence of aflatoxins (AFs), ochratoxin A (OTA), zearalenone (ZEA), deoxynivalenol (DON) and fumonisins (FBs). *Based on the analysis of 19,757 samples of feed and feed raw materials sourced globally in the period 2004-2012 (NA= not analysed) (based on [358]).*

As shown in Figure 3, DON and FBs are the most prevalent mycotoxins in our region, therefore this doctoral thesis will focus on these mycotoxins.

2.2 Deoxynivalenol and Fumonisins

2.2.1 Chemical structure

2.2.1.1 Deoxynivalenol

DON, also known as vomitoxin, is a mycotoxin synthetized by many species of fungi, mainly *F. graminearum* and *F. culmorum* [377]. It is a polar organic compound, which belongs to the type B trichothecenes and its chemical name is 12,13-epoxy-3α,7α,15-trihydroxytrichothec-9-en-8-one (Figure 4) [289]. All trichothecenes contain the sesquiterpene ring characterized by a 12,13-epoxy-trichothec-9-en nucleus, which is responsible for their toxicity [195,445]. According to their functional groups, trichothecenes are divided in four classes, i.e. A, B, C, and D. Members of group B differ from group A (e.g. T-2 toxin (T-2) and HT-2 toxin) by the presence of a carbonyl functional group at the C8 position. The most frequently detected mycotoxins of type B are nivalenol (NIV), DON and some derivatives as 3-acetyldeoxynivalenol (3-ADON), and 15-acetyldeoxynivalenol (15-ADON). Group C members have another epoxy group between the C-7 and C-8 or C-8 and C-9 positions, respectively (e.g. crotocin). Compounds in group D include a macrocyclic ring between C-4 and C-15 (e.g. satratoxin) [445].

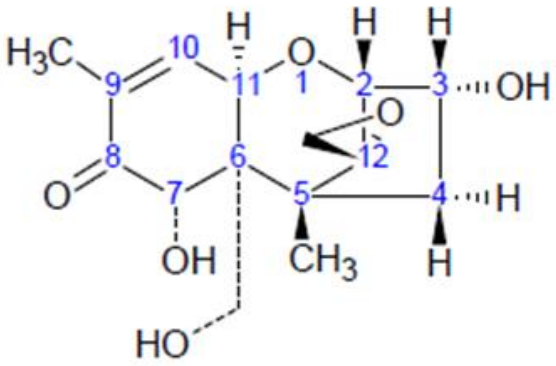

Figure 4. Chemical structure of deoxynivalenol (DON).

One of the most important physicochemical properties of DON is its ability to withstand high temperatures up to 350°C. Due to this stability during processing and cooking, DON can easily enter the food and feed chain with potential to cause human and animal toxicity [58,376]. The International Agency for Research on Cancer (IARC) has listed DON as group 3 substance (non-carcinogenic) [197].

2.2.1.2 Fumonisins

FBs are a group of structurally related secondary metabolites of *F. verticillioides* (formerly *F. moniliforme*), *F. proliferatum*, and other *Fusarium* species [152,431]. Fumonisin B_1 (FB_1), B_2 (FB_2), and B_3 (FB_3) are the most abundant of the more than 15 FBs analogues that have been described [194]. These different FBs have been categorized into four main categories (A, B, C, and P) [385]. However, toxicological assessment of FBs has mainly focused on FB_1, the most prevalent analogue. FB_1 is a diester of propane-1,2,3-tricarboxylic acid (TCA) and has a long-chain aminopolyol backbone, i.e. 2-amino-12,16-dimethyl-3,5,10,14,15-pentahydroxyeicosane (Figure 5) [194]. FB_2 and FB_3 are less prevalent, and differ structurally from FB_1 in the number and placement of hydroxyl groups, i.e. loss of a free hydroxyl group on C-10 or C-5 position, respectively [431]. The primary amino group is necessary for the biological activity of FBs, since acetylation of FB_1 to fumonisin A_1 blocks the cytotoxicity and the ability to inhibit ceramide synthase [194]. Alkaline hydrolysis and microbial degradation by FB carboxylesterase cleave the tricarballylic acid side chain of FB_1, leading to the less toxic metabolite hydrolyzed FB_1 (HFB_1) or aminopentol [172,431].

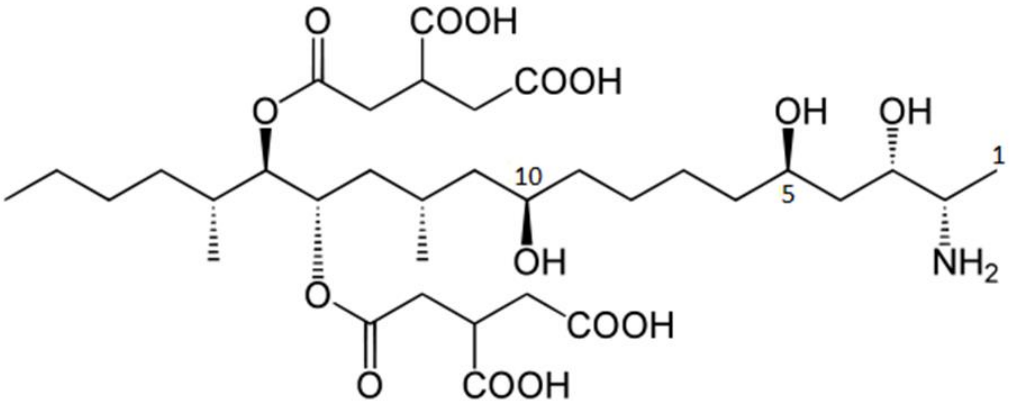

Figure 5. Chemical structure of fumonisin B_1 (FB_1)

FBs are relatively heat stable (up to 100-120°C) and therefore survive many of the conditions used in food and feed processing. Although decomposition of FBs begins at higher temperatures (≥ 190°C) [194]. For instance, N-(1-deoxy-D-fructos-1-yl) fumonisin B_1 (NDF) has been identified as thermal reaction product of FB_1 with glucose [329]. The IARC has classified FB_1 as a group 2B compound (possibly carcinogenic to humans) [197].

2.2.2 Toxicokinetics

2.2.2.1 Deoxynivalenol

Generally, the chicken is considered to be one of the farm animal species most resistant to the toxic effects of DON. Differential sensitivity might be related to differences in toxicokinetic properties (absorption, distribution, metabolism, and excretion (ADME)) among animal species [317,349]. A low absolute oral bioavailability (F) of 19.3% and 20.9% was observed in broiler chickens and turkey poults, respectively [98,300]. In sheep even a lower oral F has been reported (5.9-9.9%) [335]. In the pig, the animal species most sensitive for DON toxicity, the bioavailability was much higher after single oral bolus administration (54%) [170]. In pigs an increased systemic DON absorption is seen after chronic exposure (F= 89%) [170], while in broilers a decreased absorption is observed, due to intestinal morphological and functional adaptations [300,452]. After a single oral bolus administration of 0.75 mg DON/kg bodyweight (BW) to broiler chickens, DON is rapidly absorbed (time to maximal plasma concentration (T_{max})=0.58 h). Next, DON was rapidly eliminated after both oral (elimination half-life after oral intake ($T_{1/2el\ PO}$)= 0.64 h) and intravenous (IV) administration (elimination half-life after IV administration ($T_{1/2el\ IV}$) = 0.47 h) [300]. Compared to pigs a higher plasma clearance is observed in broilers after IV administration, 0.0038 L/min/kg versus 0.12 L/min/kg, respectively [170,300].

The gastrointestinal metabolism of DON includes the conversion of DON to various degradation derivatives. DON is de-epoxidized to de-epoxy-DON (DOM-1) by intestinal commensal bacteria [86,185]. In pigs and humans it was suggested that pre-exposure of the microbiota to DON is necessary to induce the bacterial detoxification activity [256]. Accordingly, in turkeys DOM-1 is detected only after chronic exposure to 4.0 – 6.5 mg DON/kg feed, and not after single oral bolus administration of 0.75 mg DON/kg BW [98,103]. In broiler chickens, no DOM-1 is detected in plasma after single oral bolus administration (0.75 mg DON/kg BW), nor after chronic feeding (7.5 mg DON/kg feed) [300,301]. The total concentration of DOM-1 is substantially higher in bile than in blood. DOM-1 is detected in higher concentrations in bile (51.6 ng/mL) after chronic exposure of broilers to 7.5 mg DON/kg feed [301]. Also in turkey poults, DOM-1 is detected in bile after chronic exposure to 5.4 mg DON/kg feed [90]. In contrast, plasma levels of DOM-1 in broilers are higher compared to DON after a single oral administration of feed naturally contaminated with DON (0.19 mg DON/kg BW) [454]. The DOM-1 proportion in broilers accounts for 7-11% of the total level of DON and DOM-1 in excreta of pullets. In contrast to pigs, no DOM-1 is detected in jejunal and ileal chyme [85].

Besides microbial mediated degradation, DON is also metabolized by phase II biotransformation in the intestinal mucosa and liver. Again, remarkable species differences in phase II biotransformation are responsible for differences in sensitivity to DON. Conjugation with glucuronic acid and sulfatation of DON and DOM-1 are considered as detoxification pathways which increase the water solubility, thereby facilitating their excretion via urine and bile. Glucuronic acid or sulphate are enzymatically transferred to a hydroxyl group of DON at C-atoms 3, 7 and 15 [86]. Glucuronidation of DON has been identified as a major biotransformation pathway in mammals [265]. In contract, DON-3α-glucuronide is only a minor phase II metabolite in broilers and turkey poults. It seems that poultry biotransform DON more extensively to the sulphate conjugate, such as DON-3α-sulphate [98,434].

2.2.2.2 Fumonisins

In different avian and mammalian species it has been observed that FB_1 is poorly absorbed after oral exposure. In laying hens, only 0.71% (F) is absorbed after oral administration of 2 mg [14C]FB_1/kg BW [432]. In turkeys and ducks, also a low F was demonstrated after dosing 100 mg FB_1/kg BW, namely 2-2.3% and 3.2%, respectively [398,399]. Similar oral bioavailability can be found in mammals, i.e. 2-4% of oral administered dose in rats, monkeys and pigs [262,334,364]. No FB_1 could be recovered in plasma of Holstein cows administered an oral bolus of 1 or 5 mg FB_1/kg BW [333]. In contrast to DON, the higher sensitivity of pigs to FBs compared to poultry is not due to differences in oral absorption. Furthermore, the poor intestinal absorption of FBs has been designated as the "fumonisin paradox", or how a toxin can induce liver failure although it is not effectively absorbed after oral intake [366].

In pigs, FB_1 shows higher systemic distribution when compared to avian or other mammalian species. After IV administration of 0.4 mg [14C]FB_1/kg BW to pigs, a volume of distribution (Vd) of 2.41 L/kg was observed [334]. Poultry species on the other hand show a lower Vd after IV administration of FB_1 compared to pigs, and also differences between avian species exist. The Vd of laying hens was lower compared to ducks and turkeys, namely 0.08, 0.8 and 1 L/kg, respectively [398,399,432]. FBs are also rapidly eliminated in all animal species [431]. Different poultry species can be ranked in order of increasing total body clearance (Cl) as follows: laying hens, turkeys and ducks, with a Cl of 1.18, 7.6, and 19.3 mL/min/kg, respectively. Consequently, the elimination half-life was the much longer in laying hens ($T_{1/2el\ IV}$ = 40-69 min, $T_{1/2el\ PO}$ = 60-173 min), and turkeys ($T_{1/2el\ IV}$ = 85 min, $T_{1/2el\ PO}$ = 214 min), compared to ducks ($T_{1/2el\ IV}$ = 26 min, $T_{1/2el\ PO}$ = 70 min) [398,399,432]. Specific toxicokinetic data of FBs in broilers are lacking.

Following oral intake, FB_1 can be converted by intestinal microbial degradation, through the use of FB carboxylesterases, to partially hydrolyzed FB_1 ($pHFB_1$) or full HFB_1 by cleavage of the tricarballylic acid esters at the C-14 and/or C-15 position [172]. In pigs fed 45 mg FB_1/kg BW for 10 days, 41%, 47% and 12% of total FB1 in faeces were recovered as FB_1, $pHFB_1$ and HFB_1, respectively [142]. In rodents and pigs a reduced intestinal and hepatotoxicity of HFB_1 compared to FB_1 was observed after oral administration [72,172,192]. There are no data regarding avian ability to hydrolyze FBs. In contrast to DON, there is currently no evidence concerning phase II biotransformation of FBs in the intestinal mucosa and liver [63,380].

2.2.3 Toxicodynamics

2.2.3.1 Deoxynivalenol

The underlying molecular mechanisms of toxicity of DON and other trichothecenes to eukaryotic cells is characterized by their ability to target ribosomes [256,317,418,458]. DON binds to the 60S ribosomal subunit through the reaction of the epoxide group of DON with the nucleotides forming ribosomal RNA (rRNA) [256,318]. It is suggested that only rRNA possesses both the correct spatial organization and chemical functions, present either on nucleotides or rRNA-associated proteins, allowing their selective interaction with DON [256]. The mechanism of translation inhibition by DON is characterized by impairment of the peptidyl transferase function, consequently affecting protein synthesis at the initiation, elongation or termination level [318,346]. Furthermore, the binding of DON to ribosomes can rapidly activate mitogen-activated protein kinases (MAPKs) and induce cell apoptosis by ribotoxic stress response (RSR) [198]. It is hypothesized that DON enters the cell and binds to active ribosomes, leading to the activation of RNA-activated protein kinase (PKR) and hematopoietic cell kinase (Hck), which then activate MAPKs (Figure 6) [256,317]. MAPKs phosphorylate specific serines and threonines of target proteins substrates and regulate cellular activities ranging from immunomodulation, proliferation, gene expression, mitosis, differentiation, and apoptosis [70,112,205]. The primary MAPK subfamilies include: p44 and p42 MAPKs or extracellular signal regulated protein kinase 1 and 2 (ERK 1/2), p54 and p46 c-Jun N-terminal kinase 1 and 2 (JNK 1/2) or stress-activated protein kinases (SAPK 1/2), and p38 MAPK [70,317]. The type of activated MAPK is dose dependent. In macrophages, low doses (nM) activate preferentially ERK, causing cell survival and increased immune gene expression, whereas high doses (µM) activate p38 leading to apoptosis, rRNA cleavage and protein synthesis inhibition. At low doses, DON has been shown to regulate the expression of various genes involved in the innate immunity and the inflammatory reactions through selective transcription, mRNA stabilization and translational regulation [69,183,184,278,279].

In addition to the effect of PKR, Hck and MAPKs, it was recently demonstrated that DON affects the phosphorylation of 188 proteins, including proteins involved in transcription, epigenetic modulation, cell cycle, RNA processing, translation, ribosome biogenesis, cell differentiation and cytoskeleton organization [311,312].

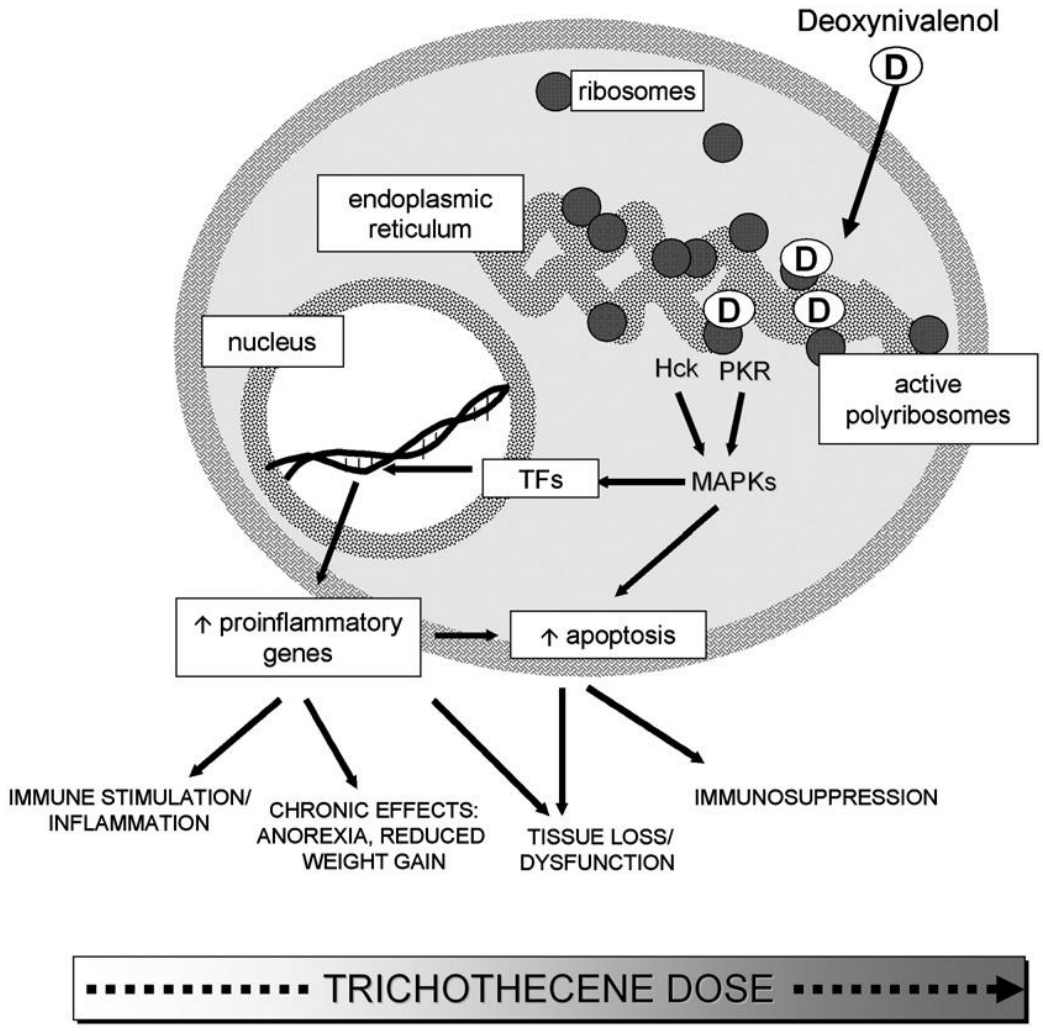

Figure 6. Molecular mechanism of action of DON and other trichothecenes. *DON enters cell and binds to active ribosomes, inducing the activation of RNA-activated protein kinase (PKR) and hematopoietic cell kinase (Hck). Subsequently, phosphorylation dependent mitogen-activated protein kinases (MAPKs, a.o. ERK 1/2, JNK 1/2, p38) modulate transcription factor (TF) activation. Depending on exposure level of DON, this MAPKs activation induces an increased pro-inflammatory cytokine gene expression or cellular apoptosis resulting in anorexia and reduced weight gain, immune stimulation and inflammation, tissue loss and dysfunction, and immunosuppression (adapted from [317]).*

2.2.3.2 Fumonisins

FBs have a clear structural similarity to the long-chain base backbones of sphingolipids. Hence, FBs competitively inhibit sphinganine N-acyl transferase (ceramide synthase) and consequently disrupt the ceramide and sphingolipid metabolism (Figure 7) [270,344,436]. The primary biological consequences of ceramide synthase enzyme blockage are inhibition of sphingolipids synthesis, and an accumulation of free sphinganine (Sa), and to a lesser extent of sphingosine (So) in tissues, serum and urine [385,431]. The accompanying increase in the Sa:So ratio in tissues and body fluids following FBs exposure is a suitable biomarker of exposure in humans and animals [34,337,431]. This biomarker can be useful for confirming FBs exposure in farm animals, however, changes are reversible, and dose and time dependent [431]. Accumulated sphingoid bases are subsequently phosphorylated by kinase enzymes to their 1-phosphate (1-P) metabolites, which also accumulate. Sa 1-P and So 1-P exert their biological effect by acting as a ligand for G-protein coupled cell surface receptors. These receptors are critical for extracellular signal transduction, mitosis, regeneration and cell migration modulating diverse processes including immunomodulation and cell to cell adhesion [431].

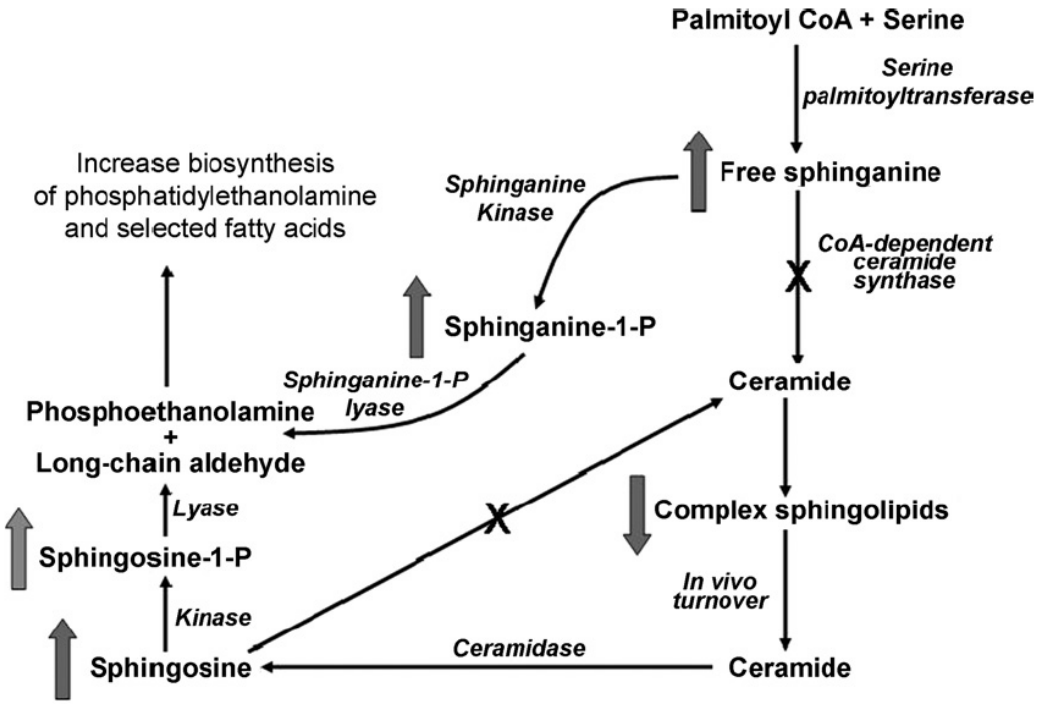

Figure 7. Disruption of sphingolipid metabolism by FBs. *The FBs-induced inhibition of ceramide synthase (X) results in an increased tissue and serum concentration of sphinganine, sphingosine and their 1-phosphate metabolites and a decreased level of complex sphingolipids (adapted from [431]).*

2.2.4 Impact on broiler performance

2.2.4.1 Deoxynivalenol

The acute form of DON mycotoxicosis rarely occurs in poultry flocks under field conditions [17]. In broiler chickens the lethal dose for 50% of subjects (LD_{50}) is set at 140 mg DON/kg BW [193]. However, chronic exposure to DON at low or moderate levels can cause anorexia, diarrhea, decreased bodyweight gain (BWG) and altered nutritional efficiency [319]. A reduced feed intake, BWG and feed efficiency was observed in broilers fed a diet contaminated with a moderate level of DON (10-15 mg DON/kg feed) for 3 to 6 weeks [20,88,157,448]. Under experimental conditions, no adverse effects on performance parameters have been demonstrated at contamination levels below the European maximum guidance level of 5 mg/kg feed [16,131].

DON may affect animal feed intake and weight gain by modulating central and peripheral neuroendocrine control mechanisms [42,323]. The increased activity of the central serotoninergic system may play a role in the DON-induced anorectic response [42,140]. Serotonin or 5-hydroxytryptamine (5-HT) is synthesized and released from the enterochromaffin cells in the gastrointestinal tract, and acts in a paracrine pattern on the terminals of vagal efferent neurons of the enteric nervous system. This action can affect both neuronal signaling and downstream secretion of both anorexigenic and orexigenic hormones. Serotonin from the gut might also enter into the circulation where it can evoke central effects [318,323]. In rodents, it has been shown that DON impairs gastric emptying and gut motility. The impact of DON on gut motility in rats and mice is inhibited by the selective $5HT_3$ receptor antagonists ondansetron and granisetron, implying the involvement of this receptor [140,323]. In broiler chickens, feeding a *Fusarium* mycotoxins contaminated diet induced an increase of 5-HT and its main metabolite 5-hydroxyindoleacetic acid (5-HIAA) concentrations in the pons and cortex. In contrast to pigs, higher cerebral levels of norepinephrine were observed in broilers after DON exposure, antagonizing the effect of serotonin on appetite suppression [391]. Furthermore, it has been demonstrated in mice that DON-induced cytokine upregulation corresponds to an increased expression of several suppressors of cytokine signaling (SOCs), associated with impairment of growth hormone signaling [7]. DON also induces the release of satiety hormones, a.o. peptide YY (PYY) and cholecystokinin (CCK) [141,447]. In a mouse model, it was recently described that in addition to its peripheral action, DON can reach the brain after oral administration, and impair centrally the anorexigenic/orexigenic balance [161]. Besides, DON-induced decreased zootechnical parameters can also be associated with the modulation of intestinal functions [171].

2.2.4.2 Fumonisins

In general, poultry are relatively resistant to FBs toxicity when compared to pigs and horses [202,225,230,397,409,438]. The LD_{50} of FB_1, when injected into the air cell of embryonating broiler chickens eggs, was determined to be 18.7 µg per egg. Comparative embryotoxicity assessment demonstrated a higher toxicity of FB_1 compared to FB_2 or FB_3 [187]. Liver, kidneys and the intestinal tract are target organs of FBs toxicity in most animal species [48,171,431]. Feeding a FB_1 contaminated diet (100-400 mg/kg feed) to broiler chicks caused black, sticky diarrhea during the first 2 weeks of life [230].

In poultry important differences in sensitivity towards the deleterious effects of FBs are observed depending on the age and species. Increased mortality due to FB_1 has only been observed in broiler chickens during the first three days of life (≥125 mg/kg feed) [202], and in growing ducks of 12-14 weeks old (20 mg/kg feed) [397]. No mortality has been recorded in laying hens, turkeys or older broiler chickens fed high doses of FB_1 (≥ 200 mg/kg feed) for several weeks [225,229,230,438].

The presence of high concentrations of FBs in broiler feed (≥ 100 mg/kg) affects performance. Feeding broiler chicks a FBs contaminated diet in doses ranging from 100 to 400 mg FB_1/kg feed for 2 to 3 weeks induced a dose dependent decrease in BWG and feed intake [202,230]. Under experimental conditions, no negative effects on performance parameters have been observed at contamination levels below the European maximum guidance level of 20 mg FB_1+FB_2/kg feed [131]. However, the increased Sa:So ratio in broilers fed 20 mg FB1/kg feed for three weeks, clearly demonstrated animal toxicity at this level [188].

2.2.5 Effect of DON and FBs at the gastrointestinal level

2.2.5.1 Cell viability, proliferation, and intestinal morphology

Following the ingestion of DON or FBs contaminated food or feed, the intestinal epithelial cells (IEC) can be exposed to high concentrations of these toxins [47]. The low oral bioavailability of DON and FBs in poultry [98,300,398,399,432] highlights the importance of the gastrointestinal tract as target organ. It has been suggested that DON can reach the enterocytes in the more distal regions of the intestine via the systemic circulation [256]. DON and FB_1 have been shown to negatively affect intrinsic components of the intestinal barrier and the viability of human and animal enterocytes [45,106,212,223,258,272,344,359,363,425]. Furthermore, studies on porcine IEC (IPEC-1 and -J2) and human colon adenocarcinoma (Caco-2 and HT-29) cells have shown that actively dividing cells are more sensitive compared to fully differentiated cells [45,272,425]. Chronic ingestion of DON (3 mg/kg feed) and FBs (6 mg/kg feed) in piglets for

5 weeks, decreased the number of mitotic figures in jejunal enterocytes [50]. This higher sensitivity of proliferating cells is probably associated with the inhibition of protein synthesis by DON and, subsequently, nucleic acid synthesis [43,425]. Proliferating cells are more sensitive than confluent cells to toxic effects of FBs, because there is a continuous dynamic turnover of sphingolipids in growing cells as well as an ongoing demand for their *de novo* synthesis [272].

Morphometric analyses of histological alterations have shown that by affecting enterocyte viability and proliferation, DON and FBs affect the intestinal morphology in exposed animals. The small intestinal epithelium is continuously regenerated by proliferating cells that migrate up the villi. These cells differentiate, and apical microvilli and absorptive functions develop during their movement toward the tip of the villi [371]. In contrast to mammals, proliferation of enterocytes in poultry is not confined to the crypt, but can also occur along the villus [419]. Reduced villus height in the duodenal and jejunal segment was observed in broiler chickens after 3-6 weeks feeding a DON contaminated diet (7.5-10 mg DON/kg feed) [18,303]. Three weeks feeding DON at 5 mg/kg feed reduced only duodenal villus height and crypt depth [19]. A linear correlation was suggested between increasing levels of DON and the decrease in villus height in duodenum and jejunum [453]. A decreased villus height was also observed in the jejunum of broiler chickens fed high concentrations of FBs (>100 mg FB_1/kg feed) [57,340]. In pigs exposed to FBs for 9 days (1.5 mg FB_1/kg BW), however, ileal villi tended to be longer [235]. No effect on villus height or crypth depth was observed after feeding piglets FBs (6 mg/kg feed) for 5 weeks. No additive or synergistic effects of DON and FBs were shown on intestinal morphological changes in pigs [50].

2.2.5.2 Epithelial integrity

The integrity of epithelial intercellular structures, including tight junctions, adherence junctions and desmosomes, is crucial for sealing the intestinal barrier. These tight junctions regulate the paracellular transport, which refers to the transfer of substances across the epithelium by passing through the intercellular space between the cells [47]. DON and FBs have shown to modulate the intestinal paracellular transport, leading to an increased passage of macromolecules and bacteria (Figure 8 and 9). The measurement of passage of tracers, such as fluorescein isothiocyanate-dextran, and the transepithelial electrical resistance (TEER) of *in vitro* cell monolayers or *ex vivo* intestinal explants are considered as good indicators of epithelial integrity and the degree of organization of the tight junctions [258,322]. Several *in vitro* studies using Caco-2 or IPEC-1 cells indicate that long term exposure to DON and FB_1 decreases TEER and increases paracellular permeability of tracers [45,212,258,363,420]. These alterations are associated with an altered expression of specific tight junction proteins (Figure

8). DON treatment of IPEC-1 or IPEC-J2 cells led to disintegrated zona occludens 1 (ZO-1) structures and a decreased total amount of ZO-1, claudin 3 (CLDN3), and claudin 4 (CLDN 4) [106,322]. As a result of intestinal damage, a compensatory up-regulation of mRNA expression of tight junction protein claudin 5 (CLDN5) was observed in the jejunum of broilers fed DON (7.5 mg/kg feed) for 3 weeks [303]. Furthermore, it has been shown *in vivo* and *ex vivo* that DON and FBs, alone or in combination induced changes in intestinal junction complexes and in E-cadherin and occludin protein expression [31,50]. The impact of DON can be explained by the fact that the structure and function of these tight junction proteins can be regulated by signaling molecules involved in MAPKs pathways [264], which can be activated by DON [318]. The molecular mechanism underlying FB_1 disruption of epithelial integrity remains to be investigated. Consequently, the impaired intestinal integrity could lead to the translocation of luminal antigens and bacteria, including *Escherichia coli*, that are normally restricted to the gut lumen [306,322]. Loss of intestinal integrity can also affect absorption of toxins and drugs, such as antibiotics doxycycline and paromomycin [169].

2.2.5.3 The intestinal mucus layer

The surface of the intestinal epithelium is lined with a layer of mucus, produced as a result of mucin secretion by goblet cells. Mucins are a group of glycoproteins of which the mucus lining is composed. They protect the epithelium in several ways [325]. The mucus layer is a component of the extrinsic barrier and forms a physical barrier against mechanical, chemical and physical aggressors from the intestinal lumen. Furthermore, in this layer, mucus-associated microbiota can be established. Some of these commensal mucus-associated bacteria have a positive impact on host immunity, nutrient digestion, and may influence detoxification of ingested xenobiotics, including mycotoxins [321]. A few studies suggest that DON and FBs may affect the intestinal mucus production. Goblet cell hyperplasia was observed in broiler chickens and piglets exposed to FB_1 (300 and 30 mg/kg feed, respectively) [57,326]. A reduction of the number of mucus-producing goblet cells in jejunum and ileum was seen in piglets fed a DON (3 mg/kg) or a DON+FBs (3 and 6 mg/kg, respectively) contaminated diet for 5 weeks. No effect was observed in piglets fed a FBs contaminated diet [50]. Recently, it was demonstrated that subtoxic doses of DON decreased mucin production in human colonic goblet cells (HT-29 16E) and porcine intestinal explants. This inhibition was due to a specific decrease in the level of mRNA encoding for the intestinal membrane-associated (MUC1), the secretory mucins (MUC2 and MUC3), and resistin-like molecule beta (RELMβ), which regulates intestinal mucus secretion. The effect of DON on the mRNA expression of mucins and RELMβ relies on the activation of PKR and MAPK p38 [321].

2.2.5.4 Production of antimicrobial peptides

Antimicrobial peptides are present at the intestinal epithelial surface and serve as another innate defense mechanism. These peptides are cationic molecules and act by disrupting the integrity of the microbial membranes [47,52]. Defensins are a highly evolutionarily conserved group of antimicrobial peptides which are present in mammals, birds, invertebrates and plants. Three subfamilies of defensins exist, α, β and θ defensins. Thirteen avian β-defensins or gallinacins have been described. Furthermore, also three different cathelicidins or fowlicidins have been described in chickens [52]. Exposure of porcine intestinal epithelial cells (IPEC-J2) to DON and FBs increased their mRNA expression of porcine β-defensins 1 and 2 (pBD1 and pBD2). Although, no decreased level of secreted pBD1 and pBD2 proteins could be demonstrated, a decreased antimicrobial activity was observed against *E. coli* in the supernatant of mycotoxin treated cells [435]. To the best of our knowledge, no reports are available studying the effect of mycotoxins on intestinal antimicrobial peptides in poultry.

2.2.5.5 Cellular oxidative stress

Additionally, both DON and FBs may affect cell homeostasis and viability by the induction of cellular oxidative stress (Figure 8). Oxidative stress is defined as an imbalance between the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS), on the one hand, and the ability to readily detoxify or repair the resulting damage, on the other hand [121,274]. Disturbances in the intracellular redox equilibrium may cause toxic effects through oxidative injuries, such as oxidation of proteins, lipid peroxidation, and DNA damage leading to genotoxicity [126,263]. Oxidative stress might play a role in DON- and FBs-induced toxicity by increased production of ROS and lipid peroxidation after DON and/or FBs exposure [274,328,455]. However, differences exist between both mycotoxins in the importance of oxidative stress as contributor to their toxicity. Along with aflatoxin B_1 and T-2 toxin, FBs are classified as moderate oxidant mycotoxins, while DON is classified as a non-oxidant mycotoxin [121]. Assessment of the production of malondialdehyde (MDA) in human colon adenocarcinoma (Caco-2) cells following mycotoxin exposure, revealed that FB_1 has a higher capability to induce lipid peroxidation compared to DON [223]. The accumulation of sphingoid bases induced by FBs stimulate the production of ROS [111]. Feeding a FB_1 contaminated diet (100 mg/kg feed) for 21 days to broiler chickens increased the hepatic level of thiobarbituric acid reactive substances (TBARS), an indicator of lipid peroxidation [328]. Moreover, it has been suggested that FBs increase the susceptibility of cells to lipid peroxidation because the reduction in complex sphingolipids contributes to membrane dysfunction resulting in cellular damage [1]. In contrast to the increased expression of heat shock protein (Hsp) 70, a

cytoprotectant, following FBs exposure [150,350], no such an effect was observed with DON [36,121]. Accordingly, DON did not alter plasma and liver MDA level of broiler chickens fed a diet containing 10 mg DON/kg for 17 days [145]. Osselaere *et al.* demonstrated an up-regulation of mRNA of sensitive markers of oxidative injury, i.e. hypoxia inducible factor 1 subunit alpha (HIF-1α) and hemeoxygenase (HMOX), in the jejunum of broilers fed a DON contaminated feed (7.5mg/kg) for 3 weeks [303]. Similarly, DON increased the TBARS level in jejunal tissue of broilers exposed to 10 mg DON/kg feed for 5 weeks [21]. In conclusion, recent research could suggest that also the oxidative pathway may be one of the mechanisms by which DON and FBs induce intestinal toxicity.

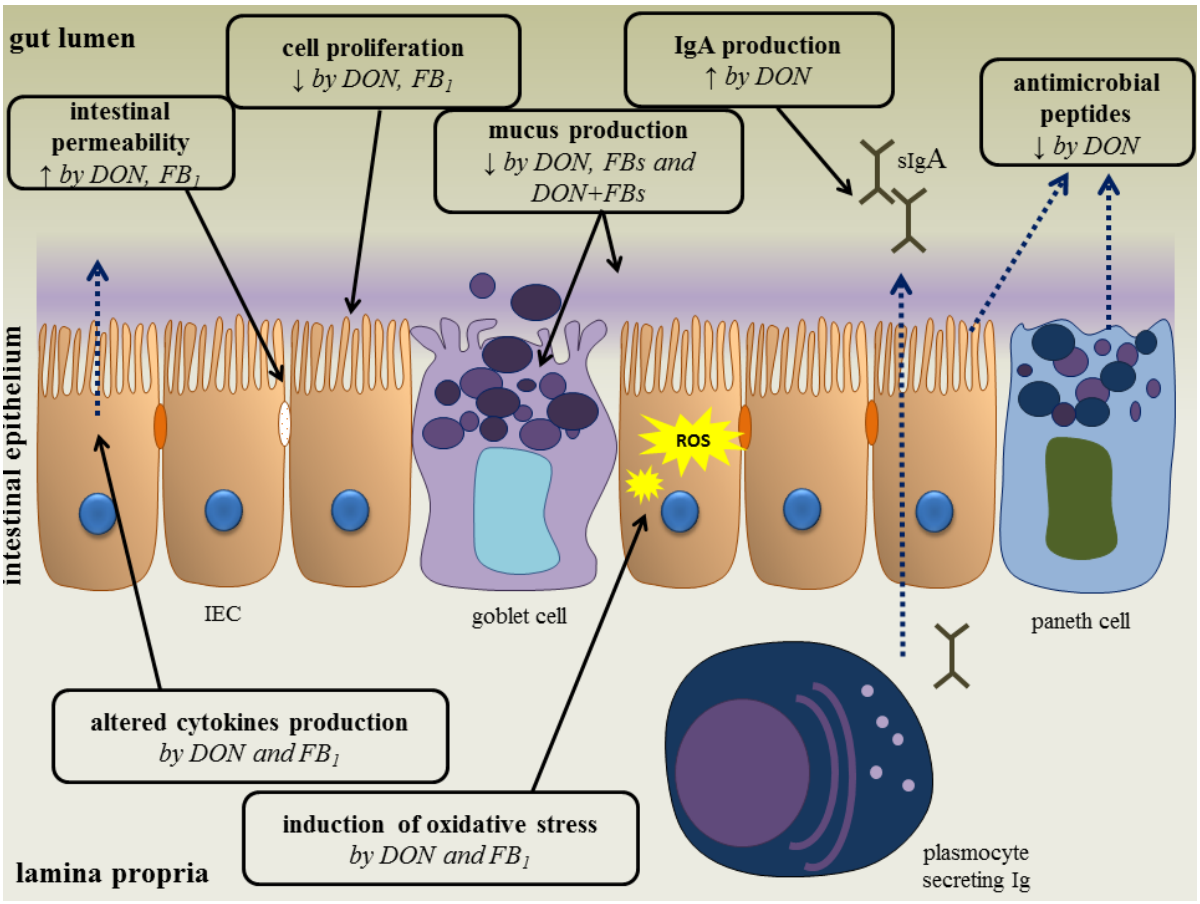

Figure 8. Overview of the effects of DON and FBs on the intestinal cell-derived innate immune response*. DON and FBs alter the different intestinal defense mechanisms including epithelial integrity, cell proliferation, mucus layer, antimicrobial peptides, immunoglobulins (Ig), cytokine production and induction of oxidative stress (IEC: intestinal epithelial cell) (based on [47]).*

2.2.5.6 Modulation of digestive and absorptive processes

DON and FBs also modulate digestive and absorptive processes [171]. The activity of aminopeptidase N was lower in piglets fed 1.5 mg FB_1/kg BW for 9 days compared to control [235]. This enzyme is a zinc-dependent membrane-bound exopeptidase that degrades preferentially proteins and peptides with a N-terminal neutral amino acid [396]. Additionally, aminopeptidase N has shown to be a molecular target of cholesterol absorption inhibitors in the enterocyte brush border membrane [224]. Plasma cholesterol was increased in pigs that were chronically exposed to FB_1, probably as a result of alterations in the hepatic lipid metabolism [108,282]. DON decreased glucose absorption by inhibiting the active Na^+-dependent glucose transporter 1 (SGLT1) in broiler chickens (Figure 9) [23-25]. DON exposure of human intestinal (HT-29) cells revealed that SGLT1 is the most DON-sensitive transporter, followed by the passive fructose transporter GLUT5 [258]. However, passive sugar transporters of the GLUT family, such as GLUT2, were only slightly affected by DON [27,258]. Since this transporter mainly mediates the basolateral exit of glucose, unlike SGLT1 which is attached on the apical membrane, GLUT2 would not be as exposed to DON (Figure 9) [171].

It has been shown that DON also affects intestinal amino acid uptake. An up-regulation of the SLC7A10 transporter was observed in the liver and jejunum of broiler chickens fed a DON contaminated diet (5 mg/kg feed) for 23 days [107]. The SLC7A10 protein transports D- and L-serine, and several other neutral amino acids [290]. In contrast, in HT-29 cells the active and passive L-serine uptake was inhibited by DON [258]. This difference could suggest a direct inhibition of the L-serine transporter by DON, resulting in the up-regulation of SLC7A10 [107]. Based on the electrophysiological changes in the jejunal mucosa of laying hens following addition of L-proline on the luminal side, it is suggested that DON inhibits the Na^+-amino acid co-transport [26]. Furthermore, also a decreased mRNA expression of transporters of palmitate and monocarboxylates (e.g. butyrate, lactate and pyruvate) was observed in the jejunum of DON exposed (5 mg/kg feed) broilers [107].

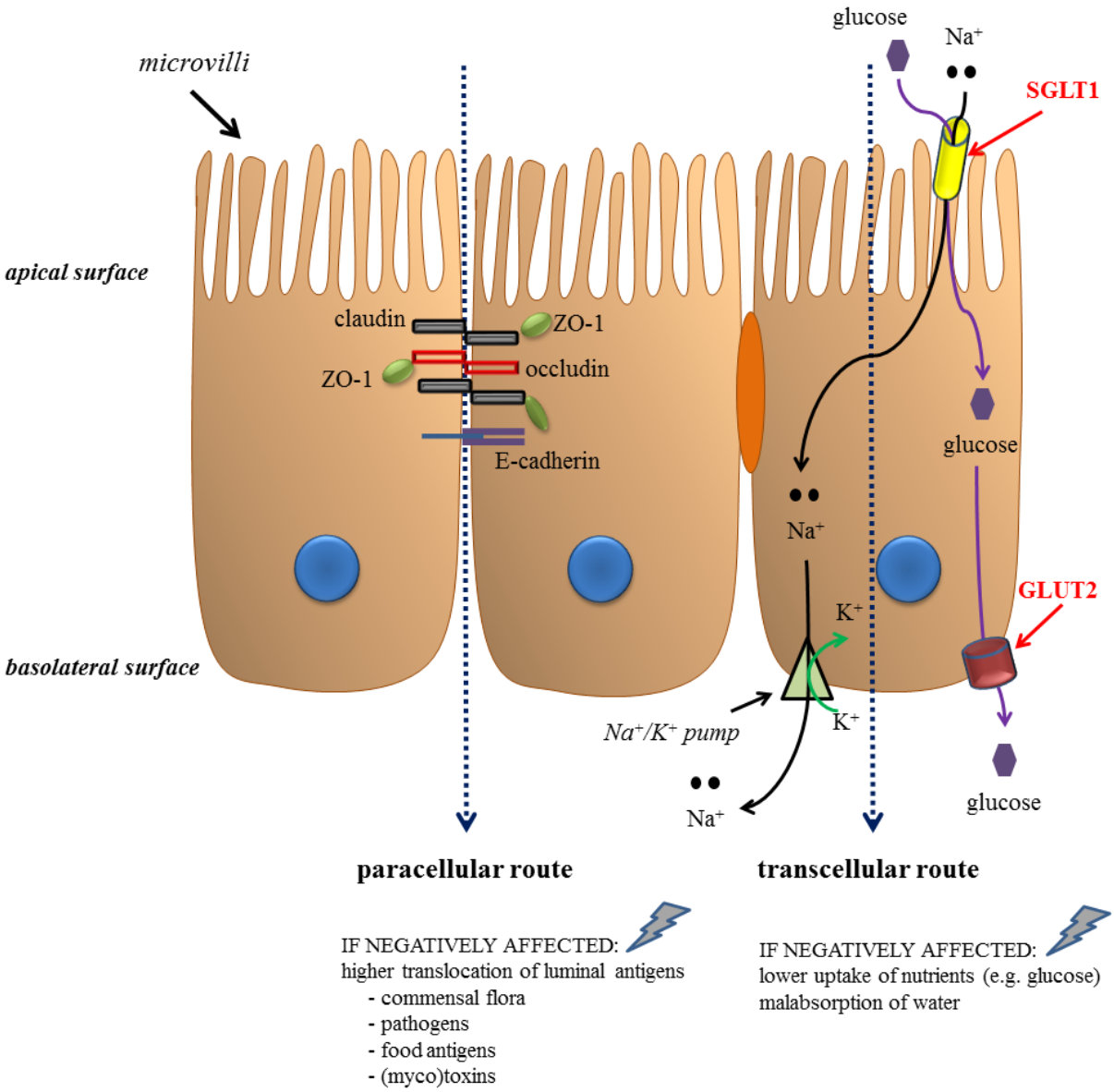

Figure 9. DON and FBs modulate intestinal epithelial transcellular and paracellular transport. *The left side of the figure represents the tight junction complex involved in the paracellular route of absorption. It is a draft of the tight junction complex, including the proteins claudin, occludin and zonula occludens-1 (ZO-1). Additionally, E-cadherin also participates to cell adhesion. The right side of the figure represents the glucose transport through the transcellular route of absorption. The main apical transporter for active glucose uptake in small intestine is the Na^+-dependent glucose transporter 1 (SGLT1). The basolateral transporter GLUT2 facilitates diffusive transport of intracellular glucose into bloodstream (adapted from [171]).*

2.3 Mycotoxin management

Three main strategies can be used to help prevent the toxic effects of mycotoxin-contaminated food and feed. Firstly, prevention of fungal infections, and subsequent mycotoxin production, during plant growth, harvest, storage and distribution. Therefore, a range of recommended strategies are based on Good Agricultural Practice and Good Manufacturing Practice, a.o. planting varieties of cereals that are more resistant to fungal infection, appropriate measures toward insect invasions, appropriate field management, optimal harvesting procedures, and optimal storage conditions [39,201,208,222]. Secondly, in spite of the efforts to prevent mycotoxin contamination by the agricultural industry, the absence of mycotoxins in rations of farm animals cannot be fully assured. In Europe, maximum levels for products intended for animal feed production have been set by the European Commission (Table 1). Several post-harvest approaches are described for the mycotoxin detoxification of animal feed. The physical method is mostly based on either removal of the contaminated commodities or inactivation of the toxins present in these commodities. Physical treatment includes for example mechanical sorting and separation, density segregation, washing, polishing, flotation, milling, thermal inactivation, extrusion, and irradiation. Decreasing mycotoxin levels by mixing batches of feed and the use of chemical decontamination processes are not authorized within the EU [222]. Biological detoxification methods involve microbial enzymatic degradation or modification of the mycotoxins [208,266]. Physical, chemical and biological treatments of contaminated feed are generally not efficient, and are also expensive [207]. The last strategy, inhibition of mycotoxin absorption in the gastrointestinal tract by *in vivo* decontamination using feed additives, is frequently applied in livestock. Thes additives are called mycotoxin detoxifying agents. Their extensive usage has led to the establishment of a new category of feed additives in 2009: “substances for reduction of the contamination of feed by mycotoxins: substances that can suppress or reduce the absorption, promote the excretion of mycotoxins or modify their mode of action” [133]. These additives are divided in two categories; adsorbing agents and biotransforming agents. Adsorbent materials diminish the absorption of mycotoxins from the gastrointestinal tract by physically or chemically binding these toxins. Ideally, this complex does not dissociate in the gastrointestinal tract, resulting in an efficient elimination via faeces and hereby preventing or minimizing exposure of animals to mycotoxins. Mycotoxin biotransforming agents or modifiers, such as bacteria, yeast, fungi and enzymes, biotransform mycotoxins in less toxic metabolites [222]. Further reviews were published by Kolosova and Stroka [222], and Devreese *et al.* [100].

Table 1. The guidance values on the presence of DON and FBs in feed material and finished feed for animals, as determined in the Commission Recommendation 2006/576/EC [131].

Mycotoxin	**Feed materials / finished animal feed**	**Maximum levels** (µg/kg)
Deoxynivalenol (DON)	*feed materials:*	
	cereals and cereal products with the exception of maize by-products	8,000
	maize by-products	12,000
	complementary and complete feedingstuffs:	
	all animal species with the exception of [(2)]	5,000
	[(2)]complementary and complete feedingstuffs for pigs	900
	[(2)]complementary and complete feedingstuffs for calves (< 4 months), lambs and kids	2,000
Fumonisins (FB_1+FB_2)	*feed materials:*	
	maize and maize products	60,000
	complementary and complete feedingstuffs:	
	complementary and complete feedingstuffs for pigs, horses (*Equidae*), rabbits and pet animals	5,000
	complementary and complete feedingstuffs for fish	10,000
	complementary and complete feedingstuffs for poultry, calves (< 4 months), lambs and kids	20,000
	complementary and complete feedingstuffs for adult ruminants (> 4 months) and mink	50,000

2.4 The impact of *Fusarium* mycotoxins on animal host susceptibility to infectious diseases

A wealth of research papers clearly indicate a negative influence of *Fusarium* mycotoxins on the intestinal function and immune system. Since the intestinal tract is a major portal of entry to many enteric pathogens and their toxins, mycotoxin exposure could increase the animal susceptibility to these pathogens. Furthermore, mycotoxin-induced immunosuppression may also result in decreased animal or human host resistance to infectious diseases. Tables 2 and 3 attempt to summarize the impact of *Fusarium* mycotoxin exposure on the animal and human host susceptibility to infectious diseases. More specifically, the effect of *Fusarium* mycotoxins on enteric, systemic and respiratory infectious diseases in livestock animals and animal models for human diseases are highlighted.

Table 2. Interaction between Fusarium mycotoxins and infectious diseases: in vitro approach.

mycotoxin	exposure dose	exposure period	cell line (host species)	pathogen	effect	reference(s)
DON or T-2	> 25 ng DON/mL or 5 ng T-2/mL ≥ 0.75 µg DON/mL or ≥ 2.5 ng T-2/mL	24 h	undifferentiated IPEC[1]-J2 differentiated IPEC[1]-J2 (pig)	*Salmonella* Typhimurium	↑ invasion	425,429
DON or T-2	0.5 µg DON/mL or ≥ 1.0 ng T-2/mL	24 h	differentiated IPEC[1]-J2 (pig)	*Salmonella* Typhimurium	↑ translocation	425,429
DON or T-2	0.025 µg DON/mL or 1 ng T-2/mL	24 h	PAM[2] (pig)	*Salmonella* Typhimurium	↑ invasion	426,429
DON	5- 50 µM (1.5 - 15 µg/mL)	48 h	IPEC[1]-J1 (pig)	*E. coli* (SEPEC)[3]	↑ translocation	322
T-2	0.001 µM	6 h	peritoneal macrophages (mouse)	*P. aeruginosa*[4]	↓ phagocytosis	430
T-2	0.01- 0.05 µM	20 h	alveolar macrophages (rat)	*S. cerevisiae*[5]	↓ phagocytosis	156
T-2	0.1 µM	6 h	alveolar macrophages (rat)	*S. aureus*[6]	↓ phagocytosis	156
T-2	1-5 ng/mL 2-5 ng/mL	24 h	HD-11 cell line[8] (chicken)	*A. fumigatus*[7]	↓ phagocytosis ↑(A) immune response ↑ germination	239

DON=deoxynivalenol; T-2=T2-toxin

[1]IPEC=Intestinal Porcine Epithelial Cell, [2]PAM=porcine alveolar macrophage, [3]septicemic Escherichia coli, [4]Pseudomonas aeruginosa, [5]Saccharomyces cerevisae, [6]Staphylococcus aureus, [7]Aspergillus fumigatus, [8]chicken macrophages

(A)= increased gene expression of pro-inflammatory cytokines interleukin (IL)-1β, IL-6, IL-18, IL-12β and chemokines C-C motif, ligand 1 inflammatory (CCLi1), C-X-C motif ligand 1 inflammatory (CXCLi1); and C-X-C motif ligand 2 inflammatory (CXCLi2)

Table 3. The influence of Fusarium mycotoxins on infectious diseases in animals: in vivo approach.

mycotoxin	**exposure dose**	**exposure period**	**animal species**	**age**	**pathogen**	**effect** compared to negative control	**reference(s)**
DON, 15-ADON, ZEN and fumonisins	6.5 mg DON, 0.44 mg 15-ADON, 0.59 mg ZEN and 0.37 mg fumonisins/kg feed	6 w	chicken (broiler)	1 d	*E.*[1] *maxima*	↓ percentage of $CD4^+$ and $CD8^+$ T cells in jejunal mucosa	[163]
DON, 15-ADON and ZEN	3.8 mg DON and 0.3 mg 15-ADON and 0.2 mg ZEN/kg feed	10 w	chicken (broiler)	1 d	*E.*[1] *acervulina, E.*[1] *maxima, E.*[1] *tenella*	↓ level of blood monocytes at end of challenge period percentage of $CD8^+$ T-cells not restored at end of recovery period ↑ IFN-γ gene expression	[164]
DON, 15-ADON and ZEN	3.8 mg DON, 0.3 mg 15-ADON and 0.2 mg ZEN/kg feed	10 w	chicken (broiler)	1 d	*E.*[1] *acervulina, E.*[1] *maxima, E.*[1] *tenella*	↓ intestinal recovery: duodenal villus height and apparent villus surface area	[162]
DON, 3-ADON and ZEN	5.5 mg DON, 0.17 mg 3-ADON and 0.26 mg ZEN	3 w	chicken (pullet)	13 w	*A.*[2] *galli*	↑ faecal excretion of *A. galli* eggs ↓ proportion of pullets with *A. galli* antibodies	[85]
DON	1 µg/mL	6 h	pig	5 w	*Salmonella* Typhimurium	synergistic ↑ gene expression IL-12, TNF-α, IL-1β, IL-8, MCP-1 and IL-6	[425]
T-2	15 and 83 µg/kg feed	23 d	pig	3 w	*Salmonella* Typhimurium	↓ colonization of the cecum	[429]
FB_1 and FB_2	8.6 mg FB_1 and 3.2 mg FB_2/kg feed	9 w	pig	4 w	*Salmonella* Typhimurium	synergistic transient effect digestive microbiota balance	[59]

[1]Eimeria (E.), [2]Ascaridia (A.), s.a.=single administration, DON= deoxynivalenol, 3- and 15-ADON= 3- and 15-acetyldeoxynivalenol, T-2= T-2 toxin, ZEN= zearalenone, FB_1= fumonisin B_1, FB_2= fumonisin B_2, FB_3= fumonisin B_3, IFN-γ=interferon γ, IL=interleukin, TNF-α= tumor necrosis factor α, MCP-1= monocyte chemoattractant protein-1

Table 3 (cont.): The influence of Fusarium mycotoxins on infectious diseases in animals: in vivo approach

mycotoxin	**exposure dose**	**exposure period**	**animal species**	**age**	**pathogen**	**effect** compared to negative control	**reference(s)**
T-2	2 mg/kg BW	2 d	chicken (broiler)	1 d	*Salmonella* Typhimurium	↑ mortality	[460]
T-2	1 mg/kg BW	3 w	mouse	5 - 6 w	*Salmonella* Typhimurium	↑ mortality	[394]
T-2	1 mg/kg BW	10 d	mouse	5 - 6 w	*Salmonella* Typhimurium	↑ bacteria-related organ lesions	[393]
T-2	2 mg/kg BW	s.a.	mouse	-	*Salmonella* Typhimurium	↑ mortality	[430]
DON	1 mg/L drinking water	3 w	mouse	7 w	*Salmonella* Enteritidis	↑ translocation to mesenteric lymph node, liver and spleen	[179]
FB_1	150 mg/kg feed	6 w	Japanese quail	1 d	*Salmonella* Gallinarum	↑ clinical signs and mortality ↓ blood lymhocyte number	[96]
FB_1	0.5 mg/kg BW	6 d	pig	3 w	*E. coli* (SEPEC)[3]	↑ intestinal colonization ↑ translocation to the mesenteric lymph node, lung, liver and spleen	[306]
FB_1	1 mg/kg BW	10d	pig	3-4 w	*E. coli* (ETEC)[4]	intestinal infection prolonged impaired function of intestinal antigen presenting cells	[104]
fumonisins and aflatoxin	[a]50 - 350 ng fumonisins /mL and 1 - 3 ng aflatoxin/mL	-	calf	< 1m	*E. coli* (STEC)[5]	↑ susceptibility to hemorrhagic enteritis	[29]

BW=bodyweight, [3]septicemic Escherichia coli, [4]enterotoxigenic Escherichia coli, [5]shiga toxin producing Escherichia coli, [a]mycotoxin level detected in the hemorrhaged mucosa

Table 3 (cont.): The influence of Fusarium mycotoxins on infectious diseases in animals: in vivo approach

mycotoxin	**level of exposure**	**exposure period**	**animal species**	**age**	**pathogen**	**effect** compared to negative control	**reference(s)**
moniliformin	75 - 100 mg/kg diet	3 w	chicken (broiler)	0 d	*E. coli* (APEC)[6]	↓ bacterial clearance	242
moniliformin and FB_1	100 mg moniliformin and 200 mg FB_1/kg diet	3 w	turkey	0 d	*E. coli*[3] (APEC)[6]	↓ bacterial clearance	241
DON	5 - 10 mg/kg feed	10 w	channel catfish	juvenile	*E. ictaluri*[7]	↓ mortality	254
T-2	1 - 2 mg/kg	6 w	channel catfish	juvenile	*E. ictaluri*[7]	↑ mortality	255
FB_1, FB_2 and FB_3	20 mg FB_1, 3.5 mg FB_2 and 1.9 mg/kg feed	42 d	pig	3 d	*M. hyopneumoniae*[8]	↑ severity of the pathological changes	331
FB_1	10 mg/kg feed	24 d	pig	3 d	*B. bronchiseptica*[9] *and P. multocida*[10] *(type D)*	↑ extent and severity of the pathological changes	330
FB_1	0.5 mg/kg BW	7 d	pig	piglets	*P. multocida*[10] *(type A)*	↓ growth rate and ↑ coughing ↑ total number of cells, number of macrophages and lymphocytes in bronchoalveolar lavage ↑ gross pathological lesions and histopathological lesion of lungs	178

BW=bodyweight, [6]avian pathogenic Escherichia coli, [7]Edwardsiella ictaluri, [8]Mycoplasma hyopneumoniae, [9]Bordetella bronchiseptica, [10]Pasteurella multocida

Table 3 (cont.): The influence of Fusarium mycotoxins on infectious diseases in animals: in vivo approach

mycotoxin	**exposure dose**	**exposure period**	**animal species**	**age**	**pathogen**	**effect** compared to negative control	**reference(s)**
T-2	0.1 mg/mouse ≈ 3.3 mg/kg BW	20 d	mouse	adult	*M. tuberculosis*[11]	↑ bacterial count in spleen	211
T-2	0.1 mg/mouse ≈ 3.3 mg/kg BW	20 d	mouse	adult	*M. bovis*[12]	↓ mouse survival time	211
T-2	0.5 mg/kg BW	21 d	rabbit	-	*A.fumigatus*[13]	↓ phagocytosis by alveolar macrophages	293
T-2	0.93 mg/kg feed	25 d	chicken (broiler)	0 d	*A.fumigatus*[13]	pre-exposure of *A. fumigatus* to T-2 increased severity aspergillosis in chickens fed T-2 diet	240
T-2	2 mg/kg BW	s.a.	mouse	-	*P. aeruginosa*[14]	↓ phagocytosis by peritoneal macrophages	430
DON	25 mg/kg BW	s.a.	mouse	7-10 w	reovirus (serotype 1)	↓ viral clearance and ↑ fecal shedding ↓ T-helper (Th)1 response by ↓ IFN-γ expression ↑ intestinal IgA and ↑ Th2 response: by ↑ IL-4, IL-6 and IL-10 gene expression	237
T-2	1.75 mg/kg BW	s.a.	mouse	7-10 w	reovirus (serotype 1)	↓ viral clearance and ↑ fecal shedding ↓ Th1 response by ↓ IFN-γ gene expression	238
FB1	12 mg/kg BW	18	pig	1 m	PRRSV[15]	↑ histopathological lesions of lungs	338

BW=bodyweight, s.a.=single administration, [11]Mycobacterium tuberculosis, [12]Mycobacterium bovis, [13]Aspergillus fumigatus, [14]Pseudomonas aeroginosa, [15]PRRSV = Porcine Reproductive and Respiratory Syndrome Virus, IgA= immunoglobulin A

In recent years, research investigating the effects of *Fusarium* mycotoxins on the intestinal and immune functions has made substantial progress. However, only limited information is available on the interaction between mycotoxins and infectious diseases. The aforementioned literature data (Table 2 and 3) indicates that *Fusarium* mycotoxins may influence the animal and human host susceptibility to enteric, systemic and respiratory infectious diseases. Depending on host, pathogen and mycotoxin characteristics, exposure to *Fusarium* mycotoxins can generally exacerbate infectious diseases. On the other hand, T-2 has been shown to decrease the colonization capacity of *Salmonella* in the pig intestine. *Fusarium* mycotoxins may influence the host–pathogen interaction by negatively affecting the intestinal barrier function and the innate and adaptive immune response [41,47,304]. *Fusarium* mycotoxins affect the morphology and barrier function of the intestinal layer [47], leading to increased translocation of different bacterial species including *Salmonella enterica* and *E. coli*, to the systemic compartment. Moreover, the negative influence of these mycotoxins on the function of macrophages results in impaired phagocytosis of bacterial and fungal pathogens. However, also the adaptive immune response is targeted, demonstrated by the effect on gene expression of several cytokines, leading to an altered Th1 and Th2 response.

The economic impact of mycotoxins on animal production is generally considered to be mainly due to losses related to direct effects on animal health and trade losses related to grain rejection [443]. It is clear, however, that the indirect influence of myocotoxins on animal health, by enhancing infectious diseases, should also be taken into account. These effects occur even at low to moderate mycotoxin contamination levels of feed [386]. Some publications showed that these effects can even occur at contamination levels below the European maximum guidance levels, suggesting that the legislation may not cover all deleterious health effects of mycotoxins.

In conclusion, *Fusarium* mycotoxins may alter the human and animal susceptibility to infectious diseases by affecting the intestinal health and the innate and adaptive immune system. Further research will be necessary to investigate the impact of mycotoxins on other enteric infectious diseases, such as necrotic enteritis in poultry.

3 Necrotic enteritis in broiler chickens

Enteric diseases are one of the most important issues to the poultry industry, because they reduce animal welfare and cause high economic losses worldwide due to increased mortality rates, decreased weight gain, increased feed conversion ratio (FCR), and increased medication cost [78,176,421,422]. Worldwide, necrotic enteritis (NE) is one of the economically most important enteric diseases in poultry production [267,308,421]. This disease may present as acute clinical disease or as a subclinical condition [404]. In broiler chickens, total mortality rates associated to a clinical NE outbreak in a broiler flock are often between 2 and 10%, however, rates as high as 50% have been reported [267,308]. Subclinical NE is estimated to result in a 12% reduction in BW and 10.9% increase in FCR [374]. Surveys of veterinarians and poultry producers globally have estimated a morbidity rate of 20% and an average loss of 0.05 EUR per broiler chicken [77,267,442]. The majority of naturally occurring outbreaks of NE in broiler flocks are reported between the second and fifth week of age [84,308,404]. Furthermore cases of NE have also been reported in 7 to 12 week old turkey poults [151], and 3 to 6 month old layers [308]. An increased occurrence of the disease is observed in adult laying hens [3].

3.1 Etiology

NE in broilers may arise when changes in the gut microbial homeostasis allow colonization of virulent *Clostridium perfringens* type A strains containing the NetB toxin gene [125,159,247,315,423]. *C. perfringens* is a Gram-positive rod-shaped anaerobic bacterium, a commensal in the gastrointestinal tract of animals and humans, that also has been isolated in the feed and environment [78,367,378]. *C. perfringens* has no flagella but is motile by way of type IV pilli [427]. Although classified as anaerobic, *C. perfringens* can tolerate aerobic conditions. *C. perfringens* can survive under extreme conditions, due to its differentiation from vegetative cells to highly resistant dormant spores. This bacterium can grow over a wide pH range, varying from 5.0 to 8.0, whereas optimum growth occurs at pH 6 to 7 [295]. *C. perfringens* utilizes a variety of sugar compounds by degrading these compounds into monosaccharides that can enter the anaerobic glycolysis pathway to generate energy. In contrast to the variety of sugar-utilization enzymes, many genes encoding enzymes required for amino acid biosynthesis are lacking in *C. perfringens*. Therefore, the bacterium actively degrades and imports various materials from the host tissues to grow and survive in the host [367]. It is suggested that the intestinal colonization of broilers by *C. perfringens* is an early event in the life of animals, and that the bacterium can be transmitted within the integrated broiler chicken operation, starting from the hatchery [76,77].

3.2 Pathogenesis

The ability of *C. perfringens* to cause disease is mediated via the production of toxins and extracellular enzymes [320,367].

3.2.1 Toxin production

Based on the production of the four so-called major toxins (α-, β-, ε- and ι-toxin) *C. perfringens* strains are classified into five toxinotypes (A, B, C, D and E) (Table 4) [320,378]. NE is caused predominantly by *C. perfringens* type A, and to a lesser extent by type C [125,378]. Besides, some strains of *C. perfringens* type A produce an enterotoxin at the moment of sporulation, which is responsible for foodborne disease in humans [159,354,423].

Table 4. Toxinotypes of C. perfringens *(based on [320]).*

Type	Major toxins			
	α	**β**	**ε**	**ι**
A	X			
B	X	X	X	
C	X	X		
D	X		X	
E	X			X

Alpha (α)-toxin is a zinc-dependent phospholipase C sphingomyelinase that hydrolyzes phospholipids, which negatively affects the cellular membrane organization [267,408]. Cellular substrates for α-toxin are phosphatidylcholine and sphingomyelin, both components of the cellular membrane of epithelial cells in the gastrointestinal tract. Hydrolysis of cell membrane phospholipids induces the formation of diacylglycerol, activating protein kinase C, and subsequent the arachidonic acid cascade. This induces the synthesis of inflammatory mediators, such as leukotrienes, thromboxane, platelet-agglutinating factor and prostacyclin, which cause blood vessel contraction, platelet aggregation, and myocard dysfunction, leading to acute death [353,407,423]. However, an α-toxin-negative mutant of *C. perfringens* strain from a NE outbreak was still able to produce NE in broilers. These findings suggest that α-toxin is not an essential virulence mediator for NE in broiler chickens [215].

It has been shown that the pore-forming NE B-like (NetB) toxin is a key virulence factor in the pathogenesis of avian NE [214,424]. A *netB* null mutant is avirulent in a chicken NE model, and virulence is restored upon complementation with the wild-type *netB* gene [214].

Furthermore, the majority of NE outbreak strains carry the *netB* gene, whereas most of *C. perfringens* isolates from healthy broilers were found to be *netB*-negative [214,216,260,424]. NetB toxin is identified as a heptameric β-pore-forming toxin (β-PFT), showing high similarity to the structure of the heptamer formed by *Staphylococcus aureus* alpha-hemolysin (αHL) [356,449]. The expression of *netB* is regulated at the transcriptional level by the VirSR two-component system [66,367]. Proteins belonging to the family of αHL-like β-PFT are organized into three main domains according to their structural and functional characteristics, i.e. rim, stem and β-sandwich [80,356]. Binding to the target cell membrane is mediated by the rim domain, which contains loops rich in aromatic residues that are proposed to interact with the outer leaflet of the lipid bilayer. However, differences in the rim domain between clostridial and staphylococcal members of β-PFT may suggest different binding mechanisms. In particular, NetB does not interact efficiently with phosphatidylcholine or sphingomyelin [356]. The stem domain consists of the characteristic β-hairpin involved in the penetration of the toxin into the cellular membrane and formation of the transmembrane β-barrel structure. The β-sandwich domain forms the protein backbone with key functions in toxin oligomerisation. Recently, it was demonstrated that cholesterol and the amino acid K41 are key factors affecting NetB oligomerization and pore-formation [80,356]. In addition to promoting cell lysis through an osmotic imbalance, it has been suggested that the cellular calcium influx caused by NetB may also lead to a programmed cellular necrosis [308]. Occasional *netB*-negative NE isolates could be explained by a sequence divergent from the published *netB* toxin or the presence of a yet unidentified toxin. Moreover, the characteristics of isolates might change during the culturing process or there might even be a mixture of pathogenic and non-pathogenic strains present in some diseased birds [424].

3.2.2 Single strain dominance

In healthy animals, a high degree of genetically different *C. perfringens* isolates can be found, even within individual birds and within the same intestinal segment. In contrast, isolates from bird with NE are usually genetically identical [125,291]. NE outbreak strains are more capable of secreting factors inhibiting growth of other *C. perfringens* strains compared to strains isolated from the gut of healthy chickens [30,406]. Bacteriocins are antimicrobial peptides produced by bacteria that generally inhibit the growth of closely related strains [199]. Recently, a novel bacteriocin, called perfrin, was shown to be associated with *netB* positive *C. perfringens* strains from NE outbreaks [403]

3.2.3 Adhesion to the intestinal epithelium

In the gastrointestinal tract, *C. perfringens* can adhere to the host's epithelium or to extracellular matrix molecules (ECMM). Intestinal damage, caused by coccidiosis, toxins, or collagenolytic enzymes secreted by *C. perfringens,* increases the accessibility of the ECMMs for epithelial binding of *C. perfringens* [261,404]. Occurrence and severity of NE lesions are associated with the efficiency of the *netB* positive strain to bind to collagen type III, IV and V, fibrinogen, laminin and vitronectin [404]. Recently, it has been suggested that adherence of *C. perfringens* to the intestinal epithelium is facilitated by the production of sialidases [67,236]. Genome sequencing has revealed that *C. perfringens* strains can typically possess up to three sialidase-encoding genes, *nanH*, *nanI* and *nanJ*, respectively [285,367]. However, *C. perfringens* has the ability to produce many glucoside hydrolases directed at the muco-oligosacharides part of the mucosal layers of the intestine [136,137]. Two prominent chitinase genes present on the major pathogenicity locus (NELoc1) of NE isolates are speculated to have mucin degradation functions [233]. For example, *C. perfringens* can produce hydrolases with an affinity for specific mucin sugar residues, a.o. galactose, N-acetylgalactosamine and N-acetylglucosamine [135].

3.2.4 Predisposing factors

The intestinal tract of NE affected chickens contains large numbers of *C. perfringens* (>10^5 colony forming units (cfu)/g ileal digesta), although high numbers of *C. perfringens* in intestinal tract are not sufficient to produce NE [73,308,369]. Therefore, it is generally accepted that predisposing factors or risk factors are required, allowing the proliferation of *C. perfringens* by either providing nutrients or favorable ecological niches [441].

3.2.4.1 Concurrent infectious diseases

The best known predisposing factor for NE is intestinal damage caused by coccidial pathogens [347,423,441]. The role of coccidial infections, caused by *Eimeria* spp., in the occurrence of NE has been hypothesized from field observations [186,189,246]. Moreover, it is shown that administration of *Eimeria* oocysts or coccidial vaccines are important for the induction of NE in an experimental *C. perfringens* infection model [2,158,368]. Coccidial pathogens can damage the intestinal mucosa by intracellular multiplication of the parasite in the intestinal wall, which inhibit epithelial regeneration and rupture of the cells of the intestinal lining [4]. Subsequently, intestinal damage induces leakage of plasma proteins, which can provide necessary growth substrate for extensive proliferation of *C. perfringens* [423].

Intestinal coccidial infection in poultry is characterized by a T cell-mediated inflammatory response that enhances intestinal mucogenesis [71]. This enhanced mucus production provides a growth advantage to *C. perfringens* due to its ability to use mucus as substrate [71,95]. Recently, it was demonstrated that an *Eimeria* infection modulates the caecal microbiota of broiler chickens [381,446]. The gut microbial diversity was reduced in *Eimeria* infected birds, mainly characterized by a reduced number of the *Ruminococcaceae* groups and increased abundance of three unknown *Clostridium* species [446]. It was also suggested that *Eimeria* is capable of modulating the gut mucosal immunity by reducing the abundance of *Candidatus* Arthromitus [381], recently renamed to *Candidatus* Savagella [402]. This segmented filamentous bacterium (SFB) modulates the host immune response by inducing IgA secreting cells and influence epithelial lymphocyte maturation [127,375,402]. In conclusion, coccidiosis induces a compromised intestinal microbial homeostasis, which correlates with the ability of *C. perfringens* to cause an infection [381].

Besides coccidiosis, it has been suggested that also other enteric infectious diseases can predispose for intestinal *C. perfringens* colonization [4]. Ascaridiasis has been established as a concurrent intestinal infection found in turkeys suffering from *C. perfringens* induced NE [114,294]. Rotavirus infection in mice and parvovirus infection in dogs enhanced the intestinal proliferation of *C. perfringens* [280,414].

3.2.4.2 Nutrition

C. perfringens is lacking many genes necessary for amino acid biosynthesis. Therefore, this bacterium cannot grow in an environment where amino acid supply is limited [367]. Feeding a protein-rich diet induced an increased ileal and caecal concentration of *C. perfringens* in broiler chickens [113]. The protein source seems to influence the effect of a higher dietary protein level. In chickens, feeding a fishmeal-based diet stimulates the intestinal *C. perfringens* overgrowth, and subsequently the induction of NE [4,113,210,368]. Fishmeal is characterized by a higher level of the amino acids glycine and methionine compared to soy protein concentrate [113]. A positive correlation was demonstrated between the dietary glycine content and *C. perfringens* population in the ileum and caecum of broiler chickens [83,440]. In contrast, a decreased intestinal population of *C. perfringens* and NE intestinal lesions was observed in broilers following dietary methionine supplementation [81]. Therefore, it is suggested that not only the level of individual amino acids but also the balance of amino acids is important for maximum growth of *C. perfringens* [146]. Recently, it was also demonstrated that feeding a high protein fishmeal diet induces a shift in the intestinal microbiota

composition. A microbial shift was identified in the species composition of the *Lactobacillus* genus where the abundance of *L. johnsonii* and *L. acidophilus* was reduced, while *L. reuteri* and *L. animalis* flourished. Although, no effect was observed on the total intestinal *Lactobacillus* count [446]. In accordance, dietary glycine supplementation induced a decreased level of lactobacilli and increased level of *C. perfringens* in the ileum and caecum of broilers [82,83].

High dietary levels of digestible carbohydrates that exceed the digestion and absorption capacity of the intestinal mucosa can be utilized by *C. perfringens* to proliferate [4], because the bacterium can produce a variety of carbohydrate degrading enzymes [367]. An increased growth of *C. perfringens* was observed *in vitro* by adding digestible carbohydrates, a.o. glucose, starch, lactose, ribose, trehalose, sucrose, maltose, triomaltose, dextrin, or fructose, to the growth medium. The addition of indigestible carbohydrates such as raffinose or cellobiose did not increase the growth rate [4,146,228,352]. In chickens, dietary addition of fructose and sucrose, but not of glucose, resulted in a higher number of *C. perfringens* in the intestinal tract [343,388].

Furthermore, diets with high levels of indigestible, water-soluble non-starch polysaccharides (NSP), increase the viscosity of the digesta and are risk factor for necrotic enteritis. This is the case for diets rich in wheat, rye, oats, and barley, whereas not for corn [8,75,210]. The increased intestinal viscosity can prolong the intestinal transit time associated with a greater intestinal clostridial count [8]. Besides, NSP also interact with glycoproteins on the epithelial surface to increase mucin production, and subsequently stimulate *C. perfringens* proliferation [219,368]. An increased number of *C. perfringens* was observed in the ileum, caeca and faeces of broilers fed a 50% rye based diet compared to corn-based diet [75].

Other nutritional factors can also influence the intestinal proliferation of *C. perfringens* and predispose for NE. The type of fat source is also likely to indirectly influence the intestinal microbiota. It was demonstrated that dietary fat of animal origin increases intestinal *C. perfringens* count compared to vegetable oil [221]. The size of the feed particles has been shown to also affect the intestinal number of *C. perfringens*. A higher count of intestinal *C. perfringens* was observed in mash-fed birds compared to pellet-fed birds [123]. NE induced mortality was higher in groups of broiler chickens fed a hammer-mill diet compared to groups fed a roller-mill diet [51]. Addition of whole wheat to mash or pellet diets strongly reduced the numbers of *C. perfringens* in the intestinal tract of birds. This finding may be related to the improvement of the gizzard function regulating the filling of the small intestine, which may result in lower concentrations of easily fermentable nutrients [40,124,451]. Trypsin inhibition is

also a well-established predisposing factor to NE, since trypsin induces cleavage of *C. perfringens* toxins in the small intestine [4,355]. The ability of trypsin to degrade alpha toxin is for example reduced by a high dietary level of zinc and the trypsin inhibitor activity of potato protein concentrate [28,310,355].

3.2.4.3 Immunosuppression and stress

Besides coccidiosis, other infectious diseases, such as infectious bursal disease virus, chicken anemia virus, and Marek's disease virus, have been reported to predispose chickens to NE [231,268]. Immunosuppression can also be stress-mediated. Environmental (heat or cold stress) and management-related (a.o. feed changes, litter conditions, stocking density, and vaccination programs) stressors have been shown to cause immunosuppression, predisposing to NE [267,268,308,404,410,411].

3.3 Clinical signs

NE clinical signs are common to enteritis in general, a.o. depression, anorexia, diarrhea, dehydration, and ruffled feathers. *C. perfringens* induced NE in broilers may occur as acute clinical or subclinical disease [73,186,423]. The acute form of NE is characterized by apparently healthy birds that may become acutely depressed and die within hours. Prior to the sudden increase in flock mortality, wet litter is sometimes an early indicator of disease [176,186,308,423]. The mild subclinical form of NE is characterized by no clinical signs or peak in flock mortality, although an overall reduction in bird performance is observed. Performance losses are associated with chronic intestinal mucosal damage, which results in poor nutrient digestion and absorption, reduction in BW gain and increased FCR [209,404,423]. Taking into account the limited symptoms of subclinical NE, diagnosis based on clinical signs is hard, resulting in an increased number of undiagnosed and untreated cases [308].

3.4 Pathological lesions

3.4.1 Macroscopic observations

Macroscopic lesions usually are confined to the small intestine, primarily the jejunum and ileum, but may extend to the duodenum and caeca [73,176,186,404]. The intestines are usually inflamed, thin walled, friable, dilated and filled with gas [56,298]. Intestinal mucosal lesions are characterized as a spectrum of mucosal erosion-to-ulceration, covering the surface with a grey-brown to yellow-green diphteric membrane or pseudomembrane, and occasionally with some blood clots in the intestinal lumen (Figure 10) [298,368,423]. Subclinical NE can also be

associated with cholangiohepatitis and gizzard erosions [247,296]. Livers are severely enlarged with a pale reticular pattern, sometimes with pale and stellate foci [247].

Figure 10. Typical gut lesions in severe broiler necrotic enteritis

3.4.2 Microscopic observations

Histopathology reveals a severe inflammatory response to *C. perfringens* colonization. The lamina propria is hyperemic and infiltrated with numerous inflammatory cells. Severe edema can be seen at the interface of the basal domain of enterocytes and lamina propria. Progression of lesions usually occurs from villi apices to crypts, and results in diffuse and severe coagulative necrosis of the mucosa. The intestinal epithelium is delimited by masses of tissue fragments, necrotic cells, cell debris, and numerous Gram-positive bacterial colonies suspended in mucus matrix comprising histological features of pseudo-membrane (Figure 11). Necrosis may extend into the submucosa and muscular layer of the intestine. Besides, blood vessel congestion can be observed in the lamina propria and submucosa. Microscopic lesions associated with *C. perfringens* induced cholangiohepatitis are bile duct hyperplasia, fibrinoid necrosis, cholangitis and occasionally focal granulomatous inflammation [2,158,176,245,297,404,423].

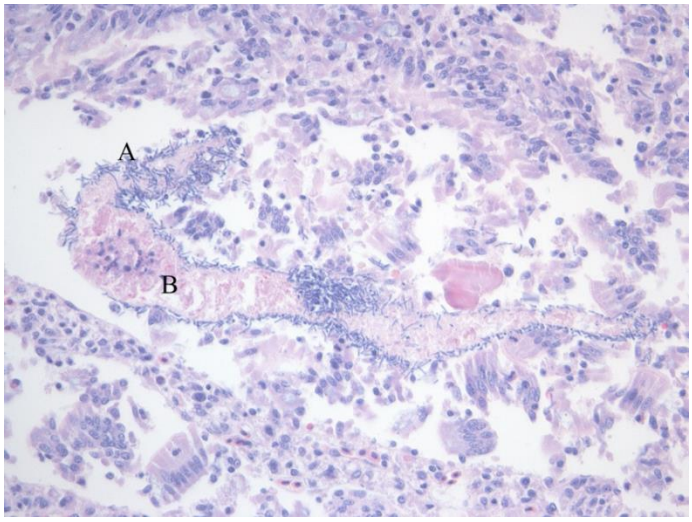

Figure 11. C. perfringens (A) colonization of an abundance of fibrin admixed with cellular debris (B) from the necrotic mucosa.

3.5 Control of necrotic enteritis

Prevention of NE is associated with measures that minimize the effects of predisposing factors that contribute to the disease development. Antibiotic growth promoters (AGP) have played a major role in the suppression of NE [424,441]. However, public concerns about the use of dietary antimicrobials and the emergence of antibiotic-resistant bacteria led to a ban on AGP in the EU in 2006 [424]. Removing AGPs and the resultant rise of NE induced an increased need for alternative methods for controlling NE, such as alternative dietary supplements (probiotics, prebiotics, acids, essential oils, …), management and vaccination strategies (discussed in detail in review articles [84,115,196,250,281]).

Probiotics have been defined as "a live microbial feed supplement, which beneficially affects the host animal by improving its intestinal microbial balance" [147]. Dietary inclusion of probiotics may modulate the intestinal environment by stimulating the immune system, maintain gut microbiota by competitive exclusion, altering intestinal metabolism through increased digestive enzyme activity, reducing bacterial enzyme activity and ammonia production, and neutralizing enterotoxins [196,250]. A number of studies have demonstrated a beneficial effect of the usage of several beneficial microorganisms, such as *Lactobacillus* spp. and *Bacillus* spp., on the development of *C. perfringens* induced NE in broilers [93,220,226,227,269].

Prebiotics are defined as indigestible feed ingredients that selectively stimulate the growth or activity of beneficial bacteria that are already resident in the gastrointestinal tract [160]. Non-starch oligosaccharides are the most commonly used group of prebiotics, and are based on any of the hexose monosaccharides, a.o. fructose, galactose, mannose and glucose [118,250]. This group of prebiotics includes a.o. fructo-oligosaccharides (FOS), galacto-oligosaccharides, mannan-oligosaccharides (MOS), arabinoxylan oligosaccharides (AXOS) and raffinose family oligosaccharides (RFOS) [115,120]. Several studies showed a beneficial effect of MOS on NE in broilers and turkey poults by reducing the intestinal number of *C. perfringens*, and subsequently NE associated lesions and mortality [218,250,275,372,450]. Non-digestible feed ingredients, such as NSPs, can induce digestive stress in broilers, causing reduction in nutrient utilization which in turn facilitates *C. perfringens* overgrowth in the gastrointestinal tract [8,75,210]. Feed supplementation with exogenous enzymes, such as xylanase and β-mannanase, has been suggested to reduce digesta viscosity, and increase nutrient digestion and digesta passage, resulting in a reduced number of *C. perfringens* [68,84,196,250,423]. Besides, other feed additives such as organic acids, herbs, spices and their essential oils have been suggested to have positive effects on controlling NE [84,115,196,250].

SCIENTIFIC AIMS

The *Fusarium* mycotoxins deoxynivalenol (DON) and fumonisins (FBs) are the two most frequently occurring mycotoxins worldwide. A long-term survey performed between 2004 and 2012 on globally sampled feed and feed raw materials, demonstrated that 56% of samples were contaminated with DON and in 54% of samples FBs were detected [358,386]. Following oral intake of DON and FBs, the gastrointestinal epithelial cell layer will be exposed first [47]. The intestinal mucosa acts as a barrier, preventing the entry of foreign antigens including food proteins, xenobiotics (such as drugs and toxins), commensal microbiota and pathogens into the underlying tissues [47,305]. As previously described, the intestinal barrier function, which consists of an intrinsic and extrinsic component, can be affected by the *Fusarium* mycotoxins DON and FBs at levels higher than the European maximum guidance levels, a.o. cell viability and proliferation, intestinal morphology, intestinal mucus layer, production of antimicrobial peptides, epithelial integrity, and modulation of digestive and absorptive processes (Figure 8 and 9) [47,305]. Since the intestinal tract is also a major portal of entry to many enteric pathogens and their toxins, mycotoxin exposure could increase the animal susceptibility to these pathogens. In addition to the effect of *Fusarium* mycotoxins on the animal host, these mycotoxins may alter the metabolism of pathogens, which may alter outcome of the infectious disease [425,429].

The development of *Clostridium perfringens* induced necrotic enteritis (NE) in broiler chickens depends on the presence of predisposing factors that alter the micro-environment of the intestinal tract and create a favorable environment for *C. perfringens* overgrowth. Well-known predisposing factors such as coccidiosis, feeding fishmeal and high dietary levels of indigestible water-soluble non-starch polysaccharides, have shown to affect the integrity of the intestinal epithelial barrier, availability of amino acids and carbohydrates, and composition of the mucus layer and microbiota composition.

Research investigating the interaction between *Fusarium* mycotoxins and infectious enteric diseases has mainly focussed on pigs [306,425,426,429]. In contrast to pigs, the chicken is considered to be one of the most resistant farm animal species to the toxic effects of DON and FBs. Therefore, the aim of this doctoral research was to investigate the interaction between *Fusarium* mycotoxins and an important enteric bacterial disease, in an animal species that is known to be rather resistant to the negative effects of mycotoxins. Although the mycotoxin level of poultry feed samples complies with the European maximum guidance levels, poultry industry may suffer from mycotoxin-associated problems. Furthermore, many studies investigating the effect of mycotoxins on animal health have been performed with high

mycotoxin contamination levels. Therefore, there is an urgent need for studies investigating the effect of mycotoxins at concentrations close to the European maximum guidance levels.

The **general aim** of this thesis was to examine whether the *Fusarium* mycotoxins DON and FBs at concentrations in the feed approaching European maximum guidance levels predispose for NE in broiler chickens, and to gain insights in the mechanisms responsible for this interaction.

The **specific objectives** of this thesis were as follows:

- To investigate the predisposing effect of DON and FBs on the development of NE in broilers in experimental *C. perfringens* infection studies.
- To examine the impact of DON and FBs on the micro-environment of the gastrointestinal tract, i.e. intestinal epithelial barrier function, microbiota composition, amino acid and protein availability for clostridial proliferation, mucus layer and oxidative stress.
- Because the mycotoxins DON and FBs frequently co-occur, and taken into account that FBs have a low oral bioavailability in healthy animals and DON impairs the intestinal barrier and/or decreases the total intestinal absorption surface area, the last study aimed to investigate whether chronic exposure to DON could influence the intestinal absorption of FBs leading to an altered systemic exposure and increased toxic effects of the latter mycotoxin in broiler chickens.

EXPERIMENTAL STUDIES

CHAPTER 1

The predisposing effect of deoxynivalenol on necrotic enteritis in broiler chickens

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Adapted from

Antonissen G., Van Immerseel F., Pasmans F., Ducatelle R., Haesebrouck F., Timbermont L., Verlinden M., Janssens G.P.J, Eeckhaut V., Eeckhout M., De Saeger S., Hessenberger S., Martel A., Croubels S. (2014) The Mycotoxin Deoxynivalenol Predisposes for the Development of *Clostridium perfringens*-Induced Necrotic Enteritis in Broiler Chickens. PLoS One 9(9): e108775.

Abstract

Both mycotoxin contamination of feed and *Clostridium perfringens*-induced necrotic enteritis have an increasing global economic impact on poultry production. Especially the *Fusarium* mycotoxin deoxynivalenol (DON) is a common feed contaminant. This study aimed at examining the predisposing effect of DON on the development of necrotic enteritis in broiler chickens.

An experimental *Clostridium perfringens* infection study revealed that DON, at a contamination level of 3,000 to 4,000 µg/kg feed, increased the percentage of birds with subclinical necrotic enteritis from 20 ± 2.6% to 47 ± 3.0% ($P<0.001$). DON significantly reduced the transepithelial electrical resistance in duodenal segments ($P<0.001$) and decreased duodenal villus height ($P=0.014$) indicating intestinal barrier disruption and intestinal epithelial damage, respectively. This may lead to an increased permeability of the intestinal epithelium and decreased absorption of dietary proteins. Protein analysis of duodenal content indeed showed that DON contamination resulted in a significant increase in total protein concentration ($P=0.023$). Furthermore, DON had no effect on *in vitro* growth, alpha toxin production and *netB* toxin transcription of *Clostridium perfringens*.

In conclusion, feed contamination with DON at concentrations below the European maximum guidance level of 5,000 µg/kg feed, is a predisposing factor for the development of necrotic enteritis in broilers. These results are associated with a negative effect of DON on the intestinal barrier function and increased intestinal protein availability, which may stimulate growth and toxin production of *Clostridium perfringens*.

Keywords

Broiler – *Clostridium perfringens* – Deoxynivalenol – Necrotic Enteritis

1 Introduction

Worldwide, necrotic enteritis (NE) leads to important production losses, increased feed consumption and mortality rates, and a reduced welfare of broiler chickens [78,421-423]. The causative agent of NE is *Clostridium perfringens*, a Gram-positive spore forming bacterium which occurs ubiquitously in the environment, in feed and in the gastrointestinal tract of animals and humans [30,405]. It has been suggested that alpha toxin production is an essential virulence factor in the pathogenesis of NE [215], but recently it was established that only strains producing NetB toxin, a β-pore-forming toxin, are capable of inducing NE in broiler chickens under specific conditions that predispose to the disease [214,404].

Acute NE is characterized by a sudden increase in mortality, often without premonitory symptoms. Nowadays, the subclinical form is becoming more prevalent, and is mainly characterized by intestinal mucosal damage without clinical signs or mortality. This leads to a decreased digestion and absorption of nutrients, a reduced weight gain and an impaired feed conversion rate [404,423].

Notwithstanding the role of *C. perfringens* in poultry production losses, the mere presence of virulent strains in the intestinal tract of broilers, or even the inoculation of chickens with high doses of these strains, does not always lead to the development of NE. Predisposing factors including dietary, husbandry and immune factors [73,75,215], are required to reproduce the disease [231,268,441]. The best-known predisposing factor is mucosal damage caused by coccidial pathogens [71,441], which could provide *C. perfringens* with essential nutrients and thus stimulate massive overgrowth [49,320]. *C. perfringens* is lacking many genes of the orthologous enzymes required for amino acid biosynthesis, among others for arginine, phenylalanine, tryptophan, tyrosine, histidine, leucine, isoleucine, valine, glutamate, lysine, methionine, serine and threonine. Therefore, *C. perfringens* growth is restricted in an environment where the amino acid supply is limited [49,146,367].

The mycotoxin deoxynivalenol (DON) is one of the most common contaminants in poultry feed worldwide. DON is a type B trichothecene produced by among others *Fusarium (F.) graminearum* and *F. culmorum*. Recent data on global mycotoxin occurrence showed that 59% of 5,819 samples of animal feed tested positive for the presence of DON. The average contamination level was 1,104 µg DON/kg feed, with a maximum observed level of 49,307 µg/kg [348]. The European maximum guidance level for poultry feed is set at 5,000 µg/kg feed [131].

Poultry is considered rather tolerant to DON. It has been suggested that concentrations higher than 5,000 µg/kg feed are necessary to negatively influence the growth performance of broilers [16,87]. This mycotoxin acts as an inhibitor of the protein synthesis at the ribosomal level whereby rapidly proliferating cells in tissues with high protein turnover rates, such as the immune system and small intestine, are most affected [87]. Accordingly, DON negatively influences small intestinal epithelial cell integrity and morphology [19,25,166,171,303,322]. As a consequence of the negative effect of DON on the gastrointestinal epithelial cells, feeding DON-contaminated diets can lead to greater susceptibility to enteric infections [171]. Only few studies have investigated the interaction between DON and enteric pathogens. In pigs, it has been shown in an intestinal ileal loop model that co-exposure to DON and *Salmonella* Typhimurium potentiates the inflammatory response in the gut [425]. *In vitro*, intestinal porcine epithelial cells (IPEC-1) show an increased translocation of a septicemic *Escherichia coli* (O75:K95) after DON exposure [322]. It is hitherto unclear whether the intestinal epithelial damage caused by contamination levels of DON below 5,000 µg/kg in feed, may act as an additional predisposing factor in broiler NE. We hypothesized that this intestinal damage may lead to higher protein availability for clostridial proliferation in the small intestine.

The objectives of this study were to examine whether DON at concentrations in the feed below the EU maximum guidance level predisposes for NE in broilers, and to gain insights in the mechanisms responsible for this interaction. Therefore, the effects of DON on the intestinal epithelial barrier function and on intestinal protein availability for clostridial proliferation were evaluated. Also, the direct effect of DON on *in vitro* bacterial proliferation, alpha toxin production and *netB* transcription was studied.

2 Materials and Methods

2.1 Deoxynivalenol

For the *in vitro* assessment of the impact of DON on growth and toxin production of *C. perfringens*, a DON stock solution of 2000 µg/mL (Fermentek, Jerusalem, Israel) was prepared in anhydrous methanol and stored at -20 °C. Next, serial dilutions of DON were prepared in tryptone glucose yeast (TGY) broth medium.

For the animal trials, DON was produced *in vitro* from cultures of *F. graminearum* in accordance to the protocol described by Altpeter *et al.* [5] (Romer Labs, Tulln, Austria), and was mixed into the experimental feed.

2.2 Bacterial strains

C. perfringens strain 56 has been used previously to induce NE in an *in vivo* model in broilers [158,405]. Originally this strain was isolated from the gut of a broiler chicken with severe NE lesions, and characterized as a *netB* toxin positive type A strain (no β_2 or enterotoxin genes) as well as a producer of moderate amounts of alpha toxin *in vitro* [159]. In addition to strain 56, a *netB* toxin negative strain (*C. perfringens* strain 6 [159]) was included as negative control for *in vitro netB* transcription measurement.

2.3 Animal experiments

2.3.1 Birds and housing

Non-vaccinated Ross 308 broilers were used that were obtained as one-day-old chicks from a commercial hatchery. Each group consisted of approximately equal numbers of males and females. All treatment groups were housed in the same room, in cages of 1.44 m², on a litter floor. All cages were separated by solid walls to prevent direct contact between birds from different treatment groups. Before each trial, the cages were decontaminated with peracetic acid and hydrogen peroxide (Hygiasept® vaporizer climasept; SARL Hygiasept, Sevrey, France) and a commercial anticoccidial disinfectant (Bi-OO-Cyst Coccidial Disinfectant; Biolink, York, United Kingdom).

Chickens had *ad libitum* access to drinking water and feed and were subjected to a 23 h/1 h light/darkness programme. The animals were not fasted before euthanasia. The environmental temperature was adjusted to the changing needs of the animals according to their age (week 1: 35°C, week 2: 30°C, week 3: 25°C).

2.3.2 Feed

All birds were given a starter diet during the first eight days of the experiment, and subsequently a grower diet until the end of the trial. The diet was wheat:rye (43%:7.5%) based, with soybean meal as the main protein source during the first 16 days. From day 17 onwards, the same grower diet was used with the exception that fishmeal (30%) was added as protein source instead of soybean meal. Further details of the feed composition were as previously described [158].

In the exposed groups, an artificially DON contaminated diet was fed from day 1 onward. The contaminated feed was produced by adding DON to a control diet. To test for DON concentrations in the feed, samples were taken at three different locations in the batch and subsequently pooled. All diets were analysed for the content of DON and other mycotoxins with a validated multi-mycotoxin liquid chromatography-tandem mass spectrometry method (LC-MS/MS) [276]. The levels of DON in the different batches of control feed was below the limit of quantification. The DON contamination level in the different batches of contaminated feed varied between 2,884 ± 800 µg/kg and 4,384 ± 1,300 µg/kg feed. All other mycotoxins tested were either absent or present in low concentrations.

2.3.3 Animal experiment 1: *C. perfringens* experimental infection study

The trial was performed following an adapted protocol based on a previously described experimental infection model, with the modification that no coccidial challenge was administered [158]. In the trial, 360 chicks were divided into 4 experimental groups, each group consisting of 3 cages of 30 chicks. The experimental groups are described in Table 6 One group was experimentally infected with *C. perfringens* and received a control diet. A second group was experimentally infected with *C. perfringens* and received a DON contaminated diet, while a third group was fed a DON contaminated feed but did not receive *C. perfringens*. A fourth group was a negative control (no *C. perfringens* and control feed). Gumboro vaccine (Nobilis Gumboro D78, MSD Animal Health, Brussels, Belgium) was administered in the drinking water on day 16 to all birds. Experimental infection with *C. perfringens* consisted of oral inoculation of the birds with 4.10^8 cfu of *C. perfringens* strain 56 at days 17, 18, 19 and 20. The bacteria for the animal experiment were cultured anaerobically overnight in brain heart infusion broth (BHI, Oxoid, Basingstoke, UK) supplemented with 0.375% glucose at 37°C. The actual number of bacteria/mL was assessed by plating tenfold dilutions on Columbia agar (Oxoid) with 5% sheep blood, incubated anaerobically overnight at 37°C. Birds that were not infected with *C. perfringens* received a sham inoculation with BHI broth.

On days 21, 22 and 23, each day one third of each group was euthanized and the birds were immediately submitted to necropsy. Single-blind macroscopic NE lesion scoring of the small intestine (duodenum to ileum) was performed as previously described by Keyburn *et al.* [215]. Birds with lesion scores of 2 or more were classified as NE positive.

In addition, contents of three small intestinal segments of 27 birds per group of the third (DON, no *C. perfringens*) and fourth (negative control) experimental group were collected

and stored at -20°C until further use for protein analysis. The three segments were duodenum, jejunum and ileum. The duodenum was defined as the segment encompassing the duodenal loop, whereas the jejunum was defined as the segment between the end of the duodenal loop and Meckels diverticulum. The ileum comprised the distal segment starting at Meckels diverticulum and ending at the ileo-cecal junction.

Contents of these three segments were used to determine intestinal nitrogen (N) concentration by the Kjeldahl method used for feeding stuffs (ISO2005). Percentage crude protein per dry matter of the intestinal content was calculated from Kjeldahl N values, using 6.25 as conversion factor to protein level.

The bodyweight (BW) of 30 identified chickens per experimental group was measured at day 1, 7, 14 and at the day of euthanasia. Bodyweight gain was determined as the differences in BW divided by the period of time. The presence of coccidiosis was excluded by faecal oocyst count, by qualitative flotation, and macroscopic coccidiosis lesion scoring of the intestines [206].

2.3.4 Animal experiment 2: Effect of DON on villus height and transepithelial electrical resistance

Eighteen birds were divided into 2 experimental groups, each group consisting of 3 cages of 3 chicks. One group was fed a control diet, and the other group was fed a DON-contaminated diet. All birds received Gumboro vaccine on day 16 and they all received a sham inoculation with blank BHI broth on day 17, 18, 19 and 20. On day 21, immediately after euthanasia of the animals, 1 cm samples from the mid-duodenum, mid-jejunum and mid-ileum were collected for evaluation of the intestinal morphology. These samples were fixed in neutral-buffered formalin, and processed afterwards using standard protocols for hematoxylin and eosin staining of paraffin sections. Villus height and crypt depth were measured using a light microscope with Leica LAS software (Leica Microsystems, Diegem, Belgium). The average of 5 to 15 measurements per segment per animal was calculated.

The remainder of the mid-duodenal segment was immersed into oxygenated (O_2/CO_2, 95/5 %) Krebs Henseleit buffer solution (Sigma-Aldrich) of pH 7.4. Before opening the intestinal segment, the underlying serosal layer was stripped off. Segments were opened along the mesenteric border and rinsed with buffer solution. Per chicken, three duodenal segments of 2 cm in length were cut and each mounted in an Ussing chamber (Mussler Scientific Instruments, Aachen, Germany). Epithelial sheets had an exposed surface area of 0.28 cm^2.

Mucosal and serosal compartments were simultaneously filled with 7 mL Krebs Henseleit buffer. Four Ag/AgCl electrodes were connected to each chamber by 3M KCl-agar bridges. The electrodes were coupled to an external six-channel microcomputer controlled voltage/current clamp. After an equilibration period of 30 minutes, the transepithelial potential difference (PD, mV) and transepithelial electrical resistance (Rt - TEER, Ω.cm²) were monitored as measures of tissue viability and integrity, respectively, with the tissue unclamped in open circuit mode. Current (Isc, µA/cm²) was calculated from Ohm's law using the following equation $\text{Isc} = \frac{\text{Pd}}{\text{Rt}}$ [292].

The *in vivo* experimental protocols and care of the animals were approved by the Ethical Committee of the Faculty of Veterinary Medicine and Bioscience Engineering, Ghent University, Belgium (EC 2011/169 EC 2012/074).

2.4 *In vitro* study of the effect of DON on *C. perfringens* growth, alpha toxin production and *netB* transcription

Following concentrations of DON were tested for its effect on *C. perfringens* growth, alpha toxin production and *netB* transcription: 0, 0.2, 2 or 20 µg DON/mL TGY medium.

C. perfringens strains 6 and 56 were grown for 24 h in TGY broth medium. Subsequently, this bacterial culture was 1:1000 diluted in the different DON concentrations and incubated anaerobically at 37°C. Clostridial growth curve was assessed by bacterial plating of a ten-fold dilution series at 0, 2, 3, 4, 5, 6, 7, 8 and 24 h after inoculation. Ten-fold dilutions were made in phosphate buffered saline (PBS) solution. Six droplets of 20 µL of each dilution were plated on Columbia agar with 5% sheep blood. After anaerobic incubation overnight at 37°C, the number of colony forming units (cfu)/mL was determined by counting the number of bacterial colonies for the appropriate dilution.

Quantitative detection of alpha toxin in the *C. perfringens* (strain 56) culture supernatants was performed as previously described by Gholamiandekhordi *et al.*, using the Bio-X Alpha Toxin Elisa Kit (Bio-X Diagnostics, Jemelle, Belgium) [159]. Positive (pure alpha toxin) and negative controls (incubation buffer) were included. All tests were performed in triplicate with two technical repeats in each experiment. Subsequently, the mean optical density (OD) value was calculated relative to the positive control value, which was set at 1.

The impact of DON on *netB* transcription was tested by qRT-PCR [66]. The transcription levels of *netB* in the presence of DON were compared to non-DON contaminated test conditions normalized to the housekeeping gene *rpoA*, encoding RNA polymerase subunit A. One mL of mid and late logarithmic growth phase was collected for all test conditions, as described above, from three biological replicates. Based on the growth curve, mid and late logarithmic growth phase were defined after 3 h and 6 h incubation, respectively. Cells were collected by centrifugation at 9,300 x g for 5 min at 4°C. Total RNA was isolated using RNAzol RT (Sigma-Aldrich, Bornem, Belgium) and 40 ng of RNA was converted to cDNA with iScript cDNA Synthesis Kit (Bio-rad, Nazareth Eke, Belgium) in accordance with the manufacturer's instructions. RT-qPCR was performed using SYBR-green 2x master mix (Bioline, Brussels, Belgium) in a Bio-Rad CFX-384 system. Each reaction was done in triplicate in a 12 µL total reaction mixture using 2 µL of the cDNA sample and 0.5 µM final qPCR primer concentration (Table 5). The q-PCR conditions used were 1 cycle of 95°C for 10 min, followed by 40 cycles of 95°C for 30 s, 60°C for 30 s, and stepwise increase of the temperature from 65° to 95 °C (at 10s/0.5°C). Melting curve data were analysed to confirm the specificity of the reaction. For construction of the standard curve, the PCR product was generated using the standard PCR primers listed in Table 5 and DNA from *C. perfringens*. After purification (MSB Spin PCRapace, Stratec Molecular, Berlin, Germany) and determination of the DNA concentration with a Nanodrop ND 1000 spectrophotometer (Nanodrop Technologies, Wilmingtom, DE, USA), the concentration of the linear dsDNA standard was adjusted to $1x10^8$ to $1x10^1$ copies per µL with each step differing by tenfold. The copy numbers of samples were determined by reading off the standard series with the Ct values of the samples.

Table 5. Primer sequences used for qRT-PCR transcription analysis of netB toxin. *Sequences are presented from 5' to 3'.*

target	forward primer	reverse primer	analysis	size (nt) of amplified products
netB	TGA TAC CGC TTC ACA TAA AGG T	ACC GTC CTT AGT CTC AAC AAA T	PCR	448
netB	TCA ATT GGT TAT TCT ATA GGC GGT A	ATA TGA AGC ATT TAT TCC AGC ACC A	qPCR	75
rpoA	ACA TCA TTA GCG TTG TCA GTT AAA G	GAG GTT ATG GAA TAA CTC TTG GTA ATG	PCR	613
rpoA	CCA TCT GTT TTT ATA TCT GCT CCA GTA	GGA AGG TGA AGG ACC AAA AAC TAT T	qPCR	81

2.5 Statistical analyses

Statistical program SPSS version 21 was used for data analysis. All *in vitro* and *in vivo* experiments were conducted in triplicate with three repeats per experiment, unless otherwise noted. To compare the number of NE positive birds (lesion score ≥ 2) between different groups, binomial logistic regression was used. Bodyweight gain was analysed by using an univariate general linear model. Total protein levels, electrophysiological parameters, villus height and crypt depth measurements, *in vitro* assessment of clostridial growth and toxin production, were assessed by independent t-test, after determination of normality and variance of homogeneity. Significance level was set at 0.05.

3 Results

3.1 Animal experiments

3.1.1 DON significantly increases the number of chickens affected by NE

The DON-contaminated diet led to a significantly increased number of chickens with NE; i.e. 20 ± 2.6% of the chickens in the group inoculated with *C. perfringens* and fed a control diet were positive for NE lesions, while in the group inoculated with *C. perfringens* and fed a DON-contaminated diet 47 ± 3.0% of the broilers were positive ($P<0.001$) (Figure 12, Table 6). No animals with NE lesions were detected in the groups without bacterial challenge. Lesion scores of individual broiler chickens challenged with *C. perfringens* are shown in Figure 12.

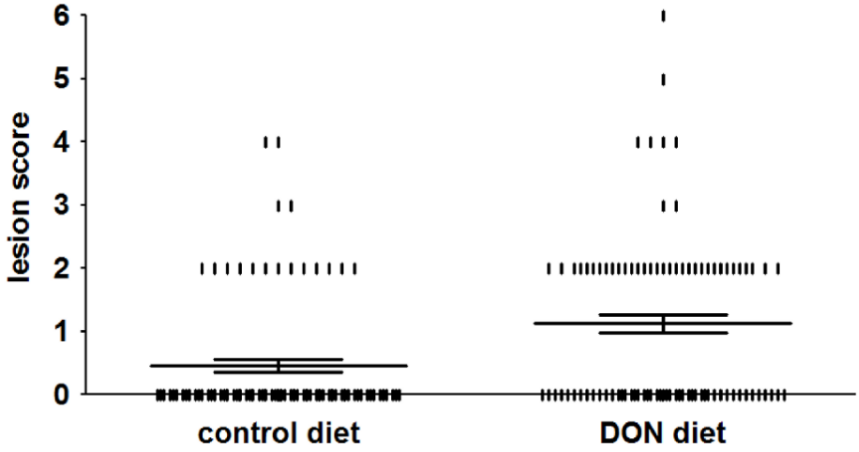

Figure 12: Lesion scores of individual broiler chickens challenged with C. perfringens. *Chickens were fed either a control or DON-contaminated diet and subsequently challenged with C. perfringens strain 56. The solid bars represent the average lesion score in each group. Error bars represent SEM. Intestinal lesions in the small intestine (duodenum to ileum) were scored as previously described [215]; 0 no gross lesions; 2 small focal necrosis or ulceration (one to five foci); 3 focal necrosis or ulceration (six to 15 foci); 4 focal necrosis or ulceration (16 or more foci); 5 patches of necrosis 2 to 3 cm long; 6 diffuse necrosis typical field cases. The score 1 used for congested intestinal mucosa was not applied here because of difficulties in scoring this characteristic objectively, and due to the lack of scientific documentation of an association between "congested intestinal mucosa" and necrotic enteritis. Birds with lesion scores of 2 or more were classified as NE positive.*

In NE positive chickens the lesions were mainly observed in the duodenum (29 ± 0.1% and 31 ± 0.1% of the NE positive chickens in the control and DON group, respectively) and jejunum (94 ± 0.1% and 96 ± 0.1%, respectively). In the ileum, only one animal in the control group and no animals in the DON group showed lesions (score 2). No statistically significant differences were observed in BW gain between the different groups (Table 6). No coccidia challenge was observed, since *Eimeria* oocysts were absent in the excreta and no macroscopic coccidiosis lesions were observed.

Table 6. Experimental groups and impact of DON on the number of chickens affected by necrotic enteritis (NE) and bodyweight (BW) gain.

					mean daily BW gain (g/day) [(2)]		
GROUP	**DON**	***C. perfringens***	**percentage of animals with NE lesions (%)**		**day 1 -7**	**day 8 – 14**	**day 15 – euthanasia**
Cp[(1)] alone	-	+	20 ± 2.6[a]	♂	20 ± 7	38 ± 7	53 ± 23
				♀	19 ± 2	37 ± 4	44 ± 21
Cp + DON[(1)]	+	+	47 ± 3.0[b]	♂	18 ± 3	41 ± 8	54 ± 24
				♀	20 ± 6	44 ± 5	56 ± 19
DON[(1)] alone	+	-	0 ± 0.0[(3)]	♂	18 ± 4	39 ± 7	60 ± 14
				♀	17 ± 5	35 ± 6	44 ± 10
negative control	-	-	0 ± 0.0[(3)]	♂	19 ± 2	44 ± 6	65 ± 19
				♀	17 ± 5	38 ± 6	67 ± 17

Four experimental groups were included, of which each experimental group consisted of 3 cages of 30 chickens. After a feeding period of 3 weeks chickens were euthanized.

(1) Cp: C. perfringens challenge strain 56; DON: deoxynivalenol challenge

(2) Results bodyweight (BW) gain based on ten animals per group in triplicate

(3) Since the mean NE lesion score in groups DON alone and negative control was zero, both groups were excluded from statistical analysis with respect to macroscopic NE lesion scoring.

[a – b] significantly different within one column (P<0.05). All data are presented as mean ± standard deviation.

3.1.2 DON increases the intestinal protein concentration

The total protein concentration in duodenal intestinal content was significantly higher in chickens fed the DON contaminated diet (*P*=0.023). However, no effect of DON on the total protein concentration in jejunal and ileal intestinal content was detected (Figure 13).

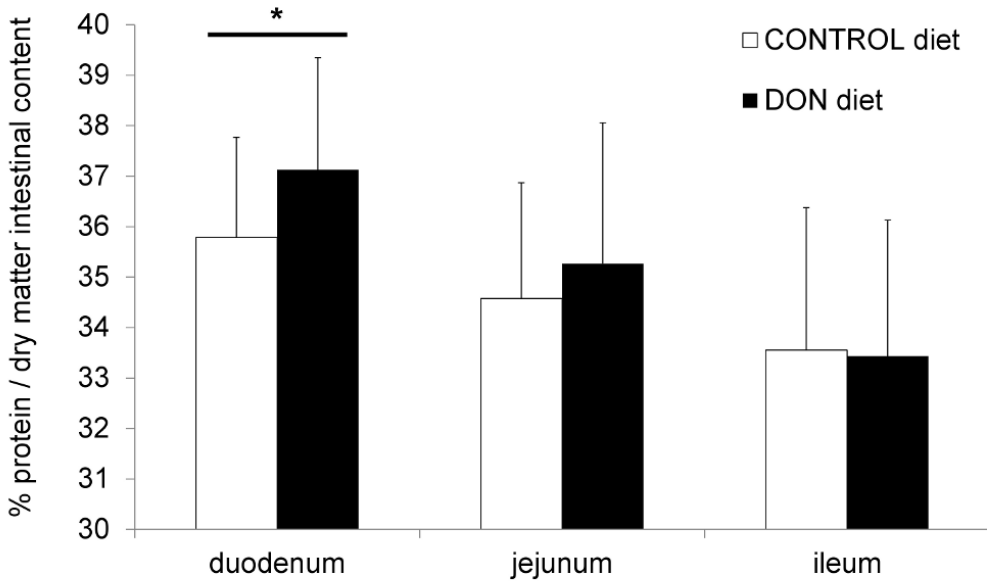

Figure 13. Protein concentration in intestinal content is significantly increased in duodenum of chickens fed a DON-contaminated diet. *Percentage crude protein per dry matter of the intestinal content was determined by the Kjeldahl method. Results are presented as the mean protein level of 27 samples per group per intestinal segment. Error bars represent SD.* $^{(*)}$ *significantly different (P<0.05) within one intestinal segment*

3.1.3 DON reduces transepithelial electrical resistance in duodenal segments

No difference was observed between the control and the DON group for PD (-2.7 ± 0.14 and -2.3 ± 0.13 mV, respectively) and Isc (7.0 ± 0.38 and 6.4 ± 0.36 µA/cm², respectively), but TEER was significantly lower (*P*<0.001) for DON fed birds (369.8 ± 5.47 Ω.cm²) compared with the control birds (392.2 ± 4.72 Ω.cm²).

3.1.4 DON reduces duodenal villus height

Results as presented in Table 7 show a significant shortening of the villi in the duodenum for the DON group compared to the control group (*P*=0.014). A trend was observed for reduction in the villus height to crypt depth ratio in the duodenum (*P*=0.073) and for the crypt depth in the jejunum in the DON group (*P*=0.052).

Table 7. Effect of DON on villus height and crypt depth measurements.

	control diet	**DON diet**	***P***
mid-duodenum			
villus height (µm)	2,175 ± 26.8	2,010 ± 52.9	0.014 (*)
crypt depth (µm)	143 ± 4.7	154 ± 9.1	0.269
villus to crypt ratio	16 ± 0.5	13 ± 1.0	0.073
mid-jejunum			
villus height (µm)	894 ± 72.6	792 ± 61.6	0.303
crypt depth (µm)	178 ± 12.3	150 ± 6.4	0.052
villus to crypt ratio	5 ± 0.3	5 ± 0.4	0.978
mid-ileum			
villus height (µm)	711 ± 63.8	689 ± 23.6	0.748
crypt depth (µm)	153 ± 10.6	144 ± 6.7	0.456
villus to crypt ratio	5 ± 0.2	5 ± 0.3	0.487

Analysis was based on 9 animals per treatment, and the mean of 5 to 15 measurements per segment per animal was calculated; data are presented as weighted mean ± SEM. (*) significantly different (P<0.05).

3.2 *In vitro* experiment

3.2.1 No impact of DON on *C. perfringens* growth, alpha toxin production and *netB* transcription

The results of the *C. perfringens* growth assay showed no influence of 0, 0.2, 2 or 20 µg DON/mL on the bacterial growth curve (Figure 14). Quantification of alpha toxin also revealed no impact of these concentrations of mycotoxin. The mean OD of the alpha toxin detection, relative to the positive control value was 1.1 ± 0.05, 1.1 ± 0.01, 1.1 ± 0.03 and 1.1 ± 0.03 in the presence of 0, 0.2, 2 or 20 µg DON/mL, respectively. Measurement of the *C. perfringens* transcription level of *netB* by qRT-PCR showed no influence of DON (Figure 15).

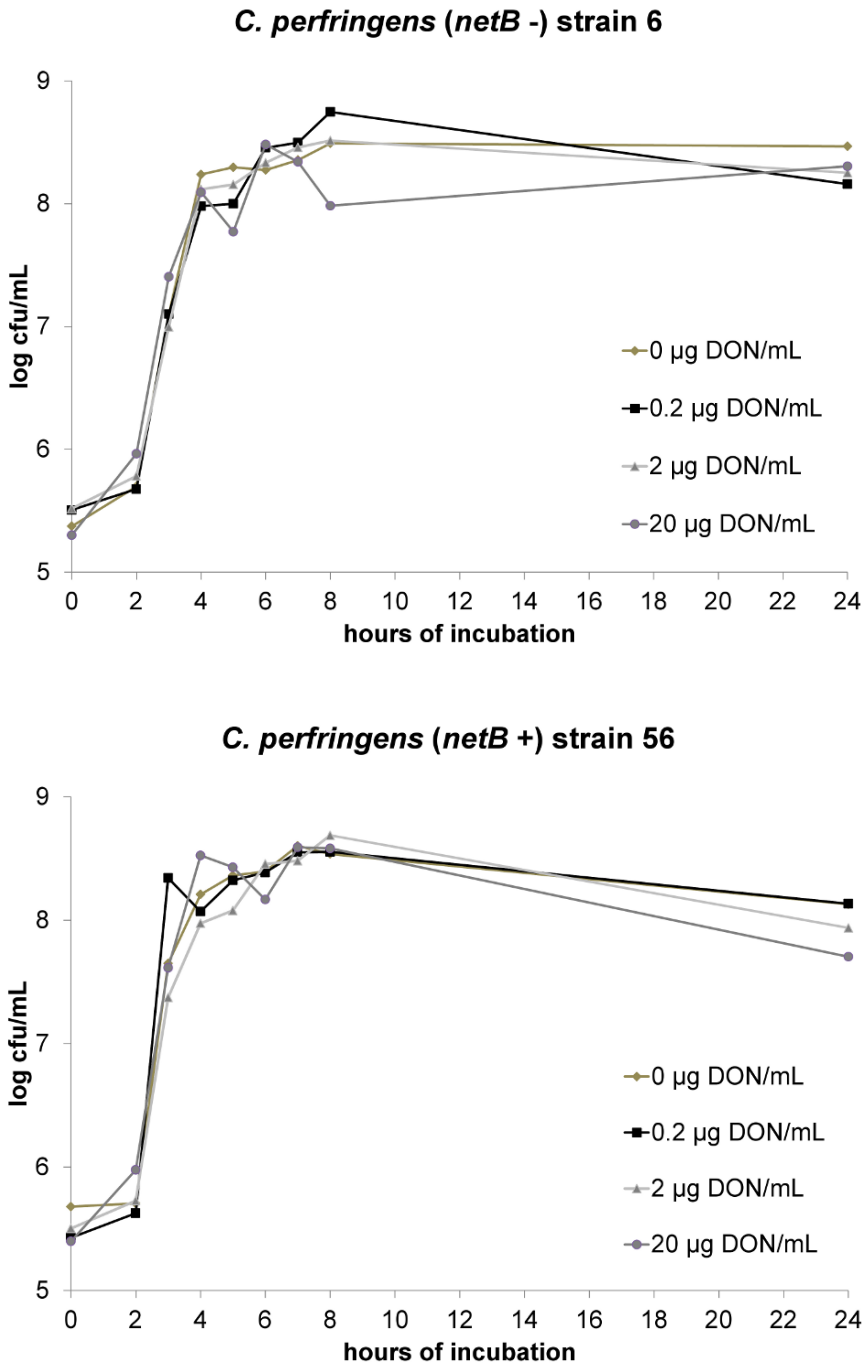

Figure 14. No impact of DON on in vitro growth of C. perfringens. *C. perfringens strains 6 and 56 were grown in TGY broth medium containing 0, 0.2, 2 or 20 µg DON/mL. Samples were taken at 0, 2, 3, 4, 5, 6, 7, 8 and 24 h after inoculation with an overnight culture of C. perfringens. The number of colony forming units (cfu) per mL was determined by bacterial plating of 10-fold dilutions. Results are presented as the mean cfu/mL. There is no significant difference between the different test conditions.*

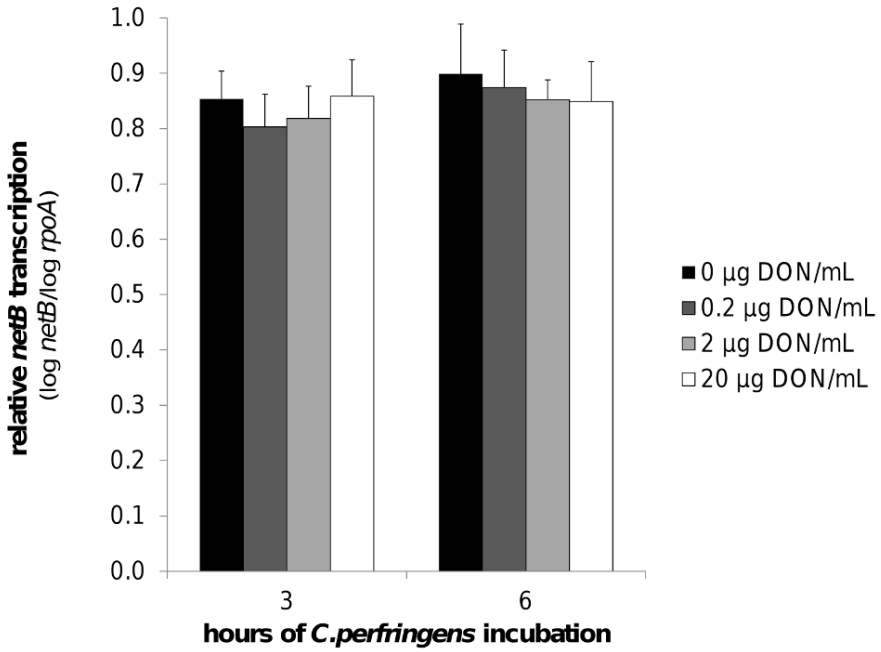

Figure 15. NetB toxin transcription is not influenced by DON. *Transcription level of netB toxin was analysed by qRT-PCR of C. perfringens strain 56 RNA samples collected from in vitro culture material in the mid (after 3 h incubation) and late logarithmic (after 6 h incubation) growth phase. C. perfringens strain 56 was grown in absence or presence (0.2, 2, 20 µg/mL) of DON. The results for the netB gene transcription were normalized to the rpoA gene transcription. Results are presented as the mean value of three biological replicates. Error bars represent SD. There is no significant difference between the different test conditions.*

4 Discussion

Our data demonstrate that the mycotoxin DON is a predisposing factor for the development of NE in broiler chickens. Indeed, contamination of the diet with DON at concentrations below the EU maximum guidance level of 5,000 µg/kg feed, significantly increased the number of chickens affected with NE.

The distribution of NE lesions in the present infection study, mainly in duodenum and jejunum, is similar as in a previously described NE infection trial, where coccidiosis was included as predisposing factor [158]. The proximal part of the intestinal tract is the main absorption site for DON [15,16,19]. Proximal intestinal epithelial cells are thus exposed to high concentrations of DON following ingestion of DON-contaminated feed, and are as such sensitive due to their high protein turnover [16,47,258]. DON negatively affected the proximal part of the intestinal tract, demonstrated by the significantly reduced villus height in the

duodenum. These results are in accordance with those observed by Awad *et al.* [19], who tested a similar contamination level and duration of exposure of DON. The decreased villus height will compromise the effectiveness of nutrient absorption due to the decreased absorption surface area [171]. Enterocytes must differentiate during their migration along the crypt-villus axis to fully express their digestive functions [14]. The sucrase and maltase activities increase for example towards the villus tip in chicks [419]. As such, the negative impact of DON on the villus height can be associated with an impaired nutrient digestion due to a reduced number of differentiated epithelial cells [171].

DON also modulates the intestinal paracellular transport leading to an increased passage of macromolecules and bacteria [322]. The intestinal barrier function is maintained by intercellular structures, including tight junctions, adherence junctions and desmosomes [47,166]. The TEER is considered as an indicator of the epithelial integrity and thus of the organization of tight junctions. In accordance with literature [258,322], we demonstrated a reduction of the TEER of the duodenal epithelium after DON exposure. These toxic effects on epithelial cells contribute to an increased protein availability in the intestinal lumen due to leakage of plasma amino acids or proteins into the gut. Consequently, this creates an environment that favors for massive overgrowth of *C. perfringens*. Indeed, in this study, the total duodenal protein level was increased. This could be caused by malabsorption, a negative effect on nutrient digestion or plasma amino acid or protein leakage in the intestine due to the altered intestinal barrier integrity. Malabsorption and maldigestion was also suggested by the decreased duodenal villus height. Furthermore, it has been shown that DON selectively modulates the activities of different intestinal transporter proteins for nutrients, and negatively influences the sodium associated amino acid co-transport for serine and proline, leading to an increased intestinal content of these amino acids [26,107,258]. We propose a negative effect of DON on the small intestinal mucosa that leads to malabsorption, maldigestion and leakage of plasma amino acids or proteins into the intestinal lumen, which provide the necessary growth substrate for extensive proliferation of *C. perfringens*.

The *in vitro* growth of *C. perfringens* was not affected by concentrations of DON up to 20 µg/mL. No influence on alpha toxin production, and *netB* transcription was demonstrated. These results suggest that the observed predisposing effect is due to the toxic effect of DON on the animal host rather than its effect on the bacterium itself.

5 Conclusion

In conclusion, as summarized in Figure 16, our results indicate that the intake of DON contaminated feed at contamination levels below the EU maximum guidance level, is a predisposing factor for the development of necrotic enteritis in broiler chickens due to the negative influence on the epithelial barrier, and to an increased intestinal nutrient availability for clostridial proliferation. We showed that DON has a cytotoxic effect on enterocytes, leading to an altered intestinal barrier function, resulting in an increased permeability of the intestinal wall. Additionally, the shortened villus height could lead to a decreased absorption of dietary proteins, resulting in an increased protein concentration in the intestinal lumen. These mechanisms lead to an increased protein content in the intestinal lumen, which is available for clostridial proliferation resulting in the development of necrotic enteritis.

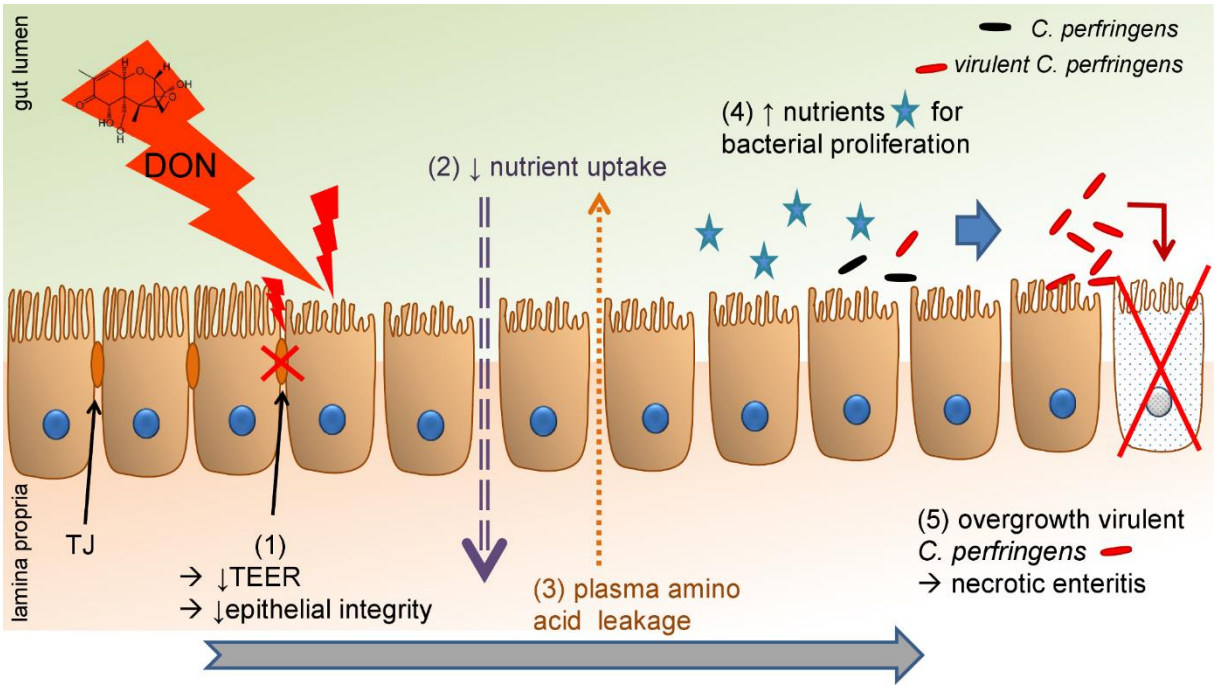

Figure 16. Deoxynivalenol predisposes for C. perfringens induced necrotic enteritis. *DON decreased villus height and reduced transepithelial electrical resistance (1), leading to a decreased absorption and digestion of dietary nutrients; and an increased intestinal barrier permeability, respectively. Taken together with an increased intestinal protein level, these results suggest an impaired nutrient uptake (2) and leakage of plasma amino acids (3) into the intestinal lumen, providing the necessary growth substrate for C. perfringens proliferation (4). Proliferation of virulent (netB positive) C. perfringens induces necrotic enteritis (5).*

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CHAPTER 2

The predisposing effect of fumonisins on necrotic enteritis in broiler chickens

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Adapted from

Antonissen G., Croubels S., Pasmans F., Ducatelle R., Eeckhaut V., Devreese M., Verlinden M., Haesebrouck F., Eeckhout M., De Saeger S., Antlinger B., Novak B., Martel A., Van Immerseel F. (2015) Fumonisins affect the intestinal microbial homeostasis in broiler chickens, predisposing to necrotic enteritis (Accepted in Veterinary Research)

Abstract

Fumonisins (FBs) are mycotoxins produced by *Fusarium* fungi. This study aimed to investigate the effect of these feed contaminants on the intestinal morphology and microbiota composition, and to evaluate whether FBs predispose broilers to necrotic enteritis. One-day-old broiler chicks were divided into a group fed a control diet, and a group fed a FBs contaminated diet (18.6 mg FB1+FB2 /kg feed). A significant increase in the plasma sphinganine/sphingosine ratio in the FBs-treated group (0.21 ± 0.016) compared to the control (0.14 ± 0.014) indicated disturbance of the sphingolipid biosynthesis. Furthermore, villus height and crypt depth of the ileum was significantly reduced by FBs. Denaturing gradient gel electrophoresis showed a shift in the microbiota composition in the ileum in the FBs group compared to the control. A reduced presence of low-GC containing operational taxonomic units in ileal digesta of birds exposed to FBs was demonstrated, and identified as a reduced abundance of *Candidatus* Savagella and *Lactobaccilus* spp. Quantification of total *Clostridium perfringens* in these ileal samples, previous to experimental infection, using *cpa* gene (alpha toxin) quantification by qPCR showed an increase in *C. perfringens* in chickens fed a FBs contaminated diet compared to control (7.5 ± 0.30 versus 6.3 ± 0.24 log10 copies/g intestinal content). After *C. perfringens* challenge, a higher percentage of birds developed subclinical necrotic enteritis in the group fed a FBs contaminated diet as compared to the control (44.9 ± 2.22% versus 29.8 ± 5.46%).

Keywords

Broiler – *Clostridium perfringens* – Fumonisins – Microbiota – Necrotic Enteritis

1 Introduction

Mycotoxins are naturally occurring secondary fungal metabolites produced both pre- and post-harvest in crops and other feed and food commodities. *Fusarium*, *Aspergillus*, and *Penicillium* are the most abundant mycotoxin producing mould genera contaminating feed and feed raw materials [39]. Fumonisins (FBs) are produced by *Fusarium verticillioides*, *F. proliferatum*, and other *Fusarium* species and are among the most widespread mycotoxins [431]. FBs are ubiquitous contaminants of corn and other grain products [386]. A global survey on the occurrence and contamination levels of mycotoxins in feed raw materials and finished feed for livestock animals showed that 54% of 11 439 tested samples were contaminated with FBs [358]. FBs were most frequently detected in South American (77%), African (72%) and Southern European (70%) samples, and less frequently in Oceania (10%) [358]. The economic impact of mycotoxins in animal feed is rather difficult to measure because information about subclinical effects on animal health and productivity losses due to chronic low level exposure is limited. Wu [443] estimated the annual economic losses in the USA due to FBs in animal feed to be US$ 1-20 million and US$ 30-46 million, in a normal and an outbreak year of *Fusarium* ear rot, respectively.

More than 28 FB homologues have been identified. Fumonisin B_1 (FB_1) is the most common and the most thoroughly investigated mycotoxin because of its toxicological importance. FB_2 and FB_3 are less prevalent, and differ structurally from FB_1 in the number and position of hydroxyl groups [431]. FBs competitively inhibit the ceramide synthase and, as a result, interfere with the biosynthesis of ceramides and sphingolipids of cell membranes [344,431]. Clinical outbreaks have been reported in horses (equine leucoencephalomalacie, ELEM) and pigs (porcine pulmonary edema, PPE). These animal species are regarded as the most susceptible to the effects of FBs [431]. In general, poultry are considered to be quite resistant toward the deleterious effects of FBs. Also species differences occur, laying hens and broilers are less sensitive to FBs compared to turkeys and ducks [225,229,397,409,438]. In broilers, systemic uptake of FB_1 after oral exposure is low, indicating that the absorption is negligible [10]. Following the consumption of FBs contaminated feed, the intestine is the first organ to be exposed to these toxins and negative effects on intestinal tissues have been reported [48]. The jejunum of broilers exposed to high FB_1 concentrations (≥100 mg/kg feed) for 28 days displays a reduced villus height (VH) and villus-to-crypt ratio (V:C) [340]. Besides a mild villus atrophy, also goblet cell hyperplasia is observed in broiler chicks exposed to high levels of FB_1 (300 mg/kg feed) for 2 weeks [57]. It has been shown in vitro that FB_1 has a toxic effect on both undifferentiated and

differentiated porcine intestinal epithelial cells (IPEC-1). The effect of FB_1 on epithelial cell proliferation correlates with a cell cycle arrest in the G0/G1 phase [46]. A negative effect of FB_1 on the expression of cell junction proteins E-cadherin and occludin, and consequently on the intestinal epithelial integrity, has been shown in vivo in pigs [31,50]. Furthermore, FB_1 modulates intestinal immunity by decreasing the expression of several cytokines, for example interleukin (IL)-1β, IL-2, IL-8, IL-12p40 and interferon (IFN)-γ in pigs [104,172,306]. It has been shown that exposure of pigs to 0.5 mg FB_1/kg bodyweight (BW) for 6 days enhanced intestinal colonization and translocation of a septicemic *Escherichia coli* (SEPEC) [306]. Feeding a FBs contaminated diet (11.8 mg FB_1+FB_2/kg feed) for 9 weeks transiently modified the faecal microbiota composition in pigs. Co-exposure to FBs and *Salmonella* Typhimurium amplified this phenomenon [59]. At present it is unclear what may be the consequences of long term exposure to low levels of FBs.

In poultry, necrotic enteritis (NE) is caused by netB producing *Clostridium perfringens* strains. *C. perfringens* is a Gram-positive spore-forming anaerobic bacterium that is commonly found in the environment and the gastro-intestinal tract of animals and humans as a member of the normal microbiota [214,378,404]. NE in chickens is still an important intestinal disease despite the application of preventive and control methods, including coccidiosis control. The acute form of the disease causes mortality without premonitory symptoms. The more frequently occurring subclinical form is characterized by intestinal mucosal damage without clinical signs or mortality, leading to decreased performance [404,423]. However, healthy birds often carry netB positive *C. perfringens* without showing any clinical symptoms of NE [404]. An outbreak of NE is a complex process requiring one or a number of predisposing factors rather than just the presence of pathogenic *C. perfringens* [368,424,441]. Pre-existing mucosal damage caused by coccidiosis, high protein feed (including fishmeal) and indigestible non-starch polysaccharides are well known predisposing factors [441]. Recently, it was shown that the mycotoxin deoxynivalenol (DON) is also a predisposing factor for the development of NE, through damage to the epithelial barrier and an increased intestinal nutrient availability for clostridial proliferation [13]. Although FBs are ubiquitous contaminants in poultry feed, information about their impact on the intestinal microbial homeostasis in broiler chickens is lacking.

The objective of this study was to evaluate the effect of FBs on the intestinal microbial homeostasis, at concentrations approaching the European Union maximum guidance levels (20 mg FB_1+FB_2/kg feed) [131]. Therefore, the influence of FBs on the intestinal microbiota composition and intestinal morphology was investigated. In addition, an attempt was made to demonstrate the consequences of the effect of FBs on the intestinal microbial homeostasis in a subclinical necrotic enteritis model.

2 Materials and methods

2.1 Fumonisins

FBs (8.64mg FB_1+FB_2/g culture material) (Biopure - Romer Labs Diagnostic GmbH, Tulln, Austria) were produced in vitro from a culture of *F. verticillioides*, and subsequently crystallized [234]. For the in vitro assessment of the impact of FB_1 on growth and toxin production characteristics of *C. perfringens*, serial dilutions were prepared in tryptone glucose yeast (TGY) broth medium of a FB_1 stock solution of 5000 µg/mL (Fermentek, Jerusalem, Israel) that had been prepared in anhydrous methanol and stored at −20 °C.

2.2 Bacterial strain and growth conditions

C. perfringens strain 56, a *netB*$^+$ type A strain, was originally isolated from a broiler chicken with NE and has been shown to be virulent in an in vivo infection model [13,158]. The inoculum for the oral infection of chickens and in vitro experiments was prepared by culturing *C. perfringens* anaerobically overnight at 37 °C in brain heart infusion broth (BHI, Bio-Rad, Marnes-la-Coquette, France) or tryptone glucose yeast (TGY) broth medium, respectively. The colony forming units of *C. perfringens*/mL was assessed by plating tenfold dilutions on Colombia agar (Oxoid, Basingstoke, UK) with 5% sheep blood, followed by anaerobic overnight incubation at 37 °C.

2.3 Animal experiment

2.3.1 Birds and housing

The animal experiment was performed using non-vaccinated Ross 308 broiler chickens, obtained as one-day-old chicks from a commercial hatchery (Vervaeke-Belavi, Tielt, Belgium). Animals of both treatment groups, control diet and FBs contaminated diet, were housed in the same temperature controlled room, in pens of 1.44 m², on wood shavings. Each

group consisted of three pens of 34 birds, with approximately equal numbers of males and females. Animal units were separated by solid walls to prevent direct contact between animals from different pens. All cages were decontaminated with peracetic acid and hydrogen peroxide (Metatectyl HQ, Metatecta, Kontich, Belgium) and a commercial anticoccidial disinfectant (Bi-OO-Cyst Coccidial Disinfectant, Biolink, York, UK) prior to the housing of the chickens. Water and feed was provided ad libitum. Chickens were not fasted before euthanasia. The animal experiment was approved by the Ethical Committee of the Faculty of Veterinary Medicine and Bioscience Engineering, Ghent University (EC 2012/194).

2.3.2 Feed preparation and experimental diets

All chickens were fed a starter diet during the first eight days of the trial, and subsequently a grower diet. The diet was wheat and rye based, with soybean meal as main protein source during the first 16 days. From day 17 onwards, the same grower diet was fed with the exception that fishmeal replaced soybean meal as main protein source [13,158].

FBs contaminated feed was produced by adding lyophilized FBs culture material to a control diet. Mycotoxin contamination of both the control and FBs contaminated diet was analyzed by a validated multi-mycotoxin liquid chromatography-tandem mass spectrometry method (LC-MS/MS) [276]. Three different batches of FBs contaminated feed were produced: a starter diet, a grower diet with soybean meal and a grower diet with fishmeal, respectively. Therefore, FBs culture material was added to 500 g of the corresponding batches of control diet. For each batch, this premix was then mixed with 5 kg of control feed to assure homogeneous distribution of the toxin and finally mixed for 20 min in the total amount of feed needed for each batch. To test mycotoxin contamination, samples were taken at three different locations in each batch, subsequently pooled per batch and analyzed for mycotoxin contamination as described above. Trace amounts of nivalenol and DON were detected in the control feed (0.059-0.116 and 0.113-0.170 mg/kg feed, respectively). Analyzed mycotoxins, their limit of detection and limit of quantification were as previously described [13,276]. The levels of FBs and all other tested mycotoxins in the different batches of control feed were below the limit of detection. The average levels of FB_1, FB_2 and FB_3 in the different batches of FBs contaminated feed were 10.4 mg/kg, 8.2 mg/kg and 2.0 mg/kg, respectively (Table 8). The average sum of FB1+FB2, 18.6 mg/kg feed was approaching the EU maximum guidance level in feed for poultry of 20 mg FB1+FB2/kg (2006/576/EC) [131].

Table 8. Concentration of FB_1, FB_2 and FB_3 in different batches of FBs contaminated diet.

type of feed	feeding period	FB_1	FB_2	FB_1+FB_2	FB_3
		mg/kg feed			
starter	day 1-8	8.0	6.6	14.6	1.6
grower (soybean meal)	day 9-16	14.5	10.6	25.1	2.5
grower (fishmeal)	day 17-end	8.8	7.4	16.2	1.8

2.4 Evaluation of the impact of FBs on broiler health

The BW of all chickens was measured on day 1 and day 8. On day 15, six birds (3♂/3♀) per pen were euthanized using an overdose of sodium pentobarbital (Natrium Pentobarbital 20%, Kela Veterinaria, Sint- Niklaas, Belgium). A blood sample was collected and subsequently a necropsy was performed. Blood samples were centrifuged (2851 × *g*, 10 min, 4 °C) and plasma was stored at ≤−20 °C until sphinganine (Sa) and sphingosine (So) concentrations were analyzed. Sa/So is suggested to be the most sensitive biomarker to FBs intoxication in many animals [431]. Plasma Sa and So concentrations were analyzed by a commercial service provider (Biocrates Life Sciences AG, Innsbruck, Austria). Briefly, Sa and So were extracted from plasma and measured in the presence of internal standards using LC-MS/MS with electrospray ionization. The BW and weight of different organs (liver, spleen, kidneys, proventriculus, ventriculus, bursa of Fabricius, heart and lungs) were recorded. The weight of each organ was converted to a relative percentage of the BW.

2.5 Evaluation of the impact of FBs on intestinal morphology

After measuring the length of the different small intestinal segments, 1 cm samples from the mid-duodenum, mid-jejunum and mid-ileum were collected and fixed in neutral-buffered formalin. Small intestinal segments were defined as duodenum encompassing the duodenal loop, jejunum between the end of the duodenal loop and Meckels diverticulum and ileum between Meckels diverticulum and the ileo-cecal junction. Villus height and crypt depth of mid-duodenum, mid-jejunum and mid-ileum were measured on hematoxylin and eosin stained histological paraffin sections using light microscopy with Leica LAS software (Leica Microsystems, Diegem, Belgium). The average of ten measurements per segment per animal was calculated.

2.6 Assessment of the impact of FBs on the intestinal microbiota

At day 15, intestinal content samples of the second half of the different small intestinal segments of all six bird per pen were collected, snap frozen in liquid nitrogen, and subsequently stored at −80 °C until further DNA extraction. DNA from intestinal content (duodenum, jejunum and ileum) was extracted using a modified QIAamp DNA Stool mini Kit (Qiagen, Hilden, Germany) protocol. An enzymatic pretreatment with lysozyme and a mechanical disruption step with a bead-beater was added to the original protocol. In brief, frozen intestinal content (250 mg) was transferred into a bead beating tube filled with 0.7 g of glass beads (Ø 100 µm), 0.6 g of ceramic beads (Ø 1.4 mm) and one glass bead (Ø 3.8 mm). Subsequently, 200 µL of TE buffer of pH 8 (10 mM Tris-HCl and 1 mM EDTA (Sigma Aldrich, Steinheim, Germany)) and 125 µL of freshly prepared lysozyme (100 mg/mL, Sigma Aldrich) was added. After homogenizing by vortex mixing (1 min), samples were incubated at 37°C for 45 min at 1000 rpm on a Thermomixer compact shaker incubator (Eppendorf, Hamburg, Germany). The final volume was adjusted to 2 mL with ASL buffer (Qiagen) and samples were bead beated for 10 s at 6000 rpm on a Precellys 24-Dual homogenizer (Bertin Technologies, Montigny le Bretonneux, France). Further DNA extraction was performed with the QIAamp DNA Stool mini kit (Qiagen) in accordance with the manufacturer's instructions. DNA integrity was evaluated by loading 3 µL of DNA on a 0.8% agarose gel stained with ethidium bromide. The purity and concentration of the extracted DNA were measured using ultraviolet absorption at 260/280 nm and 230/280 nm ratio (NanoDrop 1000 spectrophotometer, Thermo Scientific, Waltham, MA, USA).

Denaturing gradient gel electrophoresis (DGGE) separates DNA fragments of the same length but with different base-pair sequences. DNA fragments were generated from the small intestinal content DNA samples applying community PCR with universal bacterial primers targeting the variable V3 region of the 16S ribosomal RNA. The nucleotide sequences of the primers were as follows: forward primer F341 with GC clamp 5'-CGC CCG CCG CGC GCG GGC GG GCG GGG GCA CGG GGGG - CCT ACG GGA GGC AGC AG 3' and reverse primer R518 5'ATT ACC GCG GCT GCT GG-3' [284]. PCR amplification was performed in duplicate using a Mastercycler Gradient (Eppendorf, Hamburg, Germany), and each PCR reaction was done in a 45 µL total reaction mixture using 3 µL of the DNA sample (4 ng/µL), 0.125 µM of each of the primers, 100 µM deoxynucleotide triphosphate (dNTP) (Peqlab Biotechnologie GmbH, Erlangen, Germany), and 0.6 µL of peqGOLD Taq-DNA-

Polymerase (5 U/µL) (Peqlab). The PCR conditions used were 1 cycle of 94 °C for 5 min, followed by 9 cycles of 94 °C for 30 s, 64 °C for 40 s (decreased by 0.5 °C/cycle) and 72 °C for 40 s. Subsequently 19 cycles of 94 °C for 30 s, 56 °C for 40 s and 72 °C for 40 s, followed by one cycle of 72 °C for 4 min, were run. DGGE was performed as described by [284] with the INGENYPhorU-2x2 system (Ingeny, Goes, The Netherlands). Briefly, amplicons were separated using a 30 to 60% denaturating gradient [284]. 30 µL of the PCR product was loaded and electrophoresis was performed at 100 V for 16 h at 60 °C. Each gel included four standard reference lanes containing amplicons of 12 bacterial species for normalization and comparison between gels. DGGE gels were stained with 1x SYBR Green I (Sigma-Aldrich) for 30 min. Fingerprinting profiles were visualized using the Bio Vision Imaging system (Peqlab) and the Vision-Capt software (Vilber Lourmat, Marne-la-Vallée, France). The microbial profiles were processed with GelCompar II vs. 6.6 (Applied Maths NV, Sint-Martens-Latem, Belgium).

The similarity between DGGE-profiles, given as a percentage, was analyzed using the Dice similarity coefficient, derived from presence or absence of bands. On the basis of a distance matrix, which was generated from the similarity values, dendrograms were constructed using the unweighted pair group method with arithmetic means (UPGMA) as clustering-method. The microbial richness (R) was assessed as the number of bands within a profile.

Low-GC-containing operational taxonomic units (OTUs) were selected for identification based on the differences between the DGGE patterns of the control group compared to the FBs contaminated group. After extraction of the selected bands, and reapplication on DGGE to confirm their positions relative to the original sample, the respective 16S-fragments were sequenced (LGC Genomics, Berlin, Germany) and aligned to the NCBI GenBank prokaryotic 16S ribosomal RNA database using the standard nucleotide BLASTN 2.2.30+ (nucleotide basic local alignment search tool) [456].

2.7 Evaluation of the consequences of FBs exposure on necrotic enteritis

2.7.1 Quantification of total *C. perfringens* by qPCR

Total *C. perfringens* in ileal content samples, collected before experimental *C. perfringens* challenge (day 15), was quantified using the *cpa* gene (encoding alpha toxin) as target gene. qPCR was performed using SYBR-green 2x master mix (Bioline, Brussels, Belgium) in a Bio-Rad CFX-384 system. Each reaction was done in triplicate in a 12 µL total reaction mixture using 2 µL of the DNA sample and 0.5 µM final qPCR primer concentration (Table 9). The qPCR conditions were 1 cycle of 95°C for 10 min, followed by 40 cycles of 95 °C for 30 s, 60 °C for 30 s, and stepwise increase in the temperature from 65° to 95 °C (at 10s/0.5 °C). Melting curve data were analyzed to confirm the specificity of the reaction. For construction of the standard curve, the PCR product was generated using the standard PCR primers (Table 2) and DNA from *C. perfringens* strain CP56. After purification (MSB Spin PCRapace, Stratec Molecular, Berlin, Germany) and determination of the DNA concentration with a Nanodrop ND 1000 spectrophotometer (Nanodrop Technologies, Wilmingtom, DE, USA), the concentration of the linear dsDNA standard was adjusted to 1×10^8 to 1×10^1 copies per µL with each step differing by 10 fold. The copy numbers of samples (copies / g intestinal content) were determined by reading off the standard series with the Ct values of the samples.

Table 9. Primer sequences used for (q)PCR analyses.

target	forward primer / reverse primer	analysis	reference
cpa	AGT CTA CGC TTG GGA TGG AA / TTT CCT GGG TTG TTC ATT TC	PCR	32
cpa	GTT GAT AGC GCA GGA CAT GTT AAG / CAT GTA GTC ATC TGT TCC AGC ATC	qPCR	159
netB	TGA TAC CGC TTC ACA TAA AGG T / ACC GTC CTT AGT CTC AAC AAA T	PCR	13
netB	TCA ATT GGT TAT TCT ATA GGC GGT A / ATA TGA AGC ATT TAT TCC AGC ACC A	qPCR	66
rpoA	ACA TCA TTA GCG TTG TCA GTT AAA G / GAG GTT ATG GAA TAA CTC TTG GTA ATG	PCR	13
rpoA	CCA TCT GTT TTT ATA TCT GCT CCA GTA / GGA AGG TGA AGG ACC AAA AAC TAT T	qPCR	66

Sequences are presented from 5' to 3'.

2.7.2 *C. perfringens* infection trial

The remaining 28 animals per pen were used in a *C. perfringens* experimental infection trial as previously described [13]. The BW of all animals was measured on day 16 and day 21. Gumboro vaccine (Nobilis Gumboro D78, MSD Animal Health, Brussels, Belgium) was administered on day 16 in the drinking water of all cages. Both groups were experimentally infected with an oral bolus of 4.10^8 cfu *C. perfringens* strain 56 on days 17, 18, 19 and 20. On day 21, 22 and 23, each day one third of each group was euthanized by overdose sodium pentobarbital and immediately submitted to necropsy. Macroscopic NE lesion scoring of the small intestines (duodenum, jejunum and ileum) was performed single-blinded as follows; 0 no gross lesions; 1 small focal necrosis or ulceration (one to five foci); 2 focal necrosis or ulceration (six to 15 foci); 3 focal necrosis or ulceration (16 foci or more); 4 patches of necrosis of 2 to 3 cm long; 5 diffuse necrosis typical field cases, partially adapted from [215]. Chickens with a lesion score of 1 or more were classified as NE positive.

2.7.3 *In vitro* assessment of the effect of FB_1 on *C. perfringens* growth and alpha and netB toxin production

Following concentrations of FB_1 were tested for their effect on *C. perfringens* growth and toxin production: 0, 0.2, 2 and 20 µg FB_1/mL. All tests were performed in triplicate.

The *C. perfringens* inoculum was 1:1000 diluted in TGY medium, containing the different concentrations of FB_1, and incubated anaerobically at 37 °C. A growth curve was produced by bacterial plating of a ten-fold dilution series of the culture at 0, 2, 3, 4, 5, 6, 7, 8 and 24 h after inoculation. Ten-fold dilutions were prepared in phosphate buffered saline (PBS), and subsequently plated on Colombia agar with 5% sheep blood. After anaerobic incubation overnight at 37 °C, the number of colony forming units (cfu)/mL was determined.

The impact of FB_1 on *cpa* (alpha toxin) and *netB* (netB toxin) transcription was tested by qRT-PCR. The *C. perfringens* inoculum was 1:10 000 diluted in TGY medium, containing the different concentrations of FB_1, and incubated anaerobically at 37 °C until an optical density (OD) of 0.6-1.0 was measured at a wavelength of 600 nm (6h of incubation). The transcription levels of *cpa* and *netB* in the presence of FB_1 were compared to non-FB_1 contaminated test conditions and normalized to the housekeeping gene *rpoA*, encoding RNA polymerase subunit A. Total RNA was isolated using SV total RNA Isolation system (Promega, Leiden, The Netherlands). RNA was treated with Turbo DNA-free kit (Ambion,

Austin, TX, USA) per the manufacturer's instructions to remove genomic DNA contamination. Subsequently, RNA was converted to cDNA with iScript cDNA Synthesis Kit (Bio-Rad, Temse, Belgium). qRT-PCR was performed using SYBR-green 2x master mix (Bioline, Brussels, Belgium) in a Bio-Rad CFX-384 system. Each reaction was done in triplicate in a 12 µL total reaction mixture using 2 µL of cDNA sample and 0.5 µM final qPCR primer concentration (Table 9). The qPCR conditions were as described above for total *C. perfringens* determination in ileal content samples. For construction of the standard curve, the PCR product was generated using the standard PCR primers (Table 9).

2.8 Statistical analyses

Statistical program SPSS version 22 was used for data analysis. To compare the number of NE positive birds (lesion score ≥2) between different groups, binomial logistic regression was used. All other parameters, including BW relative organ weight, length of small intestines, Sa/So ratio, villus height/crypt depth measurements, concentration of *C. perfringens* in ileal digesta, in vitro assessment of clostridial growth, and *cpa* and *netB* transcription were analyzed by an independent Student's t-test, after determination of normality. Significance level was set at 0.05.

3 Results

3.1 FBs negatively affect broiler health

The inhibition of ceramide synthase by FBs causes an intracellular accumulation of sphingoid bases, mainly sphinganine. An increased Sa/So is suggested to be the most sensitive biomarker to FBs intoxication in many animal species [431]. The plasma Sa/So ratio was 1.5 fold higher in animals fed the FBs contaminated diet compared to the control animals, 0.21 ± 0.016 versus 0.14 ± 0.014, respectively ($P < 0.001$). No significant differences were observed in BW between the control group and the FBs contaminated group (Table 10). A trend ($P = 0.060$) was observed for an increased relative weight of liver in chickens fed the FBs contaminated diet (3.69 ± 0.134% of BW) compared to the control group (3.39 ± 0.081% of BW). Relative weight of bursa, spleen, proventriculus, ventriculus, kidneys, lungs and heart did not differ between both experimental groups (data not shown).

Table 10. Bodyweight of broiler chickens measured on day 1, 8, 16 and 21.

		day 1	day 8	day 16	day 21
				BW (g)	
control diet	♂	43 ± 3 (n=52)	161 ± 22 (n=52)	488 ± 78 (n=43)	794 ± 134 (n=43)
	♀	44 ± 4 (n=50)	153 ± 21 (n=50)	448 ± 53 (n=41)	754 ± 88 (n=41)
FBs contaminated diet	♂	43 ± 3 (n=51)	176 ± 26 (n=51)	519 ± 65 (n=42)	841 ± 88 (n=42)
	♀	43 ± 3 (n=51)	168 ± 23 (n=51)	476 ± 58 (n=42)	785 ± 104 (n=42)

Animals were fed a control diet or a FBs contaminated diet (18.6 mg FB1+FB2/kg feed).
Data presented as mean bodyweight (BW) (g) ± SEM. n= number of animals.

3.2 FBs reduce total small intestinal length, ileal villus height and crypth depth

The total length of the small intestine was significantly (*P=0.033*) decreased in birds of the FBs contaminated group compared to the control group (130.5 ± 2.37 and 139.0 ± 2.95 cm, respectively). No differences were observed in the relative percentage of length of the different segments of the small intestine (Table 11).

Feeding a FBs contaminated diet significantly reduced villus height *(P=0.002)* and crypt depth *(P=0.011)* in ileum (Table 12). No effect was observed on ileal villus to crypt ratio. No effect was shown in duodenum and jejunum.

Table 11. Effect of FBs on length of small intestinal segments.

organ	control diet	FBs contaminated diet	*P*	
total length small intestines[1] (cm)	139.0 ± 2.95	130.5 ± 2.37	*0.033*	*
duodenum (% of total length)[2]	16.9 ± 0.40	17.2 ± 0.35	*0.656*	
jejunum (% of total length)[2]	43.1 ± 0.69	41.7 ± 0.45	*0.118*	
ileum (% of total length)[2]	40.0 ± 0.71	41.1 ± 0.37	*0.184*	

Animals were randomly divided in two experimental groups, each group consisting of three pens. One group was fed a control diet and one was fed a FBs contaminated diet (18.6 mg FB_1+FB_2/kg feed). Six birds (3♂/3♀) per pen were euthanized on day 15 and the length of small intestinal segments was recorded. Data presented as mean ± SEM. (1) total length small intestines including all three segments: duodenum, jejunum and ileum; (2) % of total length= (length segment (cm)/ total length small intestines (cm))x100 (*) significantly different (P<0.05) / trend (P<0.10)

Table 12. Effect of FBs on villus height and crypt depth measurements.

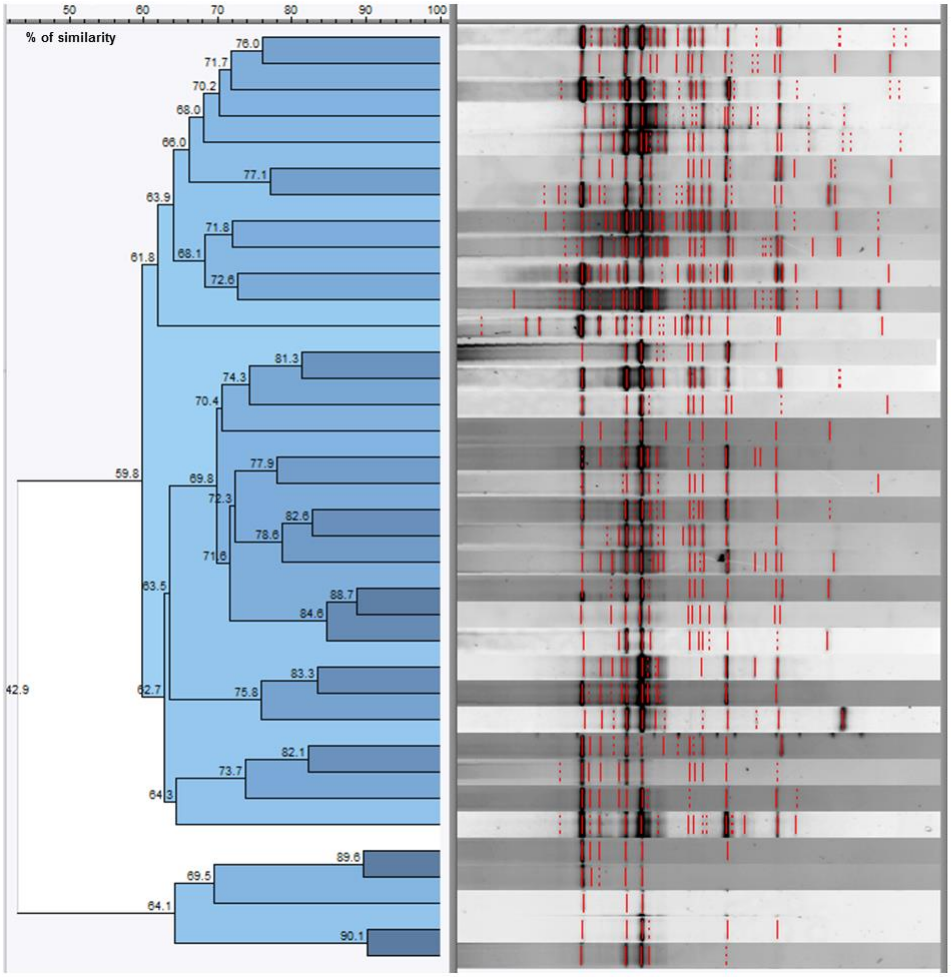
	control diet	FBs contaminated diet	*P*	
mid-duodenum				
villus height (µm)	1628 ± 39.4	1549 ± 44.8	*0.225*	
crypt depth (µm)	196 ± 7.1	197 ± 7.8	*0.999*	
villus to crypt ratio	8 ± 0.3	8 ± 0.4	*0.337*	
mid-jejunum				
villus height (µm)	842 ± 32.0	880 ± 28.6	*0.278*	
crypt depth (µm)	173 ± 6.4	182 ± 6.5	*0.385*	
villus to crypt ratio	5 ± 0.2	5 ± 0.2	*0.927*	
mid-ileum				
villus height (µm)	497 ± 31.7	393 ± 16.2	*0.002*	*
crypt depth (µm)	155 ± 4.4	131 ± 5.2	*0.011*	*
villus to crypt ratio	3 ± 0.2	3 ± 0.1	*0.349*	

Broiler chickens were fed a control diet or a FBs contaminated diet (18.6 mg FB_1+FB_2/kg feed) for 15 days. Samples of different small intestinal segments of six birds (3♂/3♀) per pen, three pens per group, were collected at day 15. Analysis based on mean of 10 measurements per segment per animal was calculated; data are presented as weighted mean ± SEM. (*) significantly different (P<0.05).

3.3 FBs affect the ileal microbiota composition

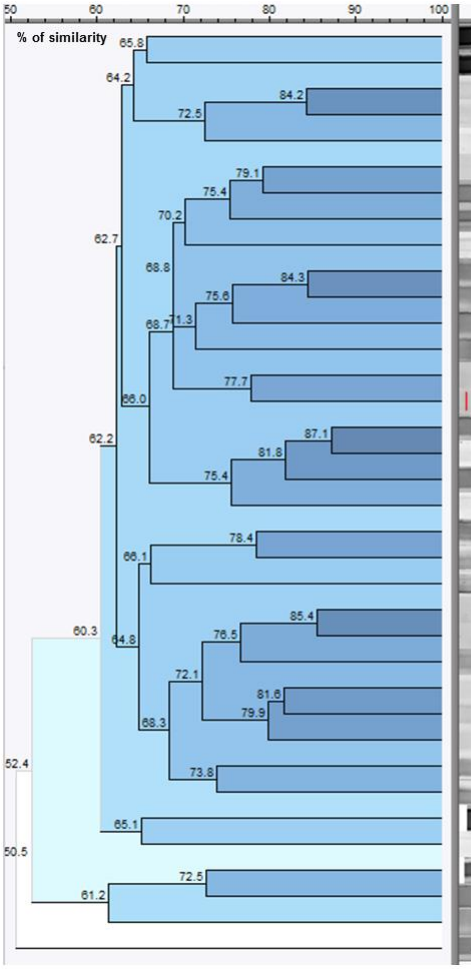
DGGE fingerprint of DNA samples of duodenal and jejunal content, applying community PCR with universal bacterial primers targeting the variable V3 region of the 16S ribosomal DNA, could not show a difference in microbiota composition between chickens fed the control diet or the FBs contaminated diet. In the duodenum, three clades were observed related to the diversity of OTUs, independent of the treatment. One clade showed five samples with a reduced number of bands in the duodenum. Most samples had an average diversity between 10 and 15 OTUs across the medium GC-range. Some samples consisted of 18 to 31 OTUs of the medium and high GC-range (Figure 17). No difference in number of OTUs between both experimental groups was shown in the jejunum. OTUs were all located in the medium range of GC-content (Figure 18). Within ileum content samples a difference of the DGGE fingerprint according to the treatment was seen. The majority of the ileum samples of the control group contained OTUs in the lower GC-range which ascribes them to one clade (Figure 19, clade A). Among the FBs group a clade of clearly reduced diversity is formed by eight samples (Figure 19, clade B). Another group of samples of this treatment group show a similar diversity of OTUs of medium GC-content compared to the control-group, but the low-GC-OTUs were absent (Figure 19, clade C). The dendrogram of DGGE profiles of four chickens (bird 20, 31, 33 and 34) of the FBs group showed a high similarity with the control group (Figure 19, clade D).

Subsequently, five low-GC-content OTUs from ileal content samples of the control group, which made the difference in their DGGE-patterns compared to the FBs group, were identified at genus level by sequencing. Based on these results the most affected groups were related to the genera *Clostridium* and *Lactobacillus*. OTUs 19 and 20 had 99.09-100% sequence similarity with the type strain of *Candidatus* Arthromitus, recently renamed as *Candidatus* Savagella [402]. OTUs 4 and 13 had 100% sequence similarity with *Lactobacillus johnsonii* and, OTU 16 was similar to the sequence of an unknown species of the genus *Lactobacillus*.



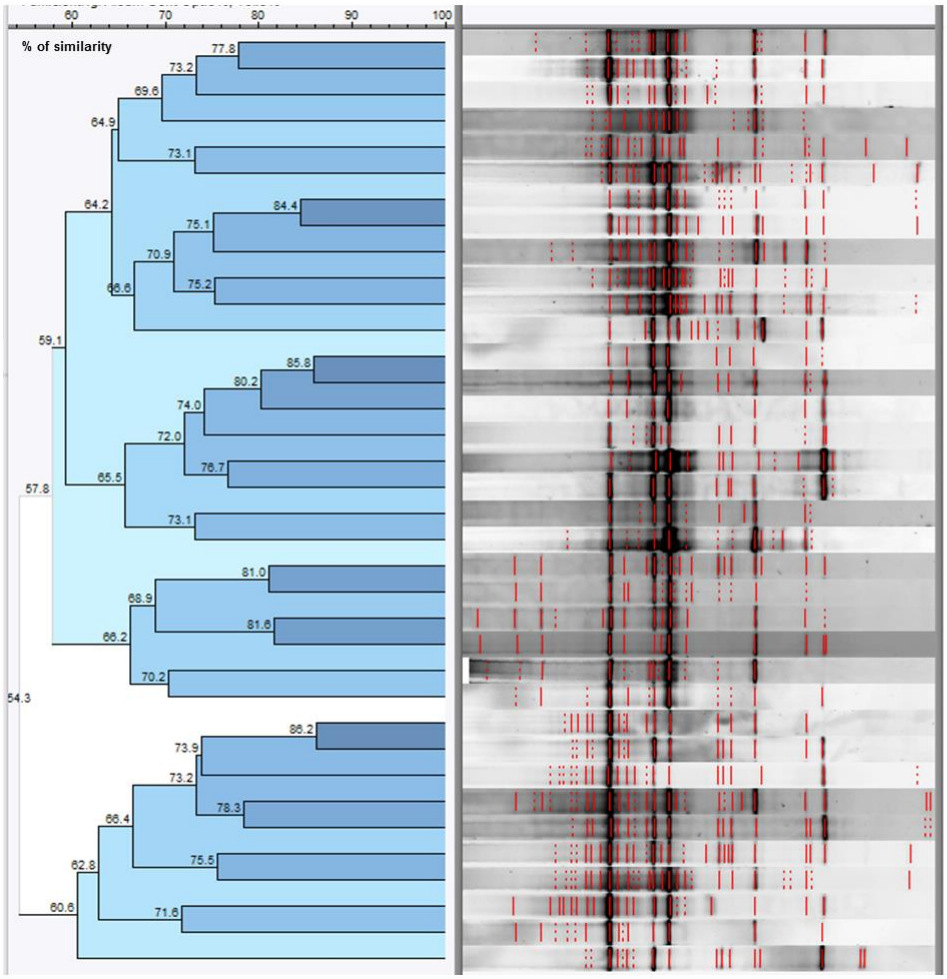
group	bird	pen	gender	bands	diversity of OTU's
control	14	3	♂	22	C
control	13	3	♂	18	C
control	12	2	♀	20	C
FBs	19	1	♂	19	C
FBs	22	1	♀	19	C
FBs	20	1	♂	18	C
control	11	2	♀	24	C
FBs	32	3	♂	28	C
FBs	25	2	♂	28	C
control	8	2	♂	22	C
control	16	3	♀	31	C
control	10	2	♀	22	C
FBs	34	3	♀	10	B
FBs	36	3	♀	13	B
control	1	1	♂	11	B
FBs	21	1	♂	10	B
control	17	3	♀	13	B
control	6	1	♀	12	B
FBs	31	3	♂	14	B
FBs	33	3	♂	15	B
FBs	27	2	♂	19	B
FBs	26	2	♂	12	B
control	15	3	♂	12	B
FBs	35	3	♀	12	B
FBs	29	2	♀	13	B
FBs	24	1	♀	12	B
FBs	28	2	♀	12	B
control	18	3	♀	13	B
control	9	2	♂	12	B
control	7	2	♂	11	B
control	5	1	♀	17	B
control	2	1	♂	6	A
control	3	1	♂	5	A
control	4	1	♀	3	A
FBs	30	2	♀	6	A
FBs	23	1	♀	5	A

Figure 17. Denaturing gradient gel electrophoresis (DGGE) fingerprint of DNA samples of duodenal content *applying community PCR with universal bacterial primers targeting the variable V3 region of the 16S ribosomal RNA (18 animals per group (3 pens / group, 6 animals / pen)). Percentage of similarity between DGGE profiles was analyzed using the Dice similarity coefficient, derived from presence or absence of bands. On the basis of a distance matrix, which was generated from the similarity values, dendrograms were constructed using the unweighted pair group method with arithmetic means (UPGMA) as clustering-method. The microbial richness (R) was assessed as the number of OTUs within a profile. Treatment is not reflected by DGGE fingerprint. Independently of treatment three clades are distinguishable concerning the diversity of OTUs:* $^{(A)}$ *one clade with reduced number of bands built by 2 FBs-samples and 3 control-samples, a second clade* $^{(B)}$ *with average diversity between 10 and 15 OTUs across the medium GC-range and a third clade* $^{(C)}$ *consisting of 18 to 31 OTUs again in the medium but also high GC-range.*



group	bird	pen	gender	bands	diversity of OTU's
control	3	1	♂	19	A
FBs	24	1	♀	18	A
control	10	2	♀	25	A
control	9	2	♂	20	A
FBs	23	1	♀	22	A
control	16	3	♀	22	A
FBs	36	3	♂	29	A
control	13	3	♂	31	A
control	15	3	♂	22	A
control	1	1	♂	29	A
control	14	3	♂	26	A
FBs	20	1	♂	22	A
FBs	19	1	♂	21	A
FBs	30	2	♀	22	A
FBs	27	2	♂	23	A
FBs	35	3	♀	18	A
FBs	36	3	♀	16	A
FBs	32	3	♂	17	A
FBs	25	2	♂	17	A
control	12	2	♀	21	A
control	7	2	♂	21	A
control	11	2	♀	15	A
FBs	22	1	♀	18	A
control	5	1	♀	16	A
control	4	1	♀	13	A
FBs	21	1	♂	14	A
FBs	28	2	♀	18	A
control	2	1	♂	13	A
FBs	34	3	♀	12	A
FBs	29	2	♀	14	A
control	18	3	♀	16	A
FBs	26	2	♂	25	A
FBs	31	3	♂	7	A
control	6	1	♀	9	A
control	17	3	♀	11	A
control	8	2	♂	29	A (1)

Figure 18. Denaturing gradient gel electrophoresis (DGGE) fingerprint of DNA samples of jejunal content *applying community PCR with universal bacterial primers targeting the variable V3 region of the 16S ribosomal RNA (18 animals per group (3 pens / group, 6 animals / pen)). Percentage of similarity between DGGE profiles was analyzed using the Dice similarity coefficient, derived from presence or absence of bands. On the basis of a distance matrix, which was generated from the similarity values, dendrograms were constructed using the unweighted pair group method with arithmetic means (UPGMA) as clustering-method. The microbial richness (R) was assessed as the number of OTUs within a profile. Treatment is not reflected by DGGE fingerprint.* $^{(A)}$ *No difference in number of OTUs was demonstrated. In general, all samples OTUs were located in the medium range of GC-content.* $^{(1)}$ *The banding-pattern of bird 8, control group, is shifted to the lower GC-range what flags it unique in comparison to the others which hardly exhibit bands in this area.*



group	bird	pen	gender	bands	diversity of OTU's
FBs	31	3	♂	16	D
control	7	2	♂	14	D
control	5	1	♀	16	D
FBs	34	3	♀	16	D
control	18	3	♀	22	D
control	1	1	♂	25	D
FBs	19	1	♂	14	D
FBs	24	1	♀	16	D
FBs	29	2	♀	19	D
FBs	27	2	♂	22	D
FBs	26	2	♂	19	D
FBs	32	3	♂	14	D
FBs	22	1	♀	9	B
FBs	25	2	♂	11	B
FBs	28	2	♀	7	B
FBs	36	3	♀	12	B
FBs	21	1	♂	13	B
FBs	35	3	♀	10	B
FBs	23	1	♀	10	B
FBs	30	2	♀	15	B
FBs	33	3	♂	18	C
control	14	3	♂	15	C
FBs	20	1	♂	15	C
control	6	1	♀	13	C
control	15	3	♂	12	C
control	2	1	♂	12	C
control	8	2	♂	16	A
control	4	1	♀	16	A
control	10	2	♀	20	A
control	12	2	♀	26	A
control	9	2	♂	19	A
control	13	3	♂	25	A
control	17	3	♀	27	A
control	16	3	♀	20	A
control	11	2	♀	14	A
control	3	1	♂	17	A

Figure 19. Denaturing gradient gel electrophoresis (DGGE) fingerprint of DNA samples of ileal content *applying community PCR with universal bacterial primers targeting the variable V3 region of the 16S ribosomal RNA (18 animals per group (3 pens / group, 6 animals / pen)). Percentage of similarity between DGGE profiles was analyzed using the Dice similarity coefficient, derived from presence or absence of bands. On the basis of a distance matrix, which was generated from the similarity values, dendrograms were constructed using the unweighted pair group method with arithmetic means (UPGMA) as clustering-method. The microbial richness (R) was assessed as the number of OTUs within a profile. Within ileum-samples a separation according to the treatment is visible,* $^{(D)}$ *although exceptions occur like samples of chicken 20, 31, 33 and 34 of FBs group which show high similarity (69.6 – 81 %) with control group.* $^{(A)}$ *The majority of the control-samples contains bands in the lower GC-range which ascribes them to one clade.* $^{(B)}$ *Among the FBs group a clade of clearly reduced diversity is formed by 8 samples.* $^{(C)}$ *The remaining of this treatment group shows a similar diversity of OTUs of medium GC-content like the control-group, however the low-GC-OTUs were absent.*

3.4 FBs increase the susceptibility for *C. perfringens* induced necrotic enteritis

Quantification of total *C. perfringens* in DNA samples of ileal content (day 15 of animal trial) by qPCR using *cpa* gene (alpha toxin) showed an increased level in chickens fed a FBs contaminated diet compared to a control diet (7.5 ± 0.30 versus 6.3 ± 0.24 log10 copies/g intestinal content).

The number of chickens with NE increased from 29.8 ± 5.46 % of the birds in the control group to 44.9 ± 2.22 % of broilers which were fed the FBs contaminated diet *(P=0.047)*. No effect was observed on the mean lesion scores of NE positive broiler chickens (Figure 20). No macroscopic coccidiosis lesions were observed.

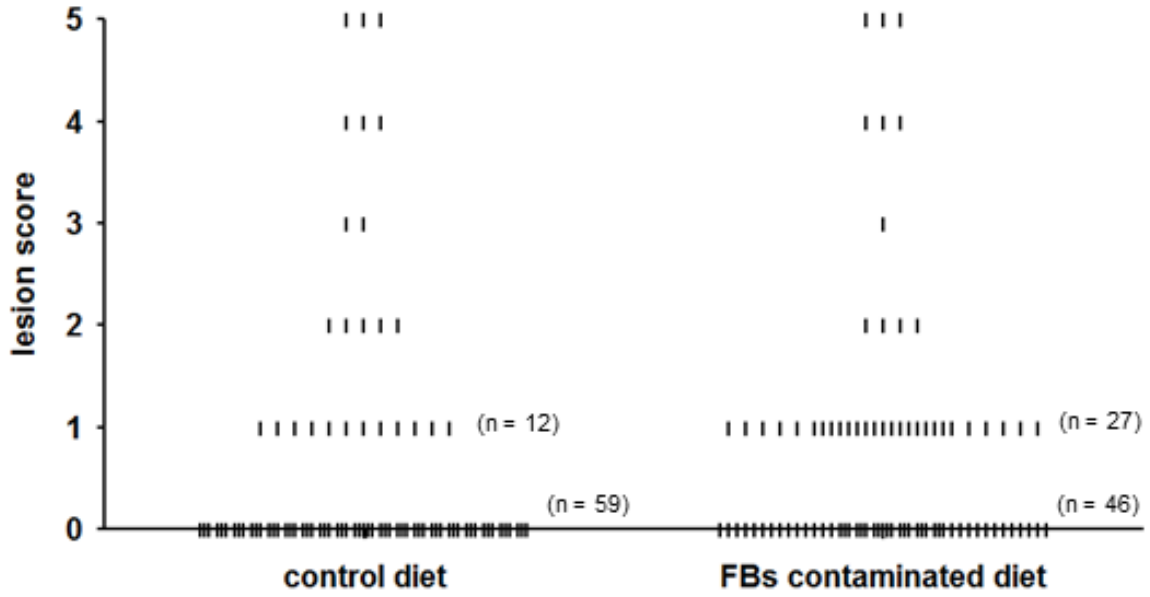


Figure 20. NE lesion score of individual broiler chickens challenged with C. perfringens. *Chickens were fed either a control diet or a FBs contaminated diet. Subsequently, birds were orally inoculated with C. perfringens strain 56. Macroscopic intestinal NE lesions in the small intestine (duodenum to ileum) were scored as follow; 0 no gross lesions; 1 small focal necrosis or ulceration (one to five foci); 2 focal necrosis or ulceration (six to 15 foci); 3 focal necrosis or ulceration (16 or more); 4 patches of necrosis of 2 to 3 cm long; 5 diffuse necrosis typical field cases. Chickens with NE lesions scores of 1 or more were categorized as NE positive. No effect was observed on the mean lesion scores of NE positive chickens.*

Results of the *C. perfringens* growth assay showed no influence of different concentrations of FB_1 on the bacterial growth curve in vitro (data not shown). qRT-PCR analyses showed no impact of FB_1 on transcription of genes encoding alpha (*cpa*) and netB (*netB*) toxin. Results for the *cpa* and *netB* gene transcription were normalized to the *rpoA* gene. The relative *cpa* transcription (log copies *cpa*/log copies *rpoA*) was 1.21 ± 0.008, 1.21 ± 0.006, 1.21 ± 0.004, and 1.22 ± 0.011 in the presence of 0, 0.2, 2 or 20 µg FB_1/mL, respectively. The relative *netB* transcription (log copies *netB*/log copies *rpoA*) was 1.03 ± 0.016, 1.05 ± 0.013, 1.04 ± 0.015, and 1.04 ± 0.007 in the presence of 0, 0.2, 2 or 20 µg FB_1/mL, respectively.

4 Discussion

The ingestion of FBs contaminated feed by broiler chickens, at a level of about 20 mg FB_1+FB_2/kg feed, affects the intestinal microbial homeostasis. Subsequently, these changes possibly predispose the birds to *C. perfringens* induced NE. To our knowledge, this is the first time such an effect has been demonstrated.

FBs negatively affect broiler health, demonstrated by the increased plasma Sa/So ratios in broiler chicks fed a FBs contaminated diet. These results suggest that the sphingolipid metabolism was impaired after exposure to levels of FBs approaching the EU maximum guidance levels [131]. FBs inhibit the ceramide synthase enzyme, causing an intracellular accumulation of sphingoid bases, mainly sphinganine. Since the disruption in the sphingolipid metabolism occurs before other indicators of cell injury, the Sa/So ratio is suggested to be the most sensitive biomarker to FB intoxication in many animal species [48,345]. A similar increase in the Sa/So ratio has been demonstrated in serum of broilers fed 80-100 mg FB_1/kg feed for 3-4 weeks [188,328,340]. Furthermore, a linear dose-dependent increase in the Sa/So ratio has been observed in the liver of broiler chickens fed 20-80 mg FB_1/kg feed for three weeks [188]. In the present study, the relative weight of liver was numerically increased in broilers fed the FBs contaminated diet. A similar effect has already been observed when broiler chickens were fed a FBs contaminated diet containing 100 mg FB_1 and 20 mg FB_2/kg feed for 2-4 weeks [340]. In broilers, this effect was not reported in other studies using low levels FB_1 (<100 mg/kg feed) [188,438]. Dietary exposure to FBs has been associated with histopathological degenerative changes in the hepatocytes including mild vacuolar degeneration and bile duct hyperplasia [401].

Consumption of a diet contaminated with FBs for 15 days reduced small intestinal length, ileal villus height and crypt depth. These results could be related to the negative impact of FB_1 on epithelial cell proliferation, reducing villus renewal and impairing intestinal absorption of nutrients [48]. This is in accordance with a previous study, where a decreased villus height was observed in the jejunum of broiler chickens fed high concentrations of FBs (>100 mg FB_1/kg feed) [57,340]. In pigs exposed to FBs for 9 days (1.5 mg FB_1/kg BW), however, ileal villi tended to be longer [235]. It remains to be determined if this effect on intestinal morphology is induced only by a direct toxic effect of FBs on intestinal epithelial cells, or also indirectly, by the microbiota shift induced by FBs. Longer villi are for example observed in the ileum of chickens treated with *L. reuteri,* indicating that the composition of the intestinal microbiota may indeed affect intestinal morphology [116]. Since the intestinal mucus layer and microbiota

are strongly associated, FBs could also modify the microbiota through modulation of the mucus production. Goblet cell hyperplasia was observed in broiler chickens exposed to very high dietary concentration of FB_1 (300 mg/kg feed) for two weeks [57]. Similarly, it was demonstrated that non-cytotoxic concentrations of DON decreased mucin production in human colonic epithelial goblet cells (HT-29 16E) and porcine intestinal explants [321]. The ingestion of FBs contaminated feed by broiler chickens for 15 days resulted in a modified composition of the intestinal microbiota of the ileum. Based on separation of DNA fragments by electrophoresis of PCR-amplified 16S ribosomal DNA fragments, using polyacrylamide gels containing a linear gradient of DNA denaturants, the DGGE technique provides a genetic fingerprint of a complex microbial community. The PCR product banding pattern is indicative of the number of bacterial species or assemblages of species that are present [284]. The results clearly indicate a reduced diversity of the ileal microbiota in broiler chickens exposed to FBs compared to the control group. The difference was mainly due to a reduced presence of low-GC-content OTUs in ileal content samples of FBs exposed animals. Feeding a FBs contaminated diet to broiler chickens was correlated with a decrease in the abundance of *Candidatus* Arthromitus, recently renamed to *Candidatus* Savagella [402]. These segmented filamentous bacteria (SFB) are a unique group of uncultivated commensal bacteria within the bacterial family of *Clostridiaceae*. These SFB are characterized by their attachment to the intestinal epithelium and their important role in modulating host immune systems [375,402]. They induce IgA secreting cells and influence the development of the T-cell repertoire [127,381]. Stanley *et al.* [381] demonstrated that the best-known predisposing factor for necrotic enteritis, coccidiosis, also eliminates or reduces the levels of this immune modulating bacterium. Since coccidiosis and FBs are both predisposing factors for *C. perfringens* induced NE in broiler chickens, the role of *Candidatus* Savagella in the pathogenesis of NE needs to be further investigated. It has been suggested that the colonization of the ileum with SFB is correlated with the population of lactobacilli [243]. Lactobacilli belong to the low GC Gram-positive group of Lactobacillales, fermenting sugars to lactic acid [336]. In this study, FBs also modulated the presence of *Lactobacillaceae* in the ileum. *L. johnsonii* (OTUs 4 and 13 with sequence similarity of 100%) was reduced in FBs exposed birds. *L. johnsonii* has been extensively investigated for its probiotic activities including pathogen inhibition, epithelial cell-attachment, and immunomodulation [336]. Similar to our results, it was recently demonstrated that *L. johnsonii* was reduced in birds fed fishmeal with or without *C. perfringens* challenge [381,446]. A positive association was demonstrated between crude protein derived from fishmeal and numbers of ileal and caecal *C. perfringens* [113]. *L. johnsonii* interferes with the

colonization and persistence of *C. perfringens* in poultry [226] and some lactobacilli can inhibit growth of *C. perfringens* [93]. It needs to be further investigated whether lactic acid bacteria (LAB) are able to counteract the negative effects of mycotoxins on the intestinal health in poultry.

5 Conclusion

In conclusion, feeding a FBs contaminated diet at contamination levels approaching the EU maximum guidance level altered the sphingolipid metabolism in broiler chickens without affecting BW gain. FBs modified the composition of the intestinal microbiota of the ileum. DGGE analysis demonstrated a reduced presence of low-GC content OTUs in ileal digesta of birds exposed to FBs, which were subsequently identified as a reduced abundance of *Candidatus* Savagella and *Lactobaccilus* spp. such as *L. johnsonii*. The ileal concentration of total *C. perfringens* was increased in chickens fed the FBs contaminated diet. Additionally, small intestinal length, ileal villus height, and crypt depth were negatively affected by FBs. The changes in the gut microbiota possibly induced an environment stimulating *C. perfringens* colonization, and predisposing the birds to necrotic enteritis. The impact of different predisposing factors for NE in broilers, among others coccidiosis, fishmeal and FBs, on intestinal microbiota shows remarkable similarities. The observed predisposing effect is due to the negative impact of FBs on the intestinal microbiota and the animal host, rather than its effect on the bacterium itself.

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CHAPTER 3

The impact of deoxynivalenol and fumonisins on the intestinal barrier

3.1 Impact of deoxynivalenol and fumonisins on the intestinal micro-environment

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Adapted from

Antonissen G., Van Immerseel F., Pasmans F., Ducatelle R., Janssens G.P.J., De Baere S., Mountzouris K.C., Su S., Wong E.A., De Meulenaer B., Verlinden M., Devreese M., Haesebrouck F., Novak B., Dohnal I., Martel A., Croubels S. (2015) The Mycotoxins Deoxynivalenol and Fumonisins alter the Extrinsic Component of Intestinal Barrier in Broiler Chickens (submitted).

Abstract

Deoxynivalenol (DON) and fumonisins (FBs) are secondary metabolites produced by *Fusarium* fungi which frequently contaminate broiler feed. The aim of this study was to investigate the impact of DON and/or FBs on the intestinal barrier in broiler chickens, more specifically on the mucus layer and induction of oxidative stress. One-day-old broiler chicks were divided into four groups, each consisting of eight pens of seven birds each, and were fed for 15 days either a control diet, a DON contaminated diet (4.6 mg DON/kg feed), a FBs contaminated diet (25.4 mg FB_1+FB_2/kg feed), or a DON and FBs contaminated diet (4.3 mg DON and 22.9 mg FB_1+FB_2/kg feed). DON and FBs affected the duodenal mucus layer by suppressing the *intestinal mucin (MUC) 2* gene expression and altering the mucin monosaccharide composition. Both mycotoxins decreased gene expression of the intestinal zinc transporter (ZnT)-1, and regulated intracellular methionine homeostasis, which are both important for preserving the cell's critical anti-oxidant activity. Feeding a DON and/or FBs contaminated diet, at concentrations close to the European Union maximum guidance levels (5 mg DON and 20 mg FB_1+FB_2/kg feed), changes the intestinal mucus layer and several intestinal epithelial anti-oxidative mechanisms.

Keywords

Broiler – Deoxynivalenol – Fumonisins – Intestinal barrier – Methionine – Mucus – Oxidative stress

1 Introduction

Mycotoxins are secondary metabolites produced by certain fungal species readily colonizing crops. They may contaminate food- and feedstuffs already in the field or post-harvest during storage [415]. Although these secondary metabolites have no biochemical significance in fungal growth and development [195], contamination of food and feed with mycotoxins is considered a worldwide problem because of its toxicity to vertebrates. Mycotoxins of importance for poultry are mainly produced by fungi of the genera *Aspergillus*, *Fusarium* and *Penicillium* [97,283,332]. Some of the most commonly found and toxicologically important mycotoxins are the *Fusarium* mycotoxins deoxynivalenol (DON) and fumonisins (FBs). A long-term global mycotoxin survey conducted during 2004-2012 indicated that the mycotoxins DON and FBs contaminated 56% and 54%, respectively, of samples taken from livestock feed and feed raw materials. Although sometimes high contamination levels were present, the majority of the feed samples was found to comply with European Union regulations and recommendations on the maximal tolerable concentrations in feed [358,386]. The maximum European guidance level for poultry feed is set at 5 mg DON/kg feed and 20 mg FB_1+FB_2/kg feed [131].

The intestinal mucosa acts as a selective barrier, on the one hand allowing the uptake of dietary nutrients, electrolytes and water from the intestinal lumen into the systemic circulation, and on the other hand preventing the passage of harmful substances from the external environment into the organism, including foreign antigens, microorganisms and their toxins [47,305,439]. The gastrointestinal barrier is divided into an intrinsic and extrinsic component. The intrinsic barrier is composed of the epithelial cells lining the digestive tract and the tight junctions that tie them together. DON and FBs impact negatively on the intrinsic component of the intestinal barrier through the modulation of intestinal epithelial integrity and epithelial cell renewal and repair [13,47,57,303,340]. This increases animal susceptibility to infectious diseases such as *Clostridium perfringens* induced necrotic enteritis in broiler chickens and colibacillosis in pigs [12,13,306]. The extrinsic barrier consists of secretions and other substances that are not physically part of the epithelium, but which affect the function of the epithelial cells and maintain their barrier function, such as the mucus layer, secretion of antimicrobial peptides, chemokines and cytokines, and reactive oxygen species (ROS) [47]. DON and FBs may affect the extrinsic component of the intestinal barrier by affecting the production of antimicrobial peptides and cytokines [46,425,435]. Only limited information is available on the impact of DON and FBs exposure on the intestinal mucus layer and on the production of ROS.

Goblet cell hyperplasia is observed in broiler chickens exposed to very high dietary concentrations of fumonisin B_1 (FB_1) (300 mg/kg feed) for two weeks [57]. In contrast, a reduction of the number of mucus-producing goblet cells in jejunum and ileum is seen in piglets fed a lower DON (3 mg/kg) or a DON and FBs (3 and 6 mg/kg, respectively) contaminated diet for five weeks [50]. Oxidative stress also might play a role in DON- and FBs-induced toxicity by increased production of ROS and lipid peroxidation after DON and/or FBs exposure [274,303,328,455]. Indeed, FBs-induced accumulation of sphingoid bases stimulates the production of ROS [111]. Moreover, it has been suggested that FBs increase the susceptibility of cells to lipid peroxidation because the reduction in complex sphingolipids contributes to membrane dysfunction resulting in cellular damage [1].

The objective of this study was to evaluate the impact of DON and/or FBs exposure on the intestinal mucus layer and the induction of oxidative stress. Therefore, the influence of both mycotoxins on the intestinal mucus production and composition was investigated. The oxidative stress effects were evaluated indirectly, by measuring the influence on different anti-oxidant mechanisms in the intestinal epithelial cells.

2 Material and methods

2.1 Animal experiment

2.1.1 Feed preparation and experimental diets

The experiment was performed with four different experimental groups of broiler chickens fed either a control diet, a DON contaminated diet, a FBs contaminated diet or a multi-mycotoxin contaminated diet including DON and FBs, respectively, from day 1 until day 15. The feed was a custom-made wheat:rye (43%:7.5%) based mash diet, with soybean meal as the main protein source [158]. A starter diet was provided during the first eight days of the experiment, and subsequently a grower diet until the end of the trial (day 15). Titanium dioxide (TiO_2) (0.5%) was added to the grower diet as an inert digestive flow marker (titanium (IV) oxide GPR Rectapur, VWR, Leuven, Belgium). Mycotoxin contaminated feed was produced by adding DON and/or FBs to a control diet. DON (15.6 mg DON/g) and FBs (5.68 mg FB_1+FB_2/g) culture materials were commercially produced *in vitro* from a culture of *F. graminearum* and *F. verticillioides*, respectively (Biopure – Romer Labs Diagnostic GmbH, Tulln, Austria), and were subsequently purified and crystallized [5,234]. Next, mycotoxins were added to 500 g of the corresponding batches of control diet (i.e. starter and grower). For each batch, this premix was then mixed with 5 kg of control feed to assure homogeneous distribution of the toxin and finally mixed for 20 min in the total amount of feed needed for each batch. Mycotoxin contamination of control and contaminated diets was assessed by a validated multi-mycotoxin liquid chromatography-tandem mass spectrometry method (LC-MS/MS) [276]. Samples were taken at three different locations in each batch, subsequently pooled per batch and analyzed for mycotoxin contamination. Trace amounts of nivalenol, DON and enniatin B were detected in the control feed (0.078-0.087, 0.190-0.223, 0.135-0.142 mg/kg feed, respectively). Analyzed mycotoxins, their limit of detection and limit of quantification were as previously described [13,276]. The average level of DON in the two batches of DON contaminated feed was 4.6 mg DON/kg feed, and the average levels of FB_1, FB_2 and FB_3 in the two batches of FBs contaminated feed were 18.4 mg/kg, 7.0 mg/kg and 1.7 mg/kg, respectively. The two batches multi-mycotoxin contaminated feed contained on average 4.3 mg DON/kg, 16.8 mg FB_1/kg, 6.1 mg FB_2/kg and 1.5 mg FB_3/kg (Table 13). Hence, dietary contamination levels of DON and FBs approached the EU maximum guidance levels for poultry feed of 5 mg DON/kg and/or 20 mg FB_1+FB_2/kg feed, respectively (2006/576/EC) [131].

Table 13. Concentration of DON, FB_1, FB_2 and FB_3 in different batches of control and mycotoxin contaminated diets. *Samples were analyzed by a validated multi-mycotoxin liquid chromatography-tandem mass spectrometry method (LC-MS/MS).*

	starter diet			
	DON	**FB_1**	**FB_2**	**FB_3**
	mg/kg feed			
control diet	0.2	ND	ND	ND
DON diet	4.6	ND	ND	ND
FBs diet	ND	19.3	7.1	1.8
DON + FBs diet	4.2	17.9	6.5	1.7
	grower diet			
	DON	**FB_1**	**FB_2**	**FB_3**
	mg/kg feed			
control diet	0.2	ND	ND	ND
DON diet	4.6	ND	ND	ND
FBs diet	ND	17.6	6.9	1.6
DON + FBs diet	4.5	15.7	5.7	1.4

(ND= not detected)

2.1.2 Experimental design and sample collection

The animal trial was performed with non-vaccinated Ross 308 broiler chickens of both sexes, which were obtained as one-day-old chicks from a commercial hatchery (Vervaeke-Belavi, Tielt, Belgium). Chicks were randomly divided into four experimental groups. Each group consisted of four pens of cockerels and four pens of pullets, with seven birds per pen. Chickens were fed, from day 1 until day 15, a control diet, a DON contaminated diet, a FBs contaminated diet or a multi-mycotoxin contaminated diet, respectively. Animals were housed in pens of 1 m² on wood shavings, and animal units were separated by solid walls to prevent direct contact between birds from different pens. All pens were decontaminated with peracetic acid and hydrogen peroxide (Metatectyl HQ, Metatecta, Kontich, Belgium) and a commercial anticoccidial disinfectant (Bi-OO-Cyst Coccidial Disinfectant; Biolink, York, United Kingdom) prior to housing of the animals. A 18 h/6 h light/darkness program was applied. The environmental temperature was adjusted to the changing needs of the animals according to their age. Water and feed was provided *ad libitum*. Bodyweight (BW) of all birds was measured at day 1, 4, 8, 11 and 15, and feed intake per pen was recorded daily. Bodyweight gain (BWG) and feed conversion ratio (FCR) were calculated as follows: $BWG_{(period\ a\text{-}b)} = [BW_{day\ b} - BW_{day\ a}]$ and $FCR_{(period\ a\text{-}b)} = [BWG_{(period\ a\text{-}b)} / (\text{cumulative feed intake}_{(period\ a\text{-}b)} \text{ per pen/number of birds per pen})]$.

After 15 days, all chickens were euthanized by intravenous injection of sodium pentobarbital (Natrium Pentobarbital 20%, Kela Veterinaria, Sint-Niklaas, Belgium). Chickens were not fasted before euthanasia. A blood sample was collected and subsequently a necropsy was performed. Blood samples were centrifuged (2,850 x g, 10 min, 4°C) and plasma was stored at ≤ -20°C until sphinganine (Sa) and sphingosine (So) concentrations were analyzed. Immediately after euthanasia, small fragments of mid-duodenum, mid-jejunum, and mid-ileum were collected, rinsed in phosphate buffered saline (PBS), and subsequently stored in RNAlater (Sigma-Aldrich, Bornem, Belgium) at -80°C until quantitative real-time PCR (qRT-PCR) analysis. The duodenum was defined as the segment encompassing the duodenal loop, whereas the jejunum was defined as the segment between the end of the duodenal loop and Meckels diverticulum. The ileum comprised the distal segment starting at Meckel's diverticulum and ending at the ileo-cecal junction. Luminal ileal content was flushed from the midpoint to the ileocecal junction with ice-cold PBS containing protease inhibitors, 1 mM phenylmethylsulfonyl fluoride, 2 mM iodoacetamide, and 10 mM ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich) (pH 7.4) [307], snap frozen in liquid nitrogen, and stored at -20°C until lyophilization. Subsequently, the intestinal segments were opened longitudinally with the mucosa side up, and the tissue was washed by immersing in ice-cold PBS containing protease inhibitors to prevent further mucolysis. The cleaned tissue was subsequently blotted on filter paper to remove excess buffer. Mucus isolation of mid-duodenum, mid-jejunum and mid-ileum was then collected by mucosal scraping with a microscope glass slide. Mucus samples were immediately snap frozen in liquid nitrogen and stored at -80°C until further mucin monosaccharide composition analysis.

The animal experiment was approved by the Ethical Committee of the Faculty of Veterinary Medicine and Bioscience Engineering, Ghent University (EC 2013/168).

2.2 Sphingolipid analysis – biomarker of FBs exposure

An LC-MS/MS method was used to quantify So and Sa in the plasma samples of 24 animals per group with three birds per pen [175]. Sa/So is suggested to be the most sensitive biomarker of FBs intoxication in many animals [365,431]. In brief, 200 µL of plasma were mixed with 600 µL of methanol/acetonitrile (ACN) (50/50, v/v), the pellet after centrifugation was re-extracted with 300 µL of methanol/water (80/20, v/v). The combined supernatants were evaporated to dryness and reconstituted in 600 µL of methanol/water (80/20, v/v). Chromatographic separation was achieved on an Agilent 1290 UHPLC system (Agilent,

Waldbronn, Germany) using a C18 Gemini column (150 x 4.6 mm, 5 µm, Phenomenex, Aschaffenburg, Germany) and a gradient from methanol/water/formic acid (40/59.9/0.1, v/v/v) to methanol/formic acid (99.9/0.1, v/v) at a flow rate of 0.9 ml/min. ESI-SRM (electrospray ionization-selected reaction monitoring) analysis was carried out on a 5500 Triple Quad mass spectrometer (AB Sciex, Framingham, MA, USA) in positive ion mode (ion spray voltage: 5500V). The declustering potential (DP), collision energy (CE) and Q1 and Q3 m/z for quantifier and qualifier transitions were for So DP 71 V, 300.3→282.3 (CE 15 eV), and 300.3→252.2 (CE 23 eV), respectively, and for Sa 146 V, 302.3→284.4 (CE 19 eV), and 302.3→60.1 (CE 21 eV), respectively. Analyst software version 1.6.2 (AB Sciex) was used for instrument control and data evaluation. Concentrations of So and Sa in plasma were determined on the basis of external standard calibration functions (So and Sa standards purchased from Avanti Polar Lipids, Inc., Alabaster, AL, USA). The limits of quantification of So and Sa were 3.9 and 4.5 ng/mL plasma, respectively, whereas the limits of detection were 1.2 and 1.8 ng/mL plasma, respectively.

2.3 RNA Isolation and Reverse Transcription

Total RNA from small intestinal segments was isolated using RNeasy Mini Kit (Qiagen Ltd., Crawley, UK) according to the manufacturer's instructions. The RNA concentration was measured by absorbance at 260 nm with a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, USA). Reverse transcription was carried out using the iScript cDNA Synthesis Kit (Bio-Rad, Temse, Belgium). This reaction was carried out in a 15 µL final volume that included 4 µL of 5x iScript Reaction Mix, 1 µL of iScript Reverse Transcriptase, 1 µL of RNA template (1 µg/µL), and nuclease-free water to complete the final volume. The reverse transcription mix was incubated at 25°C for 5 min, heated to 42°C for 30 min, and subsequently inactivated at 85°C for 5 min. The obtained cDNA was stored at -20°C until further analysis.

2.4 Impact of mycotoxin exposure on intestinal mucus layer

2.4.1 Quantitative RT-PCR analysis of mRNA expression of intestinal mucin 2 (MUC2)

Intestinal mucin 2 (*MUC2*) encodes a heavily glycosylated, gel-forming mucin, which creates a protective layer along the gastrointestinal tract [203]. The mRNA expression of MUC2 was evaluated in duodenum, jejunum and ileum tissue samples of 16 animals per group (8♂/8♀) with two animals randomly selected per pen. Primers were adopted from [203] (Table 14). Five

different reference genes were evaluated for all experimental groups and all different tissues [91]. Most stable reference genes were selected using the geNorm software. The most stable reference genes had an M-value between 0.2 and 0.5 (data not shown). To determine if the inclusion of an additional reference gene was required, the cut-off value for variation was set at 0.2. The three most stable reference genes, β-actin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and glucose-6-phosphate dehydrogenase (G6PDH) (Table 14), were selected. RT-qPCR was performed using SYBR-Green Supermix (Bio-Rad) in a Bio-Rad CFX-384 system. Each reaction was done in triplicate in a 12 µL total reaction mixture using 2 µL of the cDNA sample and 0.5 µM final qPCR primer concentration. The qPCR conditions used were 1 cycle of 95°C for 10 min, followed by 40 cycles of 95°C for 20 s, 58°C for 30 s, and a stepwise increase of the temperature from 65° to 95°C (at 0.5°C/5s). Melting curve data were analysed to confirm the specificity of the reaction. The threshold cycle values (Ct) were first normalized to the geometric means of appropriate reference mRNAs and the normalized mRNA levels were calculated according to the $2^{-\Delta\Delta Ct}$ method [244].

2.4.2 Mucin monosaccharide composition analysis

The percentage of monosaccharide components of mucin carbohydrate side chains, i.e. N-acetyl-glucosamine (GlcNac), N-acetyl-galactosamine (GalNac), galactose (Gal), fucose (Fuc), mannose (Man) and N-acetyl-neuraminic acid (NeuAc), was determined in the duodenum, jejunum and ileum of 8 animals per group (4♂/4♀) with one animal randomly selected per pen. Analysis was performed as previously described [412,413]. Briefly, intestinal mucin purification was performed by size exclusion chromatography in an XK-16 chromatographic column (GE Healthcare Bio Sciences AB, Uppsala, Sweden) filled with Sepharose CL-4B (Sigma-Aldrich, St. Louis, MO, USA) and equilibrated with elution buffer (0.2 M NaCl, 0.0015 M NaN_3). Fractions comprising the mucins peak were pooled and lyophilized. Subsequently, lyophilized mucins were appropriately diluted in PBS (100 mg/mL) and stored at -20°C until further HPLC analysis. The determination of GlcNac, GalNac, Gal, Man, Fuc and NeuAc by HPLC-fluorescence detection was performed as described by [412,413] (Figure 21 and 22). For the determination of GlcNac, GalNac, Gal, Man and Fuc the following minor modification were applied to the protocols: the concentration of internal standard was lowered to 0.5 mM xylose, sodium acetate trihydrate was used instead of sodium acetate, and for the aminobenzoic acid (ABA) reagent, ABA and sodium cyanoborohydride were dissolved in acetate-borate reaction medium (4% sodium acetate trihydrate and 2% boric acid in methanol) in concentrations of 30 mg/mL and 20 mg/mL,

respectively. For the determination of NeuAc, sodium acetate trihydrate was used instead of sodium acetate. Subsequently, an aliquot (10 µL) was injected onto the HPLC system (Hewlett Packard 1100 series, Hewlett Packard GmbH, Waldbronn, Germany) with an HP 1046A fluorescence detector (Hewlett Packard GmbH). Chromatographic separation was achieved on a C-18 reversed phase column (Hypersil ODS, 5 µm, 200x2.1 mm; Agilent Technologies Inc., Santa Clara, California, USA). Data were collected using an Agilent Chemstation chromatography data system and quantified following system calibration with appropriate standard calibration curves. Samples and standards were analysed in duplicate. GlcNac, GalNac standards were purchased from AppliChem (Bioline Scientific, Athens, Greece), Gal, Man and Fuc standards were purchased from Sigma-Aldrich (St. Louis, MO, USA), and NeuAc standard was purchased from Acros Organics.

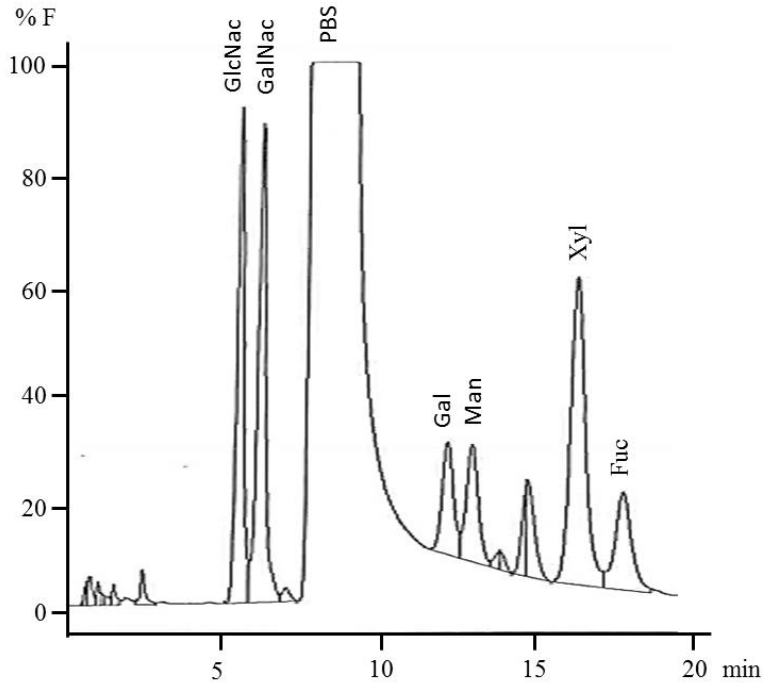

Figure 21. *Chromatogram of 0.2 mM standard mixtures of N-acetyl-glucosamine (GlcNac), N-acetyl-galactosamine (GalNac), galactose (Gal), fucose (Fuc), and mannose (Man) with 0.5 mM xylose (Xyl) added as internal standard. Purified mucin samples were diluted in phosphate buffered saline (PBS) and further analyzed by HPLC analysis with fluorescence detection.*

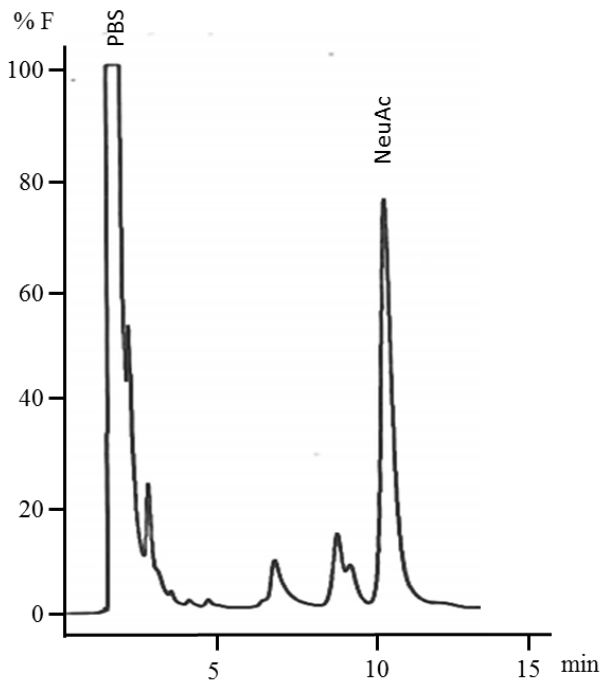

Figure 22. *Chromatogram of 0.5 mM standard of N-acetyl-neuraminic acid (NeuAc). Purified mucin samples were diluted in phosphate buffered saline (PBS) and further analyzed by HPLC analysis with fluorescence detection.*

2.5 Impact of mycotoxin exposure on the oxidative stress response

2.5.1 Quantitative RT-PCR analysis of mRNA expression of proteins involved in oxidative stress

The mRNA expression of proteins involved in oxidative stress, i.e. hypoxia-inducible factor 1 subunit alpha (HIF-1α), heme-oxigenase (HMOX) and xanthine dehydrogenase (XDH) was evaluated in the different segments of the small intestine of 16 animals per group (8♂/8♀) with two animals randomly selected per pen. Under hypoxic cellular conditions, HIF-1α activates the transcription of genes encoding proteins which increase oxygen delivery or facilitate metabolic adaptation to hypoxia [362]. HMOX modulates the cellular equilibrium between pro-oxidants such as heme and iron, and the anti-oxidant biliverdin [200]. XDH catalyzes the terminal hydroxylation in purine degradation of hypoxanthine to xanthine and of xanthine to uric acid, a potent anti-oxidant [181,384]. The primers for HIF-1α, HMOX and XDH were adopted from [303] (Table 14) and further qRT-PCR conditions were similar as described above for *MUC2*.

2.5.2 Quantitative RT-PCR analysis of mRNA expression of jejunal digestive enzymes and nutrient transporters

Maintaining a minimal level of intracellular zinc may be important for preserving critical anti-oxidant activity to counteract oxidative damage [134,382,389]. Intracellular zinc homeostasis is mediated by the expression of the basolateral zinc transporter ZnT1 (SLC30A1) [74] (Table 14, Figure 23). Methionine participates in the synthesis of cysteine, which is subsequently converted to glutathione (GSH) and taurine. GSH and cysteine can function as direct scavengers of ROS [271]. In the jejunum of chickens, methionine is transported by multiple transport mechanisms, a.o. the brushborder amino acid transporters B^0AT (solute carrier family (SLC) 6 member (A) 19), $b^{0,+}AT$ (SLC7A9) and peptide transporter PepT1 (SLC15A1), and the basolateral L-amino acid transporters y^+LAT1 (SLC7A7) and y^+LAT2 (SLC7A6) [309,379] (Table 14, Figure 23). The mRNA expression of a panel of genes encoding sucrase isomaltase (SI), the intestinal zinc transporter ZnT1, and transporters involved in methionine transport, i.e. B^0AT, $b^{0,+}AT$, rBAT, PepT1, y^+LAT1 and y^+LAT2 (Table 14, Figure 23) were evaluated by qRT-PCR in the mid-jejunum of 8 animals per group (4♂/4♀) with one animal randomly selected per pen, following a standard protocol [314]. Briefly, qRT-PCR was performed in an Applied Biosystems 7300 instrument with SYBR-Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) using the following conditions for all genes: 50°C for 2 min and 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Melting curve data were analysed to confirm the specificity of the reaction. The threshold cycle values (Ct) were first normalized to the included reference gene (β-actin). The average ΔCt of the control samples was used to calculate the ΔΔCT values according to the $2^{-\Delta\Delta Ct}$ method [244].

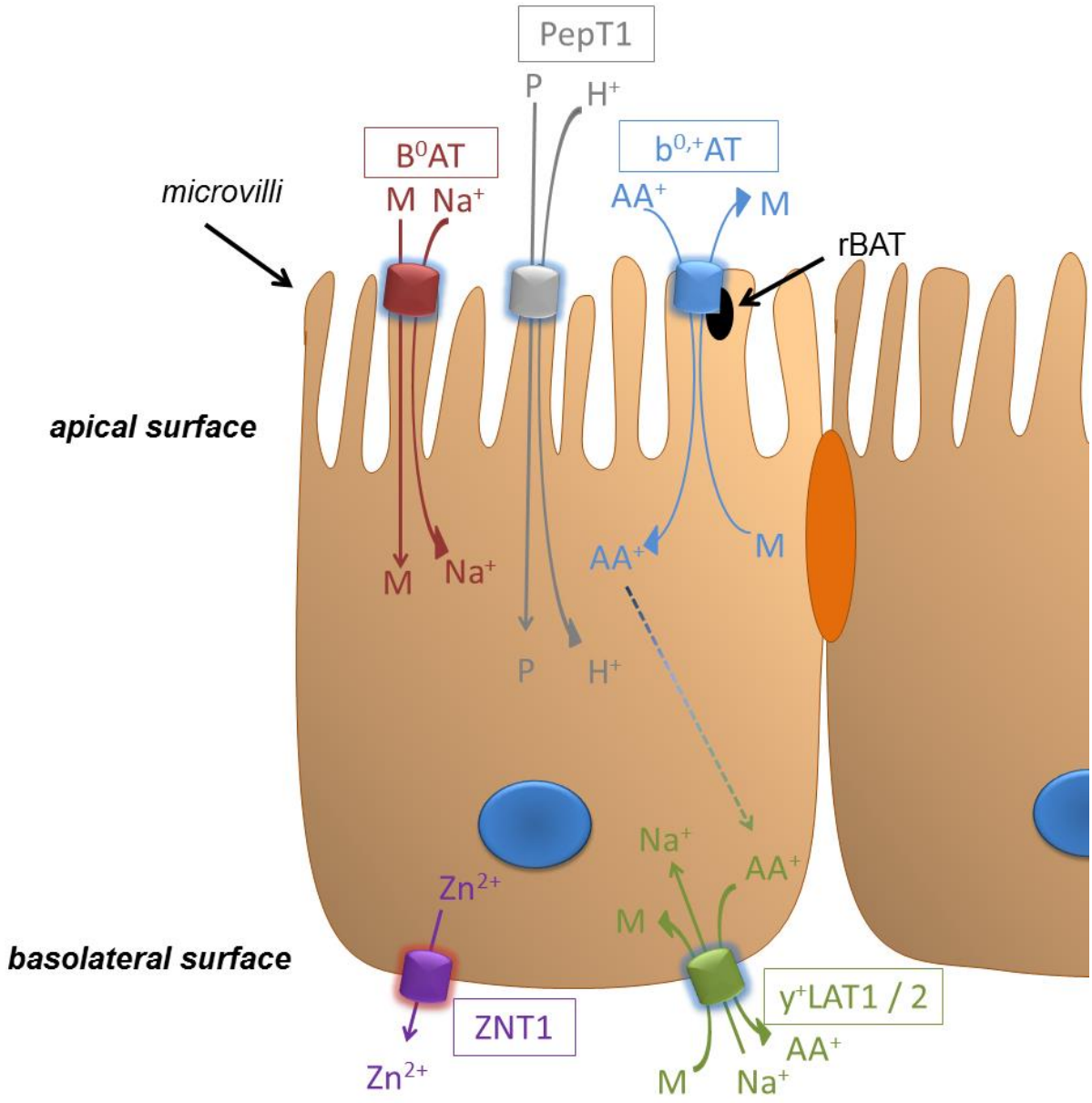

Figure 23. Transporters involved in intestinal zinc and methionine absorption. *Transporters with a probed role in intestinal methionine absorption are the brush border Na^+ dependent neutral amino acid transporter (B^0AT), Na^+-independent neutral/cysteine, cationic amino acid exchanger $b^{0,+}AT$ and peptide transporter-1 (PepT1); and the basolateral transporters y^+ L amino acid transporter-1 and -2 (y^+LAT1 and y^+LAT2). rBAT dimerizes with $b^{0,+}AT$ transporter, without having a transporter function itself. Intracellular zinc homeostasis is mediated by the expression of the basolateral zinc transporter ZNT1. (M = methionine, AA^+ = cationic amino acid, P = di- and tripeptides)*

2.5.3 Apparent ileal methionine digestibility

Samples were taken at three different locations in each batch of grower diet, subsequently lyophilized, ground, homogenized and pooled per batch. Luminal content of the ileum was lyophilized, subsequently ground and homogenized. Afterwards, equal amounts of luminal content of each bird were pooled per pen. The total concentration of methionine in the feed and luminal content samples was analyzed by automated pre-column derivatization using ortho-phthalaldehyde (OPA) in combination with high-performance liquid chromatography with fluorescence detection (HPLC-FL) [361].

In brief, 50 mg of each sample were accurately weighed in a pyrex tube, followed by the addition of 4 mL of a 6 M HCl solution (containing 1% phenol and 1% Na_2SO_3) and vortex mixed for 15 s. The tube was closed and hydrolysed for 24 h at 105 – 110 °C. Thereafter, the sample was cooled down to room temperature. The content was transferred to a 50-mL beaker that was placed in an ice-bath. The sample was carefully neutralised by the addition of 1 mL of a 10M NaOH solution. The pH was further adjusted to 2.2 by drop-wise addition of a 10 M or 1 M NaOH solution. The sample was transferred to a volumetric flask of 10 mL and water was added up to the mark. An aliquot was filtered using a 0.45 µm PolyPure II syringe filter and 950 µL was transferred to an autosampler vial. After the addition of 50 µL of the internal standard (IS) working solution (IS 1: norvaline; IS 2: sarcosine, 10 nmol/L), the samples were vortexed and placed in the autosampler of the HPLC-FL instrument for subsequent precolumn derivatization and analysis. An Agilent 1100 Series HPLC system (Agilent Technologies, Diegem, Belgium) was used for precolumn derivatization and HPLC-FL analysis.

Chromatographic separation of the derivatized methionine was achieved on a Zorbax Eclipse AAA Rapid Resolution column (4.6 x 150 mm, dp: 3.5 µm, Agilent Technologies). The mobile phase A consisted of 45 mM NaH_2PO_4 and 3.08 mM NaN_3 in water (adjusted to pH = 7.8), whereas mobile phase B was a mixture of acetonitrile/methanol/water (45/45/10, v/v/v). The flow-rate was set at 2 mL/min and a gradient elution program was applied. Detection was performed using a programmable FL detector (Agilent Technologies) that was set at excitation (λ_{ex}) and emission (λ_{em}) wavelengths of 340 nm and 450 nm, respectively. Data were collected using an Agilent Chemstation chromatography data system and quantified following system calibration with appropriate standard calibration curves.

TiO_2 concentration in luminal content and feed samples was analysed in duplicate following a previously described protocol [286]. Briefly, 0.2 g of luminal content sample or 0.5 g of feed sample were digested in 15 mL of 36 M H_2SO_4. Subsequently, 5 g of Kjeldahl catalyst (48.8% Na_2SO_4, 48.9% K_2SO_4 and 0.3% $CuSO_4$, Missouri catalyst, VWR) was added to the digestion mixture. Samples were digested at 420°C for 2 h, and after cooling for 30 min, 10 mL of 30% H_2O_2 were added. Finally, after cooling for 30 min, the total volume was made up to 100 mL with distilled water. Absorbance was measured at 406 nm. A standard curve was prepared by spiking blank samples with increasing amounts of TiO_2 (VWR). The apparent ileal methionine digestibility (AIMD) was calculated using following equation: $AIMD=[(M/TiO_2)_{diet}–(M/TiO_2)_{digesta}]/(M/TiO_2)_{diet}$, where M is the concentration of methionine [232].

Table 14. Forward and reverse primers of genes analyzed.

Gene	Gene full name	Location	Accession no.	Function	Study	Forward/reverse primer
MUC2	intestinal mucin 2	C	XM_001234581	intestinal glycosylated, gel-forming mucin	[1]	ATTGTGGTAACACCAACATTCATC/ CCTTATAATGTCAGCACCAACTTCTC
HIF-1α	hypoxia-inducible factor 1 subunit alpha	C	NM_204297	regulation of expression of a battery of hypoxia-responsive genes involved in the adaptive and cell death responses	[2]	CACCATTACCATACTTCAGCAG/ CTTCACATCATCCACACGTTC
HMOX	heme-oxigenase	C	NM_205344	limits inflammation and prevents cell death or apoptosis	[2]	CTTGGCACAAGGAGTGTTAAC/ CATCCTGCTTGTCCTCTCAC
XDH	xanthine dehydrogenase	C	NM_205127	generates reactive oxygen species	[2]	GTGTCGGTGTACAGGATACAGAC/ CCTTACTATGACAGCATCCAGTG
$b^{0,+}AT$	solute carrier family 7, member 9 (SLC7A9)	BB	XM_414130	Na^+-independent neutral/cysteine, cationic amino acid exchanger	[3]	CAGTAGTGAATTCTCTGAGTGTGAAGCT/ GCAATGATTGCCACAACTACCA
rBAT	solute carrier family 3, member 1 (SLC3A1)	BB	XM_426125	dimerizes with $b^{0,+}AT$ – no transporter function	[3]	CCCGCCGTTCAACAAGAG/ AATTAAATCCATCGACTCCTTTGC
B^0AT	solute carrier family 6, member 19 (SLC6A19)	BB	XM_419056	Na^+-dependent neutral amino acid transporter	[3]	GGGTTTTGTGTTGGCTTAGGAA/ TCCATGGCTCTGGCAGAGAT
y^+LAT1	y^+ L amino acid transporter-1 (SLC7A7)	BL	XM_418326	Na^+-dependent neutral/cationic amino acid exchanger	[3]	CAGAAAACCTCAGAGCTCCCTTT/ TGAGTACAGAGCCAGCGCAAT
y^+LAT2	y^+ L amino acid transporter-2 (SLC7A6)	BL	NM_001005832	Na^+-dependent neutral/cationic amino acid exchanger	[3]	GCCCTGTCAGTAAATCAGACAAGA/ TTCAGTTGCATTGTGTTTTGGTT
PepT1	peptide transporter-1 (SLC15A1)	BB	NM_204365	transports di- and tripeptides	[3]	CCCCTGAGGAGGATCACTGTT/ CAAAAGAGCAGCAGCAACGA
ZnT1	zinc transporter-1	BL	XM_421021	efflux of Zn^{2+}	[3]	TCCGGGAGTAATGGAAATCTTC/ AATCAGGAACAAACCTATGGGAAA
SI	sucrase isomaltase	BB	XM_422811	hydrolysis of sucrose and isomaltose	[3]	CGCAAAAGCACAGGGACAGT/ TCGATACGTGGTGTGCTCAGTT
β-actin	β-actin	C	NM_205518	reference gene	[3]	GTCCACCGCAAATGCTTCTAA/ TGCGCATTTATGGGTTTTGTT
β-actin	β-actin	C	L08165	reference gene	[1,2]	CACAGATCATGTTTGAGACCTT/ CATCACAATACCAGTGGTACG
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	C	AI981686	reference gene	[1,2]	GGCACGCCATCACTATC/ CCTGCATCTGCCCATTT
G6PDH	glucose-6-phosphate dehydrogenase	C	M11100	reference gene	[1,2]	CGGGAACCAAATGCACTTCGT/ CGCTGCCGTAGAGGTATGGGA

Location: BB brushborder, BL basolateral, C cytosol, Study: [1]qRT-PCR intestinal mucin 2 , [2]qRT-PCR oxidative stress, [3]qRT-PCR intestinal digestive enzymes and nutrient transporters

2.6 Statistical analysis

Statistical analyses of experimental data were performed using SPSS 22.0 (Chicago, IL, USA). Data were assessed using ANOVA after determination of normality and variance homogeneity. Subsequently, comparisons between all different groups was performed (Tukey HSD test). With exception of some of the genes encoding different methionine transporters, which were not normally distributed, Kruskal Wallis non-parametric testing was performed followed by a Wilcoxon-Mann-Whitney post hoc test. Differences between all experimental groups in Sa/So were analysed with each bird as the statistical unit. Results were presented as mean per experimental group ± standard deviation (SD). For the expression of genes encoding MUC2, zinc and methionine transporters, and oxidative stress, each chicken was defined as the statistical unit. Mean values and their standard errors (SE) were calculated for each experimental group separately. Differences between all experimental groups in mucin composition were analysed with each animal as the statistical unit. Mean values were calculated per experimental group, SE were expressed as pooled SEM per monosaccharide. The AIMD was compared between all experimental groups, with the pool of ileal content of seven chicken per pen as statistical unit. Mean values were calculated for each experimental group separately, standard errors of the mean (SEM) were calculated as pooled SEM. All data were first analysed per gender, and if no gender effect was present gender was excluded as a variable in the analysis. The significance level was set at 0.05 ($P \leq 0.05$). A trend was defined as P-value between 0.05 and 0.10 ($0.05 < P \leq 0.10$).

3 Results

3.1 No influence of mycotoxin exposure on bodyweight gain (BWG) and feed conversion ratio (FCR) in broiler chickens

No significant differences were observed in BWG and FCR between broiler chickens fed a control diet and chickens fed a mycotoxin contaminated diet (DON, FBs or DON+FBs) (Table 15). No mortality or macroscopic pathological findings were observed. No gender effect was observed on all measured parameters.

Table 15. Bodyweight gain (BWG) and feed conversion ratio (FCR).

	BWG (g)				FCR			
	D1-4	**D5-8**	**D9-11**	**D12-15**	**D1-4**	**D5-8**	**D9-11**	**D12-15**
control diet	35	94	90	175	1.22	1.32	1.43	1.58
DON diet	37	89	89	181	1.22	1.36	1.44	1.61
FBs diet	37	89	89	177	1.19	1.28	1.37	1.60
DON+FBs diet	35	82	91	179	1.27	1.55	1.36	1.42
SEM	0.9	1.9	1.6	2.2	0.025	0.041	0.035	0.038
P-value	*0.86*	*0.17*	*0.98*	*0.83*	*0.48*	*0.09*	*0.24*	*0.26*

Broilers were fed a control diet, DON contaminated diet (4.6 mg DON/kg feed), FBs contaminated diet (25.4 mg FB_1+FB_2/kg feed) or DON+FBs contaminated diet (4.3 mg DON/kg and 22.9 mg FB_1+FB_2/kg feed). Bodyweight (BW) of all birds was measured at day 1, 4, 8, 11 and 15, and feed intake per pen was recorded daily. Data are presented as mean BW (g) of eight pens per group with seven birds per pen. SEM represents the pooled standard error of the mean.

3.2 FBs negatively affect sphingolipid metabolism

The plasma level of Sa was significantly (*P<0.001*) increased in broilers fed a FBs (31 ± 17.2 µg/L) and a DON+FBs contaminated diet (35 ± 22.4 µg/L) compared to chickens fed a control (16 ± 8.8 µg/L) or a DON contaminated diet (11 ± 17.2 µg/L). No gender effect was observed, and no significant differences in the level of So were observed between the different experimental groups (data not shown). This also resulted in a significant (*P<0.001*) increased Sa/So ratio in chickens fed a FBs (0.27 ± 0.057) and DON+FBs (0.25 ± 0.035) contaminated diet, compared to birds fed a control (0.12 ± 0.020) or a DON (0.11 ± 0.011) contaminated diet.

3.3 Mycotoxin exposure affects duodenal mucus layer composition in broiler chickens

qRT-PCR analysis showed that the expression of the gene coding for MUC2 was significantly down-regulated in the duodenum of broilers fed DON, FBs or the multi-mycotoxin contaminated diet with DON and FBs (*P=0.04, P=0.02* and *P=0.01,* respectively) compared to the control group (Figure 24). No differences between the mycotoxin groups were observed. There was no down-regulation of *MUC2* in the jejunum or ileum.

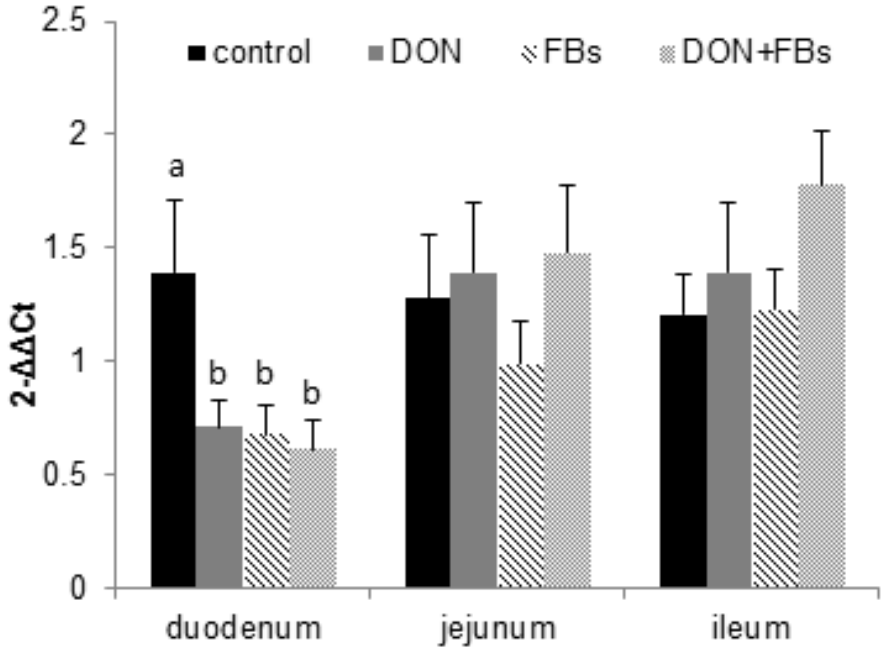

Figure 24. Effect of mycotoxins deoxynivalenol and fumonisins on mRNA expression of intestinal mucin 2 (MUC2) in broiler chickens. *Broilers were fed a control diet, DON contaminated diet (4.6 mg DON/kg feed), FBs contaminated diet (25.4 mg FB1+FB2/kg feed) or DON+FBs contaminated diet (4.3 mg DON/kg and 22.9 mg FB1+FB2/kg feed) for 15 days. After 15 days of feeding the experimental diets, mRNA expression levels in duodenum, jejunum, ileum were examined by qRT-PCR. Relative gene expression was determined using the* $2^{-\Delta\Delta Ct}$ *method. Data are presented as mean ± SE for a total of 16 animals per group.* $^{a-b}$ *significantly different (P<0.05).*

The mucin monosaccharide composition in the duodenum was also affected by mycotoxin exposure. The percentage of GalNac was significantly increased in chickens fed a FBs contaminated diet (*P=0.04*) or a DON and FBs contaminated diet (*P=0.03*) compared to animals fed a control diet (Table 16). On the other hand, a significantly decreased percentage of Gal was observed in the duodenal mucus layer of broilers fed a FBs contaminated diet (*P=0.01*) or a DON and FBs contaminated diet (*P=0.01*) compared to control (Table 16). In the DON challenged broiler chickens, numerically a similar trend for the proportion of

GalNac and Gal was observed. In all mycotoxin exposed groups a significantly increased percentage of NeuAc was demonstrated (*P=0.01*) (Table 2). Furthermore, a trend of a lower percentage of Man was observed in FBs exposed chickens (*P=0.09*), and a trend of a lower percentage of Fuc was seen in DON exposed animals (*P=0.06*). No effect of mycotoxin exposure was demonstrated on the mucin monosaccharide composition in the jejunum and ileum (Table 16). No difference between cockerels and pullets was observed for the impact of both mycotoxins on the intestinal mucus layer.

Table 16. Influence of deoxynivalenol and fumonisins on the small intestinal mucin monosaccharide composition in broiler chickens.

DUODENUM						
(%)	**GlcNac**	**GalNac**	**Gal**	**Man**	**Fuc**	**NeuAc**
control	27.5	17.2[a]	30.2[a]	4.9	8.9	11.2[a]
DON	28.1	19.4[ab]	23.8[ab]	4.2	7.3	17.3[b]
FBs	27.5	24.4[b]	20.2[b]	3.1	8.7	16.2[b]
DON+ FBs	28.8	24.3[b]	20.9[b]	3.5	6.9	15.7[b]
SEM	0.76	0.95	1.13	0.28	0.32	0.72
P-value	*0.74*	*0.01*	*0.01*	*0.09*	*0.06*	*0.01*
JEJUNUM						
(%)	**GlcNac**	**GalNac**	**Gal**	**Man**	**Fuc**	**NeuAc**
control	31.9	26.0	20.8	3.2	4.0	14.1
DON	31.7	27.6	18.9	2.4	3.8	15.7
FBs	32.5	26.7	20.9	3.4	3.8	12.8
DON+ FBs	32.3	27.3	21.4	3.0	3.5	12.5
SEM	1.08	0.85	1.12	0.19	0.17	0.74
P-value	*0.96*	*0.92*	*0.88*	*0.22*	*0.82*	*0.42*
ILEUM						
(%)	**GlcNac**	**GalNac**	**Gal**	**Man**	**Fuc**	**NeuAc**
control	24.4	24.7	32.8	4.1	4.6	9.4
DON	25.7	27.1	28.7	3.5	4.9	10.1
FBs	26.0	26.3	28.6	3.4	5.2	10.5
DON+ FBs	28.2	24.7	28.0	3.7	4.2	11.2
SEM	1.05	0.84	1.22	0.14	0.21	0.64
P-value	*0.45*	*0.71*	*0.50*	*0.39*	*0.40*	*0.83*

Broilers were fed a control diet, DON contaminated diet (4.6 mg DON/kg feed), FBs contaminated diet (25.4 mg FB_1+FB_2/kg feed) or DON+FBs contaminated diet (4.3 mg DON/kg and 22.9 mg FB_1+FB_2/kg feed) for 15 days., and subsequently small intestinal mucus samples were collected. Data are presented as means of eight animals per group with one animal per pen. SEM represents the pooled standard error of the mean. [a-b] significantly different within one column (P≤0.05).

3.4 Mycotoxin exposure modulates the oxidative stress response

Expression of *HIF-1α* was unaltered in the intestine, independently of the treatment or intestinal section (Figure 25A). An up-regulation of *HMOX* (*trend, P=0.06*) was only observed in the ileum of chickens fed a DON contaminated diet compared to control group (Figure 25B). In comparison to the control group, mycotoxin exposure altered the expression of *XDH*, demonstrated by a down-regulation (*trend, P=0.08*) in the jejunum of animals fed a FBs contaminated diet, and a similar trend (*trend, P=0.10*) was observed in the jejunum of animals fed a DON and FBs contaminated diet (Figure 25C).

The expression of a panel of digestive enzymes and nutrient transporters was examined in chickens fed DON, FBs or DON + FBs, but only a subset of genes that showed significant differences are shown in Tables 1 and 3. Aminopeptidase N (APN), amino acid (ASCT1, CAT2, LAT1) and monosaccharide (SGLT1, GLUT2, GLUT5) transporters showed no differences between control and DON, FBs or DON plus FBs treatment (data not shown). The expression of the gene encoding the basolateral zinc transporter-1 (ZnT-1) was significantly down-regulated in all mycotoxin-challenged broiler chickens, either in chickens fed DON, FBs or the multi-mycotoxin contaminated diet with DON and FBs compared to the control group (*P=0.02, P=0.01* and *P=0.05,* respectively) (Table 14). Similarly, sucrase isomaltase (SI) was also down-regulated in DON, FBs or DON plus FBs treatment compared to control (*P=0.04, P<0.01* and *P=0.01,* respectively) (Table 14). Furthermore, the transcription of the gene encoding the brushborder Na^+-independent neutral/cysteine, cationic amino acid exchanger $b^{0,+}AT$ (*P=0.05*) was significantly up-regulated in chickens fed a FBs contaminated diet compared to the other experimental groups (Table 14). In this group a similar trend of increased mRNA expression was observed for Na^+ dependent neutral amino acid transporter (B^0AT) (*P=0.07*) and y^+ L amino acid transporter 1 (y^+LAT1) (*P=0.08*) (Table 14). No effect was observed on rBAT, y^+LAT2 and the peptide transporter PepT1 (Table 14).

Feeding a DON and FBs contaminated diet significantly increased the apparent ileal methionine digestibility compared to chickens fed a control diet (82% versus 72%, respectively) (*P=0.02*). No significant differences were observed in chickens fed a DON (74%) or a FBs contaminated diet (75%) compared to chickens fed the control diet. No gender effect was observed for the impact of DON and/or FBs on the induction and modulation of intestinal oxidative stress.

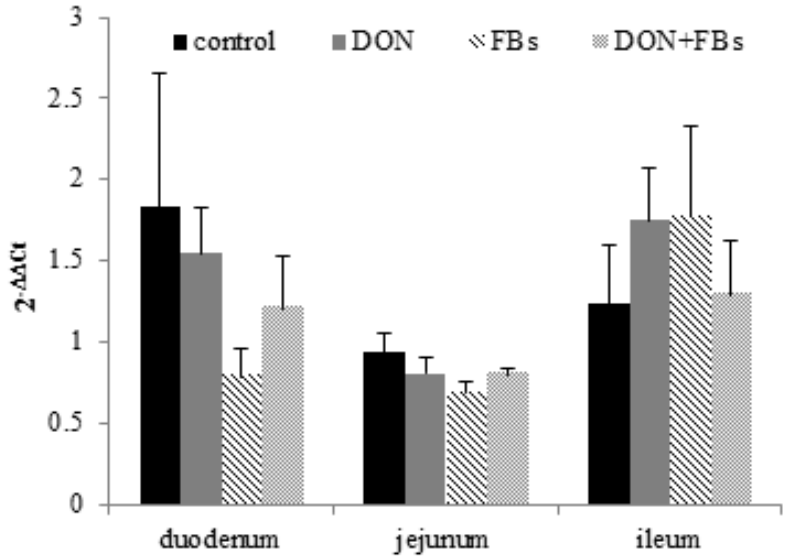

Figure 25A. Effect of mycotoxins deoxynivalenol and fumonisins on mRNA expression of hypoxia-inducible factor 1 subunit alpha in broiler chickens. *Broilers were fed a control diet, DON contaminated diet (4.6 mg DON/kg feed), FBs contaminated diet (25.4 mg FB_1+FB_2/kg feed) or DON+FBs contaminated diet (4.3 mg DON/kg and 22.9 mg FB_1+FB_2/kg feed) for 15 days. After 15 days of feeding the experimental diets, mRNA expression levels in duodenum, jejunum, ileum were examined by qRT-PCR. Relative gene expression was determined using the $2^{-\Delta\Delta Ct}$ method. Data are presented as mean ± SE for a total of 16 animals per group.*

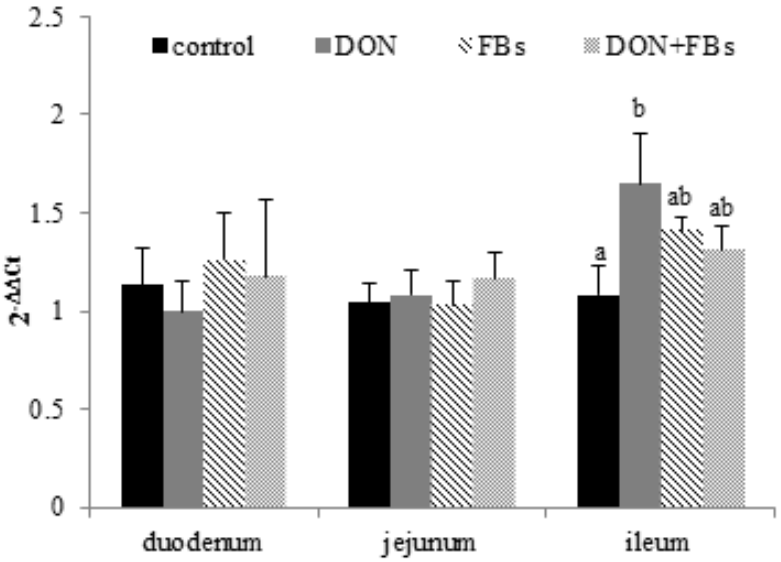

Figure 25B. Effect of mycotoxins deoxynivalenol and fumonisins on mRNA expression of heme-oxigenase in broiler chickens. *Broilers were fed a control diet, DON contaminated diet (4.6 mg DON/kg feed), FBs contaminated diet (25.4 mg FB_1+FB_2/kg feed) or DON+FBs contaminated diet (4.3 mg DON/kg and 22.9 mg FB_1+FB_2/kg feed) for 15 days. After 15 days of feeding the experimental diets, mRNA expression levels in duodenum, jejunum, ileum were examined by qRT-PCR. Relative gene expression was determined using the $2^{-\Delta\Delta Ct}$ method. Data are presented as mean ± SE for a total of 16 animals per group. $^{a-b}$ significantly different ($P<0.05$).*

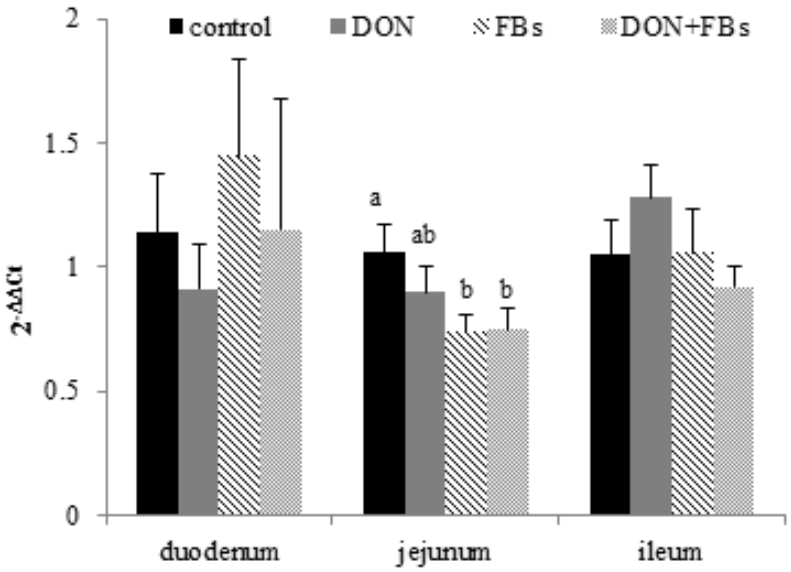

Figure 25C. Effect of mycotoxins deoxynivalenol and fumonisins on mRNA expression of xanthine dehydrogenase in broiler chickens. *After 15 days of feeding the experimental diets, mRNA expression levels in duodenum, jejunum, ileum were examined by qRT-PCR. Relative gene expression was determined using the $2^{-\Delta\Delta Ct}$ method. Data are presented as mean ± SE for a total of 16 animals per group. $^{a-b}$ significantly different (P<0.05).*

Table 17. mRNA expression of selected amino acid, peptide and mineral transporters in jejunum of mycotoxin challenged broiler chickens.

	relative gene expression ($2^{-\Delta\Delta Ct}$)			
	ZNT1	**$b^{0,+}$AT**	**rBAT**	**B^0AT**
control diet	1.1 ± 0.22^{a}	1.1 ± 0.18^{a}	1.2 ± 0.24	1.1 ± 0.18^{a}
DON diet	0.5 ± 0.10^{b}	2.2 ± 0.82^{a}	0.9 ± 0.11	3.5 ± 1.86^{a}
FBs diet	0.4 ± 0.15^{b}	22.4 ± 12.91^{b}	0.8 ± 0.31	23.3 ± 14.05^{b}
DON+FBs diet	0.6 ± 0.13^{b}	0.8 ± 0.17^{a}	0.7 ± 0.14	0.9 ± 0.28^{a}
P-value	*0.01*	*0.05*	*0.21*	*0.07^{T}*
	y^+LAT1	**y^+LAT2**	**PepT1**	**SI**
control diet	1.3 ± 0.31^{a}	1.1 ± 0.14	1.1 ± 0.20	1.2 ± 0.28^{a}
DON diet	6.8 ± 3.77^{ab}	3.7 ± 1.90	0.9 ± 0.25	0.6 ± 0.12^{b}
FBs diet	64.2 ± 39.74^{b}	19.1 ± 12.73	3.6 ± 1.91	0.3 ± 0.11^{b}
DON+FBs diet	1.5 ± 0.77^{a}	0.9 ± 0.21	0.7 ± 0.15	0.5 ± 0.11^{b}
P-value	*0.08^{T}*	*0.12*	*0.47*	0.03

Broilers were fed a control diet, DON contaminated diet, FBs contaminated diet or DON+FBs contaminated diet for 15 days, and subsequently mRNA expression was examined by qRT-PCR. Relative gene expression was determined using the $2^{-\Delta\Delta Ct}$ method. ZNT1= zinc transporter-1; $b^{0,+}$AT= Na^+-independent neutral/cysteine, cationic amino acid exchanger; B^0AT= Na^+-dependent neutral amino acid transporter; rBAT= protein related to $b^{0,+}$AT; y^+LAT1 and y^+LAT2= y^+ L amino acid transporter-1 and -2, respectively; PepT1= peptide transporter-1; SI= sucrase isomaltase. Data are presented as mean ± SE of eight animals per group with one animal per pen. $^{a-b}$ significantly different within one column (P≤0.05). ^{T} differences within one column were a trend (0.05<P≤0.10).

4 Discussion

Currently, the maximum EU guidance levels for DON and FBs in poultry feed [131], represent the concentrations at or below which no lethal effects, growth depression, nor macroscopic pathological findings are observed [16]. Consistent with this, no clinical signs of mycotoxicosis were observed in the presented animal trial. However, feeding FBs at concentrations close to the EU maximum guidance levels increased plasma Sa/So ratios, a biomarker of FBs intoxication. It is well known that FBs exposure disrupts ceramide and sphingolipid metabolism, consequently leading to an accumulation of free Sa, and to a lesser extent of So in tissues, serum and urine [365,431]. Accordingly, a dose-dependent increase in Sa/So ratio was observed in the liver, kidney, jejunum, ileum and cecum of broiler chickens fed a FBs contaminated diet [175].

Feeding broiler chickens a DON and/or FBs contaminated diet for 15 days at concentrations close to the EU maximum guidance levels affects the extrinsic component of the intestinal barrier, by modulating the intestinal mucus layer and by inducing an oxidative stress response in the intestinal epithelial cells.

The mRNA expression of *MUC2*, the gene encoding the primary gel-forming mucin, was decreased in the duodenum in all mycotoxin-exposed groups. Previously, it was demonstrated *in vitro* that non-cytotoxic concentrations of DON decreased mucin production in human colonic epithelial goblet cells (HT-29 16E) and porcine intestinal explants [321]. In accordance with the down-regulation of *MUC2* expression, significant differences in the mucin monosaccharide composition between control and mycotoxin-challenged birds were only observed in the duodenum. Mucins consist of a high proportion of carbohydrates (i.e. GlcNac, GalNac, Gal, Fuc, Man, and NeuAc) built on a protein domain rich in amino acids proline, threonine, and serine [204,395]. The O-glycan structures present in mucins are diverse and complex, consisting predominantly of α- and β-linked GlcNac, GalNac, and Gal [395]. Mycotoxin exposure affected the proportion of GalNac and Gal, but not GlcNac, in the duodenal mucus layer. The O-glycan core structures are further elongated and frequently modified by fucose and sialic acid sugar residues [395]. In all mycotoxin-exposed groups a significantly increased proportion of NeuAc was found compared to the control group, and a trend of decreased fucose fraction by DON exposure. Changes in mucin O-linked oligosaccharides composition can affect mucus layer integrity, and modulate the intestinal microbiota composition [277,395].

DON and FBs also negatively affect the cellular homeostasis by the induction of cellular oxidative stress [145,191,274,328,455]. In the present study, the response of the intestinal epithelial cells was characterized by the induction and modulation of several anti-oxidative mechanisms. Indeed, a compensatory down-regulation of *XDH* in the jejunum of chickens fed a FBs or a FBs and DON contaminated diet was observed. Of the two inter-convertible forms of xanthine oxidoreductase (XOR), xanthine oxidase (XO) and XDH, it has been shown in birds that mainly XDH is formed [61,181]. In addition to the XOR mediated degradation of hypoxanthine to the anti-oxidant uric acid, this reaction is also characterized by the formation of ROS, a.o. hydrogen peroxide and superoxide, which play an antimicrobial role in the digestive tract [149,181,259]. Therefore, our results suggest that epithelial cells convert less hypoxanthine to xanthine and to uric acid, and correspondingly, produce less ROS as a byproduct of this enzymatic reaction [61,181]. Similarly, feeding a DON contaminated diet to broilers induced an up-regulation of *HMOX* in the ileum, which results in a HO-1 mediated decrease in cellular content of pro-oxidants such as heme and iron, and an increased cellular content of the anti-oxidant biliverdin [200]. This is in accordance with the observations of Osselaere *et al.* [303], who demonstrated a similar effect in the jejunum of broiler chickens fed a DON contaminated diet (7.5 mg/kg feed) for three weeks.

Feeding a DON and/or FBs contaminated diet was associated with a down-regulation of the jejunal expression of the zinc transporter *ZnT1*, which is located at the basolateral membrane of enterocytes and functions in efflux of Zn^{2+} out of the cell [396]. Maintaining a minimal level of intracellular zinc by inhibiting its efflux may be important for preserving critical anti-oxidant activity to counteract the production of mycotoxin-induced oxidative damage [134,382,389]. Similar results were observed in broiler chickens with intestinal damage caused by the mycotoxin ochratoxin A and the intestinal protozoan *Eimeria* [339,389].

FBs-induced up-regulation of the brushborder transporter B^0AT and the basolateral transporter y^+LAT1 suggests an increased intracellular uptake of neutral amino acids, such as methionine. Intestinal uptake of methionine, could assist to counteract the mycotoxin-induced oxidative stress by different mechanisms, among others by stabilization of sulfhydryls from oxidant-induced disulphide bond formation and stabilization of membrane phospholipids [134,382,389]. In contrast, the FBs-induced up-regulation of the brushborder transporter $b^{0,+}AT$ suggests an increased intracellular uptake of cationic amino acids in exchange for neutral amino acids. However, only a small contribution of the $b^{0,+}AT$ transporter in the regulation of the intestinal uptake of methionine is suggested [379]. Increased intestinal uptake of methionine

was further documented by the increased ileal methionine digestibility. Consequently, it can be hypothesized that higher intracellular levels of methionine may increase the production of the anti-oxidants cysteine and GSH. In this way, it may stimulate the methionine sulfoxide reductase-mediated destruction of ROS [271].

The mycotoxin induced changes in gene expression are similar to those observed during trinitrobenzene sulfonic acid-induced colitis in rats [6]. Induction of colitis in rats led to a decrease in MUC2 mRNA in the jejunum and ileum and a decrease in SI and APN mRNA in the ileum but not in the jejunum. Similar down-regulation of MUC2 and SI mRNA was seen with DON and FBs mycotoxins, although the affected intestinal segments differed. This is not unexpected since colitis mainly affects the colon with some extension into the ileum. Together these results may be revealing a common response to intestinal inflammation.

5 Conclusion

In conclusion, this study shows for the first time that both DON and FBs at levels close to the EU maximum guidance levels decrease *MUC2* expression and alter mucin monosaccharide composition. Feeding a DON and/or FBs contaminated diet increased intestinal epithelial anti-oxidant mechanisms modulated among others through the intracellular levels of zinc and methionine.

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CHAPTER 3

The impact of deoxynivalenol and fumonisins on the intestinal barrier

3.2 Impact of chronic exposure to deoxynivalenol on the intestinal absorption of fumonisins

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Adapted from

Antonissen G., Devreese M., Van Immerseel F., De Baere S., Hessenberger S., Martel A., Croubels S. (2015) Chronic exposure to deoxynivalenol has no influence on the oral bioavailability of fumonisin B_1 in broiler chickens. Toxins 7: 560-571.

Abstract

Both deoxynivalenol (DON) and fumonisin B_1 (FB_1) are common contaminants of feed. Fumonisins (FBs) in general have a very limited oral bioavailability in healthy animals. Previous studies have demonstrated that chronic exposure to DON impairs the intestinal barrier function and integrity, by affecting the intestinal surface area and function of the tight junctions. This might influence the oral bioavailability of FB_1, and possibly lead to altered toxicity of this mycotoxin. A toxicokinetic study was performed with two groups of 6 broiler chickens, which were all administered an oral bolus of 2.5 mg FBs/kg BW after three-week exposure to either uncontaminated feed (group 1) or feed contaminated with 3.12 mg DON/kg feed (group 2). No significant differences in toxicokinetic parameters of FB_1 could be demonstrated between the groups. Also, no increased nor decreased body exposure to FB_1 was observed, since the relative oral bioavailability of FB_1 after chronic DON exposure was 92.2%.

Keywords

Broiler chickens – Deoxynivalenol – Fumonisin B_1 – *Fusarium* toxins – Oral bioavailability – Toxicokinetics

1 Introduction

Mycotoxins are a structurally diverse group of secondary metabolites produced by several fungal genera [39]. Moulds belonging to the *Fusarium* genus are commonly affecting feed and food in climatological moderate regions [119]. A worldwide survey on the occurrence and contamination levels of mycotoxins in finished feed for poultry, swine and dairy cows, and feed raw materials indicate that the fusariotoxins deoxynivalenol (DON) and fumonisins (FBs) are the most frequently detected mycotoxins, respectively contaminating 55% and 54% of the 17,316 investigated samples [386]. However, taking into consideration that mycotoxigenic fungi are usually capable of producing more than one mycotoxin, and that feed raw materials are commonly infected with various fungal species at a time, it is very common for feed commodities to be contaminated with different mycotoxins. A study of Streit *et al.* [386], reported that in 53% of the contaminated samples more than one mycotoxin was detected. The final mycotoxin profile of compound feed is also influenced by the levels of the different feed raw materials [387].

The intestinal tract acts as a dynamic barrier, which regulates the entry of foreign antigens into the underlying tissues including food proteins, xenobiotics (such as drugs and mycotoxins), commensal microbiota and pathogens [47]. Following the oral intake of mycotoxin-contaminated feed, the intestinal epithelium will be exposed to high concentrations of mycotoxins [47,50]. Since the main toxic effect of DON at the cellular level is the inhibition of protein synthesis, rapidly proliferating cells in tissues with a high protein turnover, such as the small intestine, are most affected [349]. Several studies demonstrated a negative effect of DON on the intestinal morphology. DON decreases the total intestinal absorption surface area for nutrients by reducing the villus height and crypt depth [13,303,452]. Furthermore, several *in vitro* and *in vivo* studies reported that DON alters the intestinal epithelial integrity and permeability, by affecting the function of the tight junctions [13,303,322]. As a result of the negative impact of DON on the intestinal integrity, DON is able to increase the translocation of septicemic *E. coli* and increase the permeability to doxycycline and paromomycin over porcine intestinal epithelial cell monolayers [169,322].

As stated above, in addition to DON, FBs are ubiquitous contaminants of corn and other grain products. FBs are produced by *Fusarium verticillioides*, *F. proliferatum,* and other *Fusarium* species [431]. More than 28 fumonisin homologues have been described, with fumonisin B_1 (FB_1) as the most thoroughly investigated because of its frequent occurrence and toxicological importance. Fumonisin B_2 (FB_2), FB_3 and FB_4 are less prevalent, and are structurally different

from FB_1 in the number and position of hydroxyl groups [101,431]. FBs mainly act by inhibiting sphinganine N-acyl transferase and consequently disrupt the ceramide and sphingolipid metabolism [344]. Liver, kidneys and the intestinal tract are target organs of FBs toxicity in most animal species [48,171,431]. However, species specific differences exist in the main affected organs. In horses, FB_1 mainly affects the brain inducing leukoencephalomalacia, while in pigs the heart and lungs are the most important target organs of FB_1, causing pulmonary edema [431]. Poultry are often considered to be quite resistant toward the deleterious effects of FBs, although important differences are observed depending on the age [202] and species [225,397,409,438]. Increased mortality due to FB_1 has only been demonstrated in broiler chicks during the first three days of life (≥ 125 mg/kg feed) [202] and in growing ducks of 12-14 weeks old (20 mg/kg feed) [397]. No mortality has been recorded in laying hens, turkeys or older broiler chickens fed high doses of FB_1 (≥ 200 mg/kg feed) for several weeks [225,229,438]. Moreover, it has been shown that FBs can reduce growth performance, and induce alterations in serum constituents and enzyme activities demonstrating hepatic toxicity in broilers, turkeys and ducks [55,229,397-399,409,438].

In different animal species it is shown that FBs are absorbed very poorly after oral administration. Vudathula *et al.* [432] showed an oral bioavailability (F) of 0.71% in laying hens administered 2 mg [14C]FB_1/kg bodyweight (BW). In turkeys and ducks, a similar F was demonstrated after administering 100 mg FB_1/kg BW, namely 2-2.3% and 3.2%, respectively [398,399]. Benlashehr *et al.* [33] demonstrated that the toxicokinetics parameters of FB_2 are not strongly different from these of FB_1 in ducks and turkeys. Furthermore, the intestinal absorption of FBs in avian species is comparable with mammalian species [262,334,364]. This poor intestinal absorption of FBs has been designated as the "fumonisin paradox" by Shier [366], or how a toxin can induce liver failure in poultry although it is not effectively absorbed after oral intake. Because the mycotoxins DON and FBs frequently co-occur, and taken into account that FBs have a low oral bioavailability in healthy animals and DON impairs the intestinal barrier and/or decreases the total intestinal absorption surface area, the aim of this study was to investigate whether chronic exposure to DON could influence the intestinal absorption of FBs leading to an altered exposure and increased toxic effects of this mycotoxin in broiler chickens. Because FB_1 is the most abundant of the FBs in feed, and toxicokinetics parameters of FB_1 en FB_2 are strongly similar [33], the impact of DON on the toxicokinetics parameters of FB_1 was investigated.

2 Materials and Methods

2.1 Chemicals, products and reagents

DON (25.9 mg DON/g culture material) and FBs (14.07 mg FB_1/g and 4.3 mg FB_2/g culture material) were produced *in vitro* from cultures of *F. graminearum* (DSMO 4258) and *F. verticillioides* (M-3125) [5,234], respectively, and subsequently purified and crystallized [5,177] (Romer Labs, Tulln, Austria). The standards of DON and FB_1 for the analytical experiments were purchased from Fermentek (Jerusalem, Israel), and Sigma-Aldrich (Bornem, Belgium) for DOM-1. Internal standards (IS) for DON, $^{13}C_{15}$-DON, and for FB_1, $^{13}C_{34}$-FB_1, were purchased from Romer Labs (Tulln, Austria). The standards were stored at ≤ -15°C. Water, methanol and acetonitrile (ACN) were of LC-MS grade and were obtained from Biosolve (Valkenswaard, The Netherlands). Glacial acetic acid and formic acid were of analytical grade and obtained from VWR (Leuven, Belgium). Millex®-GV-PVDF filter units (0.22 µm) were obtained from Merck-Millipore (Diegem, Belgium).

2.2 Feed preparation and experimental diets

Chickens were fed a starter diet during the first eight days of the experiment, and subsequently a grower diet until the end of the trial (day 21). These feeds are further referred to as control diets. The feed composition was described previously in detail [13,158]. Briefly, the diet was wheat and rye based, with soybean meal as main protein source during the first 16 days. From day 17 onwards, the same grower diet was fed with the exception that fishmeal replaced soybean meal as main protein source. Screening of the control feeds for contamination with mycotoxins was performed by a LC-MS/MS method, as described by Monbaliu *et al.* [276]. To produce a starter and grower diet experimentally contaminated with DON, purified crystallized DON was added to 500 g of control feed. This premix was then mixed with 5 kg of control feed to assure homogeneous distribution of the toxin. The premix was finally mixed for 20 min in the total amount of feed needed for each diet. To test the homogeneity of DON in the diets, a sample was taken at three different locations in the batch and analyzed for DON as described for the control diets.

Different tested mycotoxins, their limit of detection (LOD) and limit of quantification (LOQ) were as previously described by Antonissen *et al.* [13]. Trace amounts of FB_1 were detected in the control feed and the contaminated feed, but the mean level of 64 µg/kg feed was below the LOQ (116 µg/kg). The levels of DON and all other tested mycotoxins in the different batches

of control feed were below the LOQ. The average level of DON in the different batches of contaminated feed was 3.12 ± 0.234 mg DON/kg feed, which is below the EU maximum guidance level of 5 mg DON/kg feed for poultry [131]. The contaminated feed contained also 0.020 ± 0.007 mg 3-acetylDON/kg feed and 0.038 ± 0.031 mg 15-acetylDON/kg feed.

2.3 Animal experiment

Twelve one-day old broiler chickens of mixed gender (Ross 308) were randomly allocated to two different groups of 6 birds (3♂/3♀). A 18 h/6 h light/darkness program was applied. The environmental temperature was adjusted to the changing needs of the animals according to their age. The birds of one group were fed uncontaminated feed *ad libitum* (control group) whereas the birds of the other group were fed the DON contaminated feed (DON contaminated group). Drinking water was provided *ad libitum* during the entire experiment. Feed intake was measured daily per group. The BW of all animals was measured on day 1, 8, 15 and day 21. After three weeks, chickens were fasted overnight (8 h), and subsequently all birds were administered 2.5 mg FB_1+FB_2 / kg BW as an intra-crop bolus. The 2.5 mg FBs/kg BW corresponded with 1.91 mg FB_1/kg BW and 0.59 mg FB_2/kg BW. Blood was sampled by direct venipuncture from the leg vein (*vena metatarsalis plantaris superficialis*) into heparinized tubes before (0 min) and at different time points after administration, i.e. 10, 20, 30, 40, 50, 60 and 240 min. No feed was provided during the toxicokinetic experiment. Blood samples were centrifuged (2851 x *g*, 10 min, 4 °C) and plasma was stored at ≤ -15 °C until analysis. At the end of the experiment, all the animals were euthanized and a macroscopic post-mortem examination was carried out to reveal a possible pathology.

The animal experiment was approved by the Ethical Committee of the Faculty of Veterinary Medicine and Bioscience Engineering of Ghent University (EC 2012/075).

2.4 Quantification of DON, DOM-1 and FB_1 in plasma

Two LC-MS/MS methods were used to quantify DON and DOM-1, and FB_1 in the plasma samples, based on Devreese *et al.* [102]. The sample preparation procedure was the same for both methods. In brief, to 250 µL of plasma 12.5 µL of both IS and 750 µL of ACN were added, followed by a vortex mixing (15 s) and centrifugation step (8517 x *g*, 10 min, 4°C). Next, the supernatant was transferred to another tube and evaporated using a gentle nitrogen (N_2) stream (45 ± 5°C). The dry residue was reconstituted in 200 µL of water/methanol (85/15, v/v). After vortex mixing (15 s), the sample was passed through a Millex® GV-PVDF

filter (0.22 µm) and transferred into an autosampler vial. An aliquot (5 µL) was injected onto the LC-MS/MS instrument. The LC system consisted of a quaternary, low-pressure mixing pump with vacuum degassing, type Surveyor MSpump Plus and an autosampler with temperature controlled tray and column oven, type Autosampler Plus, from ThermoFisher Scientific (Breda, The Netherlands). Chromatographic separation was achieved on a Hypersil Gold column (50 mm x 2.1 mm i.d., dp: 1.9 µm) in combination with a guard column of the same type (10 mm x 2.1 mm i.d., dp: 3 µm), both from ThermoFisher Scientific. A gradient elution program was performed with 0.1 % glacial acetic acid (DON, DOM-1) or 0.1 % acetic acid (FB_1) in water and methanol as mobile phases. The LC column effluent was interfaced to a TSQ® Quantum Ultra triple quadrupole mass spectrometer, equipped with a heated electrospray ionization (h-ESI) probe operating in the negative ionization mode for DON and DOM-1, and in the positive mode for FB_1 (all from ThermoFisher Scientific). Following selected reaction monitoring (SRM) transitions were monitored and used for quantification: for DON *m/z* 355.2 > 265.1 and 355.2 > 295.1, for DOM-1 *m/z* 339.1 > 59.1 and 339.1 > 249.0, for $^{13}C_{15}$-DON *m/z* 370.2 > 279.1 and 370.2 > 310.1, for FB_1 *m/z* 722.3 > 333.9 and 722.3 > 352.4 and for $^{13}C_{34}$-FB_1 756.4 > 356.2 and 756.4 > 374.2. The LOQs of DON, DOM-1 and FB_1 were 1, 2 and 1 ng/mL, respectively, whereas the LODs were 0.05, 0.04 and 0.08 ng/mL, respectively.

2.5 Toxicokinetic and statistical analysis

Toxicokinetic analysis was performed with WinNonlin 6.3 following a *noncompartmental* model (Pharsight, St-Louis, MI, USA). The most important toxicokinetic parameters of FB_1 were calculated: maximal plasma concentration (C_{max}), time to maximal plasma concentration (T_{max}), area under the plasma concentration-time curve from time 0 to 2 h (AUC_{0-t}), elimination rate constant (k_{el}), elimination half-life ($T_{1/2el}$), mean residence time (MRT), volume of distribution divided by the absolute oral bioavailability (Vd/F), and clearance divided by the absolute oral bioavailability (Cl/F). The relative oral bioavailability (Rel F) was calculated according to the following formula:

$$[\text{Rel F} = AUC_{0-t} \text{ DON contaminated} / AUC_{0-t} \text{ control}]$$

Statistical analysis was done using a Student's t-test (SPSS 20.0, IBM, IL, USA). The significance level was set at 0.05.

3 Results and Discussion

No significant effects on BW or feed intake were seen after chronic exposure to DON (data not shown). For each diet, control and DON contaminated, no macroscopic lesions were found during gross postmortem examination.

After a single oral bolus administration of 2.5 mg FBs/kg BW (1.91 mg FB_1 and 0.59 mg FB_2), quantifiable plasma concentrations of FB_1 were detected (Figure 26). The dose was calculated based on the European maximum guidance level of 20 mg FB_1+FB_2/kg feed [131] and the daily feed intake of the birds (125 g/kg BW). As shown in Figure 26, the plasma concentration-time profile revealed that FB_1 reached the maximum plasma concentration (T_{max}) at 20 min after oral dosing in both control and DON contaminated group. This rapid appearance of FB_1 in the systemic circulation indicates that the ingested toxin is absorbed mainly in the proximal part of the intestinal tract. The T_{max} was reached more rapidly compared to studies in layers, turkeys and ducks, where a T_{max} of 60 min, 180 min, 60-120 min has been described, respectively [398,399,432]. This difference might be induced by feed deprivation prior to oral FB_1 administration in the present study, whereas in the other studies feed was not deprived. The delaying effect of feed on absorption of mycotoxins has previously been described for DON in pigs with a T_{max} of 1.3 h and 4.1 h in fasted and fed pigs, respectively [89,99].

Oral absorption of FB_1 by passive non-ionic transcellular diffusion is very limited as FB_1 is mainly negatively charged at the pH of the duodenum and jejunum in broiler chickens (pH = 6-7 [256]; pKa of the two tricarballylic acid functional groups of FB_1: 3.49-5.83 and of the amine functional group: 9.53 [253]). Besides, also limited transcellular transporter mediated FB_1 absorption has been suggested [366]. Also, paracellular transport of FB_1 is unlikely as the tight junction complex only regulates transport of very small endogenous compounds, not of xenobiotics like mycotoxins and drugs. In this study, it was hypothesized that damage evoked by chronic DON exposure could lead to less complex tight junctions or a 'leaky' epithelium thereby enhancing FB_1 transport. Indeed, DON negatively affects intestinal integrity and morphology as described previously by our group [13], where the same batches of DON contaminated diets as in the present study were used. This chronic DON exposure causes shortened intestinal villi, leading to a decreased intestinal surface area and possibly leading to a reduced transport [13,452]. This reduced surface area could thus abolish the possible increased paracellular transport.

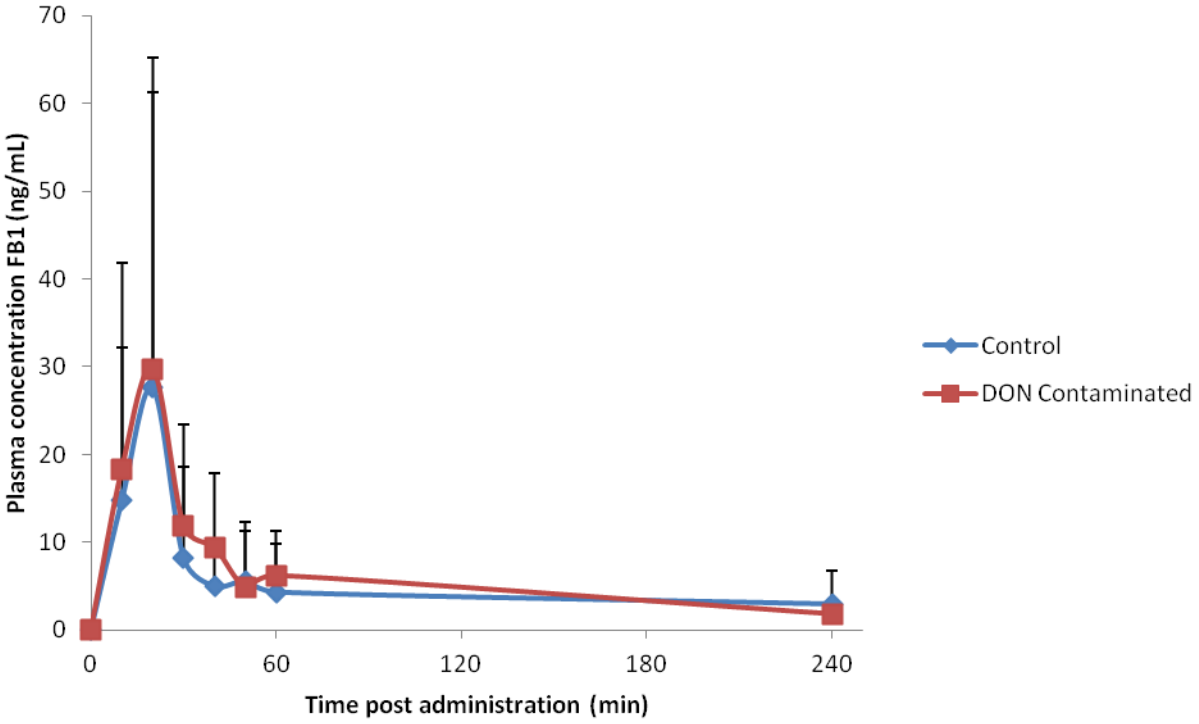

Figure 26. Plasma concentration-time profile of fumonisin B_1 (FB_1) administered as a single oral bolus of fumonisins to broiler chickens *(2.5 mg FBs/kg BW, n = 6), after 3 weeks exposure to either a diet contaminated with deoxynivalenol (DON contaminated, contamination level: 3.12 mg DON/kg feed) or uncontaminated (control) feed. Values are presented as mean ± SD.*

However, the maximum plasma concentration (C_{max}) was similar in chickens fed the control feed and the DON contaminated feed, respectively 0.033 ± 0.0213 µg/mL and 0.035 ± 0.0248 µg/mL. In accordance, Vudathala *et al.* [432] showed a C_{max} of 0.028 ± 0.103 µg/mL after oral administration of 2 mg [14C]FB_1/kg BW to laying hens. Furthermore, feeding a DON contaminated diet had no effect on the area under the plasma concentration-time profile of FB_1 from time 0 to 2 h (AUC0-2 h) when compared to the control group (Table 18), demonstrating that no effect of DON on body exposure to FB_1 was present. Since FB_1 was not administered intravenously to the broiler chickens in the present study, the actual absolute oral F remains unknown. Therefore, the actual volume of distribution (Vd) and total body clearance (Cl) are computed by the modelling software as Vd/F and Cl/F, respectively. The volume of distribution of FB_1 was similar in both experimental groups, i.e. 206.7 ± 92.37 and 234.3 ± 25.03 L/kg in the DON contaminated versus control group, respectively (Table 18).

In order to compare the Vd and Cl between poultry species, the values obtained for ducks and turkeys by Tardieu *et al.* [398,399] have been divided by their reported absolute F as well. The Vd/F of broiler chickens was higher compared to ducks and turkeys, namely 74.1-85.8 and 72.3 L/kg, respectively. The clearance (Cl/F) of FB_1 obtained after oral administration in broiler chickens was similar in both experimental groups (Table 18), and was comparable to

ducks (739-835 mL/min/kg) but was higher compared to turkeys (234 mL/min/kg) [398,399]. Consequently, the elimination half-life ($T_{1/2el}$) was twice as long in turkeys (214 min) [399] compared to broilers (106 min) and ducks (70 min) [398]. The mean residence time (MRT) was 150.8 ± 35.52 min and 165.5 ± 48.81 min in the DON contaminated group and the control group, respectively. These results are comparable with ducks (188 – 200 min) [398], but shorter compared to turkeys (408 min) [399]. This study also showed, in accordance to reports in other poultry species [398,399,432], low plasma levels of FB_1 (low ng/mL range) despite the high administered dose (2.5 mg FBs/kg BW). This low oral bioavailability suggests that the systemic exposure to this mycotoxin can therefore be enhanced when the intestinal barrier and integrity is compromised [48,171].

Table 18. Main toxicokinetic parameters of fumonisin B_1 (FB_1) administered as a single oral bolus of fumonisins to broiler chickens *(2.5 mg FBs/kg BW, n = 6), after 3 weeks exposure to either a diet contaminated with deoxynivalenol (DON contaminated, contamination level: 3.12 mg DON/kg feed) or uncontaminated (control) feed. Values are presented as mean ± SD.*

Toxicokinetic parameter of FB_1	**DON contaminated**	**Control**
C_{max} (µg/mL)	0.035 ± 0.0248	0.033 ± 0.0213
T_{max} (min)	20 ± 5.0	20 ± 5.0
AUC_{0-t} (µg/mL . min)	83.5 ± 40.21	90.6 ± 54.07
k_{el} (min^{-1})	0.0075 ± 0.00155	0.0078 ± 0.00052
$T_{1/2el}$ (min)	98.4 ± 22.74	106.2 ± 8.34
MRT (min)	150.8 ± 35.52	165.5 ± 48.81
Vd/F (L/kg)	206.7 ± 92.37	234.3 ± 25.03
Cl/F (mL/min/kg)	1544.3 ± 807.33	944.5 ± 387.33
Rel F (%)	92.2	100

C_{max} = maximal plasma concentration; T_{max} = time to maximal plasma concentration; AUC_{0-t} = area under the plasma concentration-time curve from time 0 to 2 h; k_{el} = elimination rate constant; $T_{1/2el}$ = elimination half-life; MRT = mean residence time; Vd/F = volume of distribution divided by the absolute oral bioavailability; Cl/F = clearance divided by the absolute oral bioavailability; Rel F = relative oral bioavailability

As mentioned before, no significant differences between both groups (control or DON contaminated) could be observed for any of the toxicokinetic parameters (Table 18). Also, DON and its major metabolite, de-epoxydeoxynivalenol (DOM-1), were not detected in plasma in the present study. This is in accordance to Osselaere *et al.* [48,171] where, after three-week exposure of broiler chickens to 7.5 mg DON/kg feed, no plasma levels of DON or DOM-1 were detected above the limit of quantification (LOQ = 1 ng/mL). It has been shown that DON also selectively modulates the activities of different intestinal transporter proteins for nutrients, and negatively influences the sodium associated amino acid co-transport for serine and proline [26,107,258].

Although in literature it has been demonstrated that DON negatively affects the intestinal barrier function, morphology and transporter mediated nutrient transport in different animal species, chronic exposure to concentrations respecting the European maximum guidance levels in feed did not affect the oral bioavailability of FB_1 administered as a single bolus in broiler chickens.

4 Conclusion

Previous literature reports have shown that DON impairs the intestinal morphology, integrity and transporter mediated nutrient transport, both *in vitro* and *in vivo*. Therefore, it was hypothesized that chronic exposure to DON could influence the oral bioavailability of FBs in broiler chickens, leading to altered exposure and toxic effects of this mycotoxin. In the present study, no significant effects on the main toxicokinetic parameters and oral bioavailability of FB_1 after a single oral bolus administration in broiler chickens were found after chronic exposure to DON.

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GENERAL DISCUSSION

The mycotoxins DON and FBs increase broiler chickens' susceptibility to necrotic enteritis

Different research papers indicate a negative influence of *Fusarium* mycotoxins on the intestinal function and immune system in several animal species [22,47,256,323]. Since the intestinal tract is also a major portal of entry to many pathogens and their toxins, mycotoxin exposure could increase animal susceptibility to these pathogens. Furthermore, mycotoxin-induced immunosuppression may also result in decreased animal host resistance to infectious diseases. Effects of *Fusarium* mycotoxins on susceptibility to selected enteric, systemic and respiratory infectious diseases have been reported in livestock animals such as pigs and in murine models for human diseases [12]. However, only limited information is available concerning the interactions between mycotoxins and infectious diseases in poultry. Feeding a *Fusarium* mycotoxin contaminated diet, at concentrations approaching the EU maximum guidance levels, interferes with the pathogenesis of coccidiosis, ascaridiasis and aspergillosis in broiler chickens by affecting the immune response, intestinal barrier, intestinal microbial environment and pathogen characteristics [85,162,163,240].

For the first time, this doctoral research clearly demonstrated that deoxynivalenol (DON) or fumonisins (FBs)-induced modulation of the intestinal microbial environment and barrier function predisposes for *Clostridium perfringens* induced necrotic enteritis (NE) in broiler chickens (**Chapters 1-3**). NE is a complex, multifactorial enteric disease with many known and unknown factors influencing its occurrence and severity of outbreaks. Pre-existing mucosal damage caused by coccidiosis, high protein feed such as fishmeal and indigestible non-starch polysaccharides are well known predisposing factors [441].

It was demonstrated that DON exerts a direct cytotoxic effect on enterocytes of the duodenum, resulting in an increased permeability of the intestinal barrier, which could induce leakage of plasma proteins towards the intestinal luminal compartment. Additionally, the shortened duodenal villus height could lead to a decreased absorption of dietary proteins. These mechanisms are responsible for an increased protein content in the intestinal lumen. As these proteins become available for clostridial proliferation, they increase the risk for the development of NE (**Chapter 1**). In contrast to the negative effect of DON on the intestinal villus height and crypt depth in the proximal part of the small intestine, feeding a FBs contaminated diet negatively affected intestinal morphology in the distal part (**Chapters 1**

and 2). Recently, Grenier *et al.* [175] observed a similar high sensitivity of the ileum for FBs toxicity in broiler chickens, characterized by a marked increase in the ileal sphinganine:sphingosine ratio. This suggests that the ileal segment is more exposed to FBs than any other intestinal section. Indeed, it has been shown that FB_1 can interact with cholesterol and/or bile salts while undergoing enterohepatic circulation. The molecular association between FB_1 and cholesterol involves both hydrophobic interations and a hydrogen bond between the $-NH_3^+$ group of FB_1 and the OH-group of cholesterol [252]. Bile salts are actively reabsorbed along the ileum, and therefore, this intestinal part may be more exposed to significant amounts of FB_1 [175]. It remains to be determined if this effect on intestinal morphology is induced only by a direct toxic effect of FBs on intestinal epithelial cells, or indirectly, by the FBs induced shift in microbiota (**Chapter 2**), or a combination of both.

Oxidative stress makes a substantial contribution to the pathogenesis of many gastrointestinal mucosal diseases [38]. Oxidative stress is commonly defined as an imbalance between oxidants and anti-oxidants at the cellular level or individual level [249]. Our results also suggest that intestinal epithelial cells try to cope with mycotoxin induced oxidative stress by controlling the intracellular anti-oxidant levels of zinc and methionine, in order to maintain intestinal cellular metabolism and intestinal nutrient uptake (**Chapter 3.1**). This is achieved by selective up- or down-regulation of brushborder and basolateral solute carrier (SLC) proteins, respectively. Accordingly, reactive oxygen species (ROS) have a potential etiological and/or triggering role in the pathogenesis of inflammatory bowel disease (IBD) (i.e. ulcerative colitis and Crohn's disease) in humans [341,417]. Based on the ability of mycotoxins, such as DON and FBs, of altering the intestinal barrier and the balance between oxidants and reductants, it has been stated by Maresca *et al.* [257] that is seems highly plausible that food-associated exposure to certain mycotoxins could lead to the induction and persistence of human IBD in genetically predisposed patients. Therefore, it is very likely that the demonstrated predisposing effect of DON and FBs, at concentrations close to the EU maximum guidance levels, to the development of NE in broiler chickens, can be a model for other intestinal diseases associated with oxidative stress. For example, an impairment of the anti-oxidant status was demonstrated in broiler chickens infected with *Eimeria acervulina* or *E. tenella* [154,155]. Next, basal levels of ROS were higher in epithelial cells isolated from a human gastric mucosal biopsy from *Helicobacter pylori*-infected subjects than in cells from uninfected individuals [109,180].

Furthermore, the early response to *Salmonella* Typhimurium infection is ROS generation by NADPH phagocyte oxidase with potent bactericidal effects [428].

Furthermore, the impact of two predisposing factors for NE in broilers, i.e. coccidiosis and feeding fishmeal, on intestinal microbiota composition also shows remarkable similarities to the effects of FBs. Similar to coccidiosis [381], FBs exposure reduces the ileal abundance of *Candidatus* Savagella (**Chapter 2**). *Candidatus* Savagella belongs to the segmented filamentous bacteria (SFB) which is a unique group of commensal bacteria within the family of *Clostridiaceae*. These bacteria are characterized by their attachment to the intestinal epithelium and their important role in modulating host immune systems [375,402]. More specifically, SFB colonization leads to the maturation of the gut associated lymphoid tissue, induces a strong and broad immunoglobulin (Ig)A response, activates regulatory T-cells and upregulates intestinal innate defence mediators [127,360,381]. It was also shown that FBs exposure and feeding fishmeal both reduce the ileal abundance of *Lactobaccilus* spp. such as *L. johnsonii* [446] (**Chapter 2**). *L. johnsonii* has been extensively investigated for its probiotic activities including pathogen inhibition, epithelial cell attachment, and immunomodulation [336]. *L. johnsonii* interferes with the colonization and persistence of *C. perfringens* in poultry [226] and some lactobacilli can inhibit growth of *C. perfringens* [93].

Research investigating interactions between mycotoxins and microbiota has been mainly focused on potential applications of bacteria capable of detoxifying mycotoxins present in contaminated food and feed [459]. In general, the direct impact of mycotoxins on the intestinal microbiota composition has been poorly studied. Few studies demonstrated an impact of the *Fusarium* mycotoxins DON, FBs and zearalenone (ZEN) on the gut microbiota composition of humans and pigs [59,324,351,433]. Accordingly, it needs to be investigated whether DON induces similar changes in microbiota composition in broilers, as was seen with FBs. Waché *et al.* [433] reported an increased amount of aerobic mesophilic bacteria (AMB) in fecal samples of pigs exposed to 2.8 mg DON/kg feed for 4 weeks compared to the control group. In contrast, Piotrowska *et al.* [324] observed that the AMB concentration in ascending colon content of pigs was not influenced by DON (12 µg/kg bodyweight (BW)), but decreased by ZEN (40 µg/kg BW). Feeding a moderate dietary concentration of FBs (11.8 mg/kg feed) also did not affect the intestinal concentration of AMB [59]. However, these studies used conventional microbiological methods that only identify cultivable bacteria, which is estimated to be less than 30% of the intestinal microbiota [144]. Hence, molecular methods used to qualitatively and quantitatively assess all targeted bacteria irrespective of their state (cultivable, viable non-

cultivable, non-cultivable and dead bacteria) are preferred. No impact of DON or ZEN was demonstrated on the anaerobic sulphite reducing bacteria (ASR) concentration by the culture dependent method [324,433]. However, assessment of the effect of DON in a human microbiota-associated rat model by qRT-PCR revealed an increased fecal concentration of bacteria of the *Bacteroides* /*Prevotella* group [351].

Because intestinal damage caused by coccidial pathogens is the best known predisposing factor for NE [347,423,441], it might be hypothesized that the *Fusarium* mycotoxin-induced altered immune response against coccidiosis in broiler chickens secondarily affects bird susceptibility to NE. Indeed, Girgis *et al*. [163,164] showed a negative impact of diets naturally contaminated with DON on the innate and adaptive cell-mediated immune response against coccidiosis in broilers. Furthermore, it was shown that moderate levels of *Fusarium* mycotoxins negatively affect intestinal morphology and interfere with intestinal recovery from an enteric coccidial infection, indicated by a reduced villus height and apparent villus surface area [162]. Therefore, it is important to highlight that no challenge with an overdose of live coccidial vaccine was used in the *C. perfringens* animal infection trials [158](**Chapters 1 and 2**), and no lesions associated to coccidiosis were found on necropsy. Although Girgis *et al*. [163,164] demonstrated that *Fusarium* mycotoxins impair the *Eimeria*-induced immune response, no effect was seen on fecal oocyst counts. Further data on clinical coccidiosis lesion scoring is needed in order to evaluate the effect of *Fusarium* mycotoxins on the severity of the disease.

The feeding strategy further complicates the predisposing influence of mycotoxin exposure to NE in broiler chickens. Feed structure has a strong influence on the physiological functions of the digestive tract [123]. Pelleting of the diet will usually increase feed intake of broiler chickens by 10 to 20%, compared to mash feed, and thus will increase the demands on an already high-performing digestive system [217,390]. Pellets are characterized by a higher digestibility of nutrients compared to mash diet. Subsequently, chickens fed a pelleted diet have a more poorly developed gizzard, which may lead to an increased luminal content in the upper small intestine [390]. A higher number of coliform bacteria and enterococci in the ileum and a lower number of caecal *C. perfringens* and lactobacilli was observed in pelleted-fed birds compared to mash-fed birds, probably because of a reduced amount of undigested feed remnants in the intestine [123]. In all our animal experiments broiler chickens were fed a mash diet (**Chapters 1-3**). Since DON and FBs are relatively heat stable, the pelleting of feed does not affect their concentration [110,117]. It can be hypothesized that the feed form could modulate the oral bioavailability and intestinal retention time of both mycotoxins, by affecting the release of the

toxins from the matrix and the passage rate of intestinal content. Besides, enterohepatic recycling contributes to prolonged retention times of DON and FBs in the gastrointestinal tract[171]. Döll *et al.* [110] observed no difference in the carry-over of DON to muscle, liver and kidneys in pigs fed a mash feed or pellets.

There is growing interest world-wide in the feeding of whole grains to broilers as means of lowering feed cost and because of the reported positive effects on digestive function [373]. Dietary inclusion of whole wheat has shown to decrease intestinal *Salmonella* colonization and number of *C. perfringens* in broilers. Whole grains may encourage colonization of commensal bacteria and discourage pathogenic and harmful bacteria in the intestinal tract through competitive exclusion, hydrochloric acid secretion, grinding action of the gizzard or a combination of all these [40,124,148,373,451]. However, the effect of whole wheat feeding may be variable due to different factors, such as age of birds, rate of inclusion of whole grains, and quality of wheat [373]. The latter can be negatively influenced by mycotoxin contamination. In 2007 up to 70% of analyzed European wheat samples tested positive for DON, with an average contamination level of 1.17 mg/kg feed [386]. In conclusion, it is clearly demonstrated that the feeding strategy can affect broiler susceptibility to NE. However, the impact of mycotoxins on these effects is still unknown.

Parallel to our research, Cravens *et al.* [79] showed a synergistic negative effect on BW gain in broilers challenged to induce NE and fed diets with 0.75 mg/kg aflatoxin B_1 (AFB_1), a mycotoxin produced by *Aspergillus spp.* However, the experimental setup of this study failed to demonstrate a predisposing effect of AFB_1 on NE. The used contamination level of AFB_1, 0.75 mg/kg feed, was irrelevantly high since the maximum EU level is set at 0.02 mg/kg feed [79,130]. No distinction could be made between intestinal necrosis caused by *C. perfringens* or high concentrations of AFB_1.

In conclusion, the interaction between DON or FBs and NE is based on a complex equilibrium between animal host, pathogen and mycotoxin exposure characteristics. Figures 27A and B present the main scientific contributions and realizations achieved in this doctoral thesis for DON and FBs, respectively.

Furthermore, based on research in other animal species and the negative effect of DON or FBs on the intestinal barrier, it could be hypothesized similar effects may occur for other infectious diseases, such as salmonellosis or colibacillosis. However, further research is necessary to investigate these interactions.

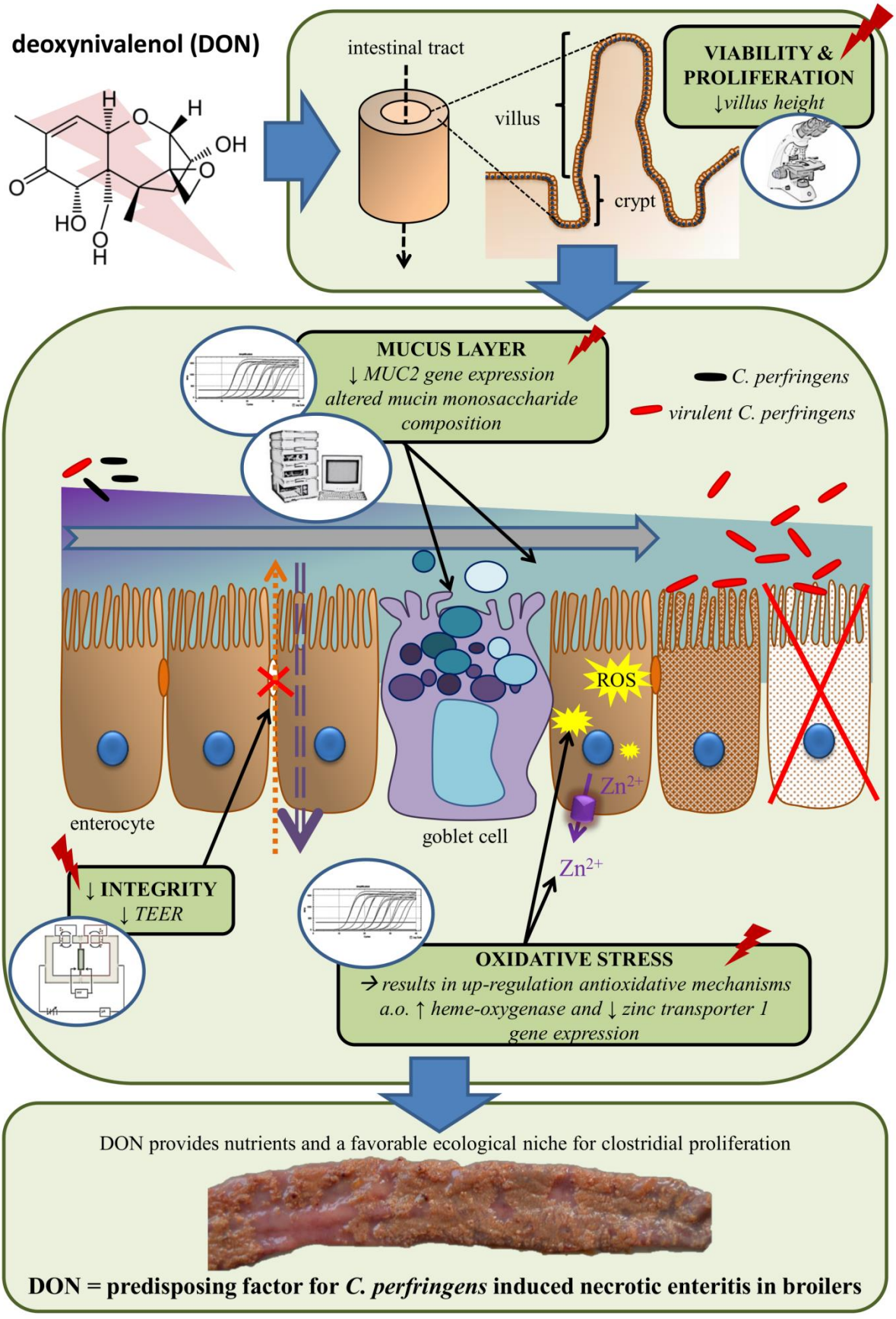

Figure 27A. Overview of the main realizations achieved in this doctoral thesis: effects of deoxynivalenol on Clostridium perfringens induced necrotic enteritis in broiler chickens.

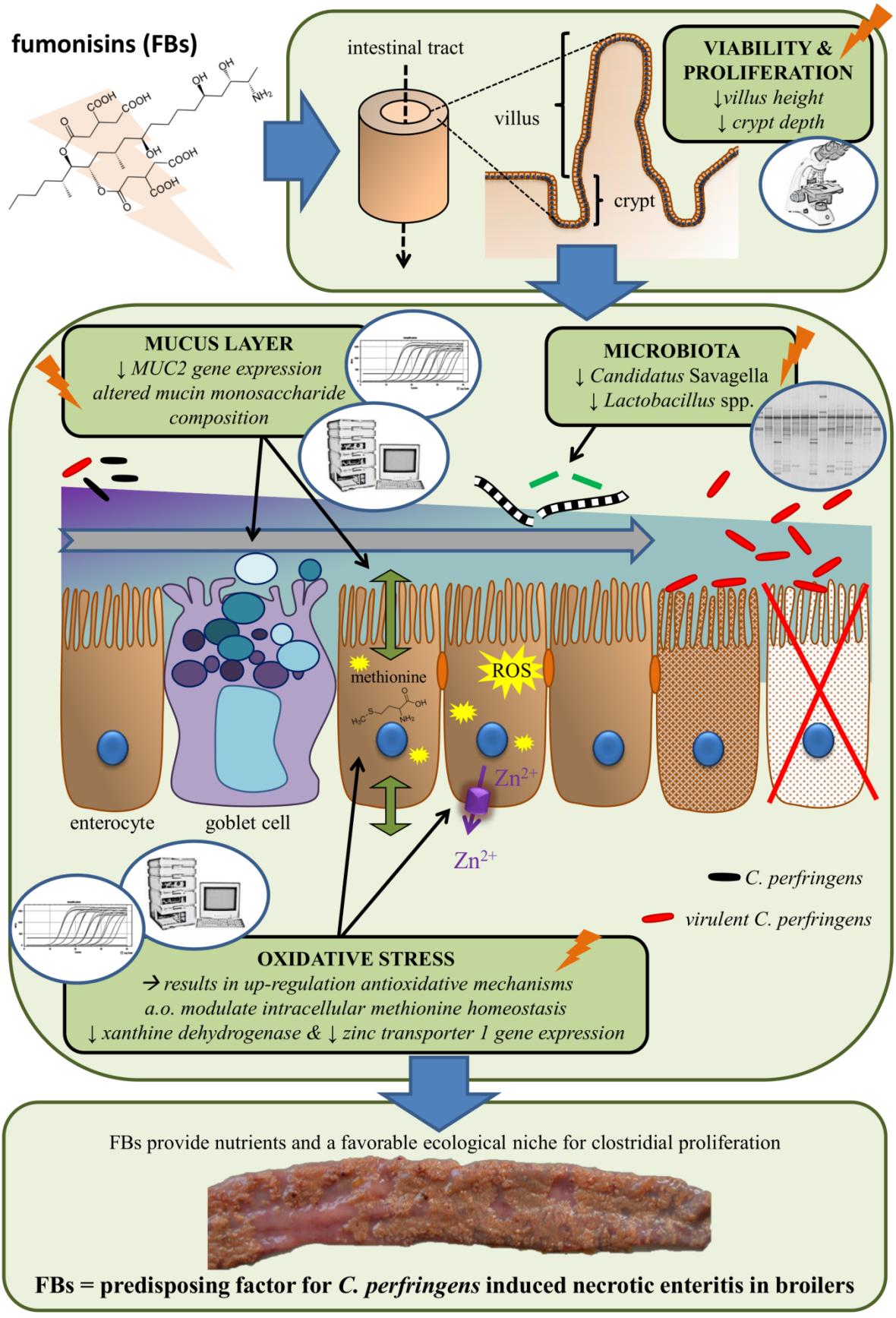

Figure 27B. Overview of the main realizations achieved in this doctoral thesis: effects of fumonisins on Clostridium perfringens induced necrotic enteritis in broiler chickens.

Treatment of infectious diseases under pressure by mycotoxin exposure?

Mycotoxins may affect poultry susceptibility to infectious diseases. However, exposure to *Fusarium* mycotoxins may also have an impact on poultry pharmacotherapy, such as an impact on the disposition and pharmacokinetics of orally administered drugs. As a drug is actively or passively absorbed from the intestinal lumen to the systemic compartment, it must cross two major barriers: the mucus layer and intestinal mucosa [277]. Feeding a DON contaminated diet to broiler chickens for 3 weeks reduced the intestinal epithelial integrity in the duodenum (**Chapter 1**), which could indeed affect the paracellular transport of drugs. *In vitro*, porcine intestinal epithelial barrier disruption by DON and T-2 toxin (T-2) promoted transepithelial passage of the antibiotics doxycycline and paromomycin [169]. *In vivo*, an increased oral bioavailability of chlortetracycline was observed in pigs after feeding a diet contaminated with T-2 [168]. Quantitative and qualitative changes in intestinal mucin induced by DON and/or FBs (**Chapter 3.1**) might also modulate drug absorption, especially via the transcellular route. This is particularly true for more lipophilic drugs, such as doxycycline, that interact with the glycoproteins and lipids in the mucus [213,370].

Besides, intestinal drug metabolizing enzymes (a.o. cytochrome P450 (CYP450) isoenzymes) and ABC drug transporter proteins (a.o. P-glycoprotein (P-gp) or MDR1 and MRP2) can also be influenced by a prolonged exposure to DON, T-2 and FBs [9,167,299,302]. Recently, we demonstrated that feeding a FBs contaminated diet for 15 days to broiler chickens, induced a significant up-regulation of the expression of *MDR1* in the jejunum. No effect was observed of mycotoxin exposure on the intestinal expression of genes encoding CYP450 isoenzymes. However, feeding a DON contaminated diet induced an increased expression of *CYP1A5* in the liver [9]. A major concern is the question whether dose adaptation is necessary for certain substrate drugs, when administered simultaneously with a *Fusarium* mycotoxin contaminated diet, in order to prevent drug toxicity, higher residues in animal tissues, prolonged withdrawal times, or treatment failure. To assess this impact, functional *in vivo* studies should be conducted using appropriate MDR1 transporter or CYP1A5 substrates.

Should the European Union adapt the maximum guidance levels for DON and FBs in feed for poultry?

The question of safe mycotoxin levels in food and feed is of major importance for human and animal health. Since on average 72% of all tested feed and feed commodity samples are positive for at least one mycotoxin [358,386], the introduction of a zero tolerance is impossible. Defining a safe mycotoxin level in livestock production should be based on a balance between the mycotoxin lowest-observed-adverse-effect level (LOAEL) for animal health, an economically feasible equilibrium for livestock industry, and trade issues[39]. However, what is the proper criterion for "safety" [39]? Currently, the maximum EU guidance levels for DON and FBs in poultry feed, 5 mg DON and 20 mg FB_1+FB_2/kg respectively [131], represent the lowest concentrations at which neither lethal effects, growth depression, nor macroscopic pathological findings are observed [16]. Indeed, no increased mortality or growth depression were observed in broiler chickens fed the experimentally contaminated diets with DON, FBs or DON+FBs, at concentrations approaching the EU guidance levels (**Chapters 1-3**). In contrast, a reduced feed intake, BW gain and feed efficiency were observed in broilers fed a diet contaminated at higher levels of DON (10-15 mg DON/kg feed) for 3 to 6 weeks [20,88,157,448]. Feeding broiler chicks a FBs contaminated diet at doses ranging from 100 to 400 mg FB_1/kg feed for 2 to 3 weeks also induced a dose dependent decrease in BW gain and feed intake [202,230].

This dissertation shows that concentrations near the EU maximum guidance levels [131] are nevertheless responsible for more distinct negative effects of DON and FBs at the intestinal level in broiler chickens. Three weeks feeding DON at 3-5 mg/kg feed reduced duodenal villus height and crypt depth (**Chapter 1**) [19]. Consumption of a diet contaminated with FBs at 18.6 mg FB_1+FB_2/kg feed for 15 days also reduced small intestinal length, ileal villus height and crypt depth (**Chapter 2**). DON and FBs negatively influenced the duodenal mucus layer in broiler chickens, characterized by a decreased intestinal mucin (*MUC*) *2* expression and altered mucin monosaccharide composition (**Chapter 3.1**), and DON also reduced the duodenal epithelial integrity (**Chapter 1**). DON and/or FBs exposure modulated the expression of selected digestive enzymes and membrane-bound SLC proteins located at the brushborder and basolateral membranes of jejunal epithelial cells, resulting in an increased apparent ileal amino acid digestibility of methionine (**Chapter 3.1**). Similarly, Dietrich *et al.* [107] demonstrated that the jejunal expression of several genes encoding nutrient uptake

mechanisms, a.o. transporters for fructose and glucose (*SLC2A5*), and D-serine and other neutral amino acids (*SLC7A10*), was affected in broiler chickens after feeding a DON contaminated diet (2.5-5mg /kg feed) for 23 days. Subsequently, this thesis also demonstrated an altered ileal microbiota composition in FBs exposed broilers, characterized by a reduced abundance of *Candidatus* Savagella and *Lactobaccilus* spp. such as *L. johnsonii*, and an increased ileal concentration of total *C. perfringens* (**Chapter 3.1**). These mycotoxin-induced alterations of the intestinal barrier and intestinal microbial homeostasis, predispose broiler chickens to NE (**Chapters 1 and 2**).

All above described effects occur at mycotoxin contamination levels approaching the EU maximum guidance levels. Hence, should safe mycotoxin levels refer to more microscopic pathological effects on the intestinal barrier and intestinal microbial homeostasis? The government and livestock industry should support the development of strategies to prevent fungal infections, and subsequently mycotoxin production, during plant growth, harvest, storage and distribution. Notwithstanding prevention of mycotoxin contamination by the agricultural industry, the absence of mycotoxins in rations of farm animals cannot be fully assured. Very high levels of mycotoxins are occasionally detected in feed and feed raw materials Streit *et al.* [386] showed a maximum contamination level of 50.3 mg DON/kg feed and 77.5 mg FBs/kg feed, respectively. The majority of feed samples is however compliant with the EU regulations and recommendations [131,132,358,386]. Decreasing the maximum guidance levels for DON and FBs would result in a major increase of rejected feed and cost for the poultry industry. It was demonstrated that 15% and 4% of feed samples exceed the lowest applicable guidance value for livestock animals for DON and FBs, respectively [386]. On the other hand, the associated decreased mycotoxin exposure would result in an enhanced broiler health and associated financial profit. The economic impact of mycotoxins on animal production is generally considered to be mainly due to losses related to direct effects on animal health and trade losses related to feedstuff rejection [443]. It is clear, however, that the indirect influence of mycotoxins on animal health, by predisposing to infectious diseases, should also be taken into account. Detailed financial evaluation of the advantage of lowering these maximum guidance levels should be performed.

Moreover, the frequently detected co-contamination of different mycotoxins [387] should also be addressed in future legislation.

Cumulative health risk assessment of co-occurring mycotoxins

Mycotoxigenic fungi are usually capable of producing more than one mycotoxin, and feed raw materials are commonly infected with various fungal species at a time [44,387,392]. Compound feed is particularly vulnerable to multiple mycotoxin contamination as it typically contains a mixture of several raw materials [387]. The response of animals to exposure to more than one mycotoxin can be the same as the response to each toxin individually (additive effect), more than the predicted sum of the responses to each individual mycotoxin (synergistic) and, more rarely, less than the predicted response to each toxin individually (antagonistic) [174]. Chronic exposure to DON impairs the intestinal morphology, integrity and transporter mediated nutrient uptake (**Chapters 1 and 3.1**), although DON does not affect the intestinal absorption of FBs (**Chapter 3.2**). Even after DON associated intestinal damage, the oral absorption of FB_1 by passive non-ionic transcellular diffusion is very limited, as FB_1 is a large and mainly negatively charged molecule at the pH of the duodenum and jejunum in broiler chickens [253,256]. Both DON and FBs exposure has a negative influence on the intestinal barrier. It has been demonstrated that both mycotoxins are associated with the induction of oxidative stress in enterocytes, and negatively affect the intestinal mucus layer (**Chapter 3.1**). For these effects no cumulative interaction was observed in the multi-mycotoxin group, fed a DON and FBs contaminated diet for 15 days. Furthermore, it was observed that FBs exposure modulated the gene expression of selected membrane bound amino acid transporter proteins located at the brushborder and basolateral membranes of jejunal epithelial cells. No similar effect was demonstrated in the multi-mycotoxin group (**Chapter 3.1**), which could suggest an antagonistic interaction between DON and FBs. Nevertheless, an increased apparent ileal methionine digestibility was observed in the multi-mycotoxin contamination group. Therefore, it needs to be investigated if this observation is influenced by differences between animals in sensitivity to mycotoxin exposure, or rather by mycotoxin-associated differences in toxicodynamics and intestinal barrier toxicity [223]. The complexity of DON and FBs interaction was also demonstrated in pigs fed a diet contaminated with DON (3 mg/kg feed), FBs (6 mg/kg feed) or both mycotoxins for 5 weeks. Following ovalbumin immunization, some parameters were not influenced by the combination of both mycotoxins (i.e. blood biochemistry, lung and kidney lesions, plasma IgA content), while other effects were (i.e. plasma IgG content, cytokines expression and liver lesions) [173].

Furthermore, also low levels of the fungal conjugated metabolites of DON, i.e. 3- and 15-acetyldeoxynivalenol (ADON), were present in the different batches of DON-contaminated feed (**Chapters 1 and 3**). These conjugated forms are currently not included in the legislation for feed. In pigs, the ingestion of 3-ADON leads to the appearance of DON (58%) and DON metabolites (42%, i.e. de-epoxy-DON (DOM-1) and DON glucuronide), but not 3-ADON, in the blood [128]. Recently, Broekaert *et al.* [54] observed a higher oral absorption of 3-ADON and 15-ADON in broiler chickens compared to DON, 18.2% and 42.2% versus 10.6%, respectively. This absorbed fraction was completely presystemically hydrolyzed for the less toxic 3-ADON to DON, and to a lesser extent (75.4%) for the more toxic 15-ADON to DON. Furthermore, in rats and pigs it was shown that the plant conjugate DON-3-glucoside (DON3G) was almost completely hydrolyzed to DON during digestion [287,288]. Data about hydrolysis of these conjugated mycotoxins in poultry are lacking. Consequently, this *in vivo* hydrolysis of conjugated forms of DON may contribute to an enhanced DON exposure and associated toxicity in broiler chickens, and thus demonstrate the possible need for appropriate risk assessment of these conjugated mycotoxins in feed, e.g. by revision of the tolerable daily intake [37,53,92,288].

Need for specific biomarkers to assess mycotoxin exposure in broiler chickens

The question remains which biomarker can be used in practice to evaluate dietary mycotoxin exposure. Currently, most methods determine the concentration of mycotoxins, their phase I and II metabolites, or other affected endogenous substances related to the mode of action of the mycotoxin, in biological fluids such as blood, bile or urine [283]. The most widely researched FBs biomarkers are those related to the disruption of the *de novo* sphingolipid biosynthesis, namely elevated levels of the sphingoid base, sphinganine (Sa), or of its ratio with sphingosine (So) [365]. A significant increased plasma Sa level and Sa/So ratio was indeed observed in broiler chickens fed a FBs or FBs and DON contaminated diet for 15 days (**Chapters 2 and 3.1**). Co-contamination with DON did not influence this plasma Sa/So ratio. Recently, Grenier *et al.* [175] also demonstrated a dose-dependent increase in Sa/So ratio in the liver, kidney, and intestinal tissue of broiler chickens fed a FBs contaminated diet for 10 to 20 days.

Following chronic exposure to DON, no DON or its metabolite de-epoxydeoxynivalenol (DOM-1) were detected in plasma (**Chapter 3.2**). Accordingly, no DOM-1 was detected in plasma after single bolus administration (0.75 mg DON/kg BW) [300], and after chronic feeding (7.5 mg DON/kg feed) [301]. Therefore, plasma DON and DOM-1 levels are not suited as a biomarker in broiler chickens. The phase II metabolite, DON-3α-sulphate, might be more suitable as biomarker for DON exposure in poultry. Recently, it was demonstrated by high-resolution mass spectrometry that DON-3α-sulphate is the major metabolite of DON in broiler chickens, with DON-3α-sulphate/DON ratios between 1,365-29,624 after oral administration. Only trace amounts of other metabolites, a.o. 10-DON-sulphonate, DOM-1, 10-de-epoxydeoxynivalenol-sulphonate and DON-3α-glucuronide, were detected [98]. In humans, DON-glucuronides were validated as biomarkers in urine [437]. Humans and other mammals more extensively glucuronidate DON, in contrast to poultry species [265].

The selectivity, sensitivity, and speed of modern LC-MS instruments not only results in the detection of novel toxins and accurate quantification of known mycotoxins, it is also a key to assess mycotoxin exposure [283]. However, future analytical developments should also investigate more easy applicable rapid test kits for mycotoxin exposure biomarkers that can be used in the field.

Future research perspectives

Comparative assessment of the predisposing effect of DON and/or FBs on clostridial enteric diseases in different avian species

Our results clearly demonstrate a predisposing effect of the mycotoxins DON and FBs on *C. perfringens* induced NE in broiler chickens by affecting the intestinal barrier and microbiota (**Chapters 1-3**). *C. perfringens* also induces enterotoxemia in other bird species such as laying hens, turkeys, ducks and quails. Recently, an increased prevalence of subclinical NE was observed in laying hens [3]. Despite this increasing prevalence, NE in laying hens and turkeys is much less investigated compared to NE in broilers [248,308]. Differences in the pathogenesis and epidemiology of NE and sensitivity to the toxic effects of mycotoxins in different poultry species highlights the need for comparative research. Turkeys are considered more sensitive to DON and FBs toxicity compared to broilers [165,229]. Furthermore, young animals are more sensitive towards the deleterious effects of mycotoxins [202]. The majority of naturally occurring outbreaks of NE in broiler and turkey flocks are reported between 2-5 and 7-12 weeks of age, respectively [84,151,308,404]. In contrast, NE in layers occurs at the age of 3-6 months [3,308].

Influence of chronic exposure to DON and/or FBs on the intestinal microbiota, and their potential role in dysbacteriosis or dysbiosis in broilers

In this doctoral thesis, it was demonstrated for the first time that FBs exposure affects the intestinal microbiota in broiler chickens. FBs exposure reduces the ileal abundance of the immunomodulating SFB (**Chapter 2**). SFB gained much interest in human and animal health because of their unique ability to educate the gut immune system and to induce a state of physiological inflammation [360]. Besides, FBs exposure also modulates the ileal abundance of *Lactobaccilus* spp. and numbers of *C. perfringens* (**Chapter 2**). Therefore, the further impact of DON and/or FBs exposure on the development and composition of intestinal microbiota in broiler chickens should be investigated. Specific microorganisms of interest of which their abundance is affected by mycotoxin exposure should be identified, isolated and cultured. Subsequently, the impact of DON and/or FBs and the identified microorganisms of interest on dysbacteriosis, or more in general intestinal health, in broilers should be evaluated. Dysbacteriosis in broiler chickens has been defined as the presence of a qualitatively and/or

quantitatively abnormal microbiota, inducing a cascade of reactions in the gastro-intestinal tract, a.o. reduced nutrient digestibility, impaired intestinal barrier function, increased risk of bacterial translocation and inflammatory responses [400].

Are DON and FBs also a predisposing factor for other infectious diseases in poultry?

Although demonstrated in pigs [306,425], currently, no data are available suggesting a similar increased intestinal colonization or translocation of avian pathogenic *Escherichia coli* (APEC), *Salmonella* Enteritidis, *Salmonella* Typhimurium or other serovars in poultry. Following intraperitoneal inoculation with *Salmonella* Typhimurium, an increased level of *Salmonella*-related organ lesions or mortality was seen in T-2-challenged broiler chickens [460]. Recently, we demonstrated that feeding pigeons a DON contaminated diet (3.5 mg DON/kg feed) for 4 weeks increased the severity of the macroscopic lesions related to an infection with *Salmonella* Typhimurium variant Copenhagen in the small intestine, liver, spleen and kidneys, however, without showing an effect on the *Salmonella* numbers in these organs [11]. Therefore, it needs to be investigated if similar interactions between DON or FBs and salmonellosis or colibacillosis exist in poultry.

Potential for mycotoxin protection by selected pre- and probiotics

A mycotoxin-induced intestinal oxidative stress and microbiota shift has been demonstrated in this doctoral thesis. Recently, Wu *et al.* [444] demonstrated that feed supplementation with 2% glutamic acid ameliorated DON-induced oxidative stress, intestinal injury and signaling inhibition in pigs. *In vitro*, the anti-oxidant zinc protects human liver carcinoma (HepG2) and human epithelial colorectal adenocarcinoma (Caco-2) cells against the oxidative damage and DNA damage induced by ochratoxin A [339,457]. Treatment with *Lactobacillus reuteri* or *L. casei* prevented aflatoxin-induced toxicity in rats [182]. Probiotic administration of *Lactobacillus* spp. has been shown to reduce intestinal oxidative stress [143]. A reduced abundance of *Lacobacillus* spp. has been demonstrated in broilers for FBs (**Chapter 2**). Therefore, the potential role of pre- and probiotics in the symptomatic treatment of mycotoxins associated intestinal lesions needs to be investigated.

Conclusions

This doctoral thesis demonstrates for the first time that feeding a DON or FBs contaminated diet is a predisposing factor for the development of *C. perfringens* induced NE in broiler chickens. This coincides with negative effects on selected components of the intrinsic and extrinsic intestinal barrier of the chicken host, i.e. villus height, tight junctions, mucus, oxidative stress, and microbiota homeostasis. Consequently, exposure to DON and/or FBs at concentrations approaching the EU maximum guidance level in feed provides both nutrients and a favorable ecological niche for clostridial proliferation. Therefore, this doctoral thesis demonstrates that in addition to the best-known predisposing factors, coccidiosis and fishmeal, DON and/or FBs contaminated feed should be included as major risk factor for the development of *C. perfringens* induced NE in broiler chickens. Similarly to coccidiosis, contamination of poultry feed with mycotoxins, a.o. DON and FBs, is a worldwide frequently occurring problem in poultry industry. This dissertation shows that feeding a diet contaminated with mycotoxins at concentrations close to the EU maximum guidance levels is nevertheless responsible for more distinct negative effects of DON and FBs at the intestinal level in broiler chickens, and consequently respecting these maximum contamination levels does not protect against the predisposing effect of both mycotoxins on the development of NE in broilers. Accordingly, major preventive efforts should be performed to keep the animal exposure to mycotoxins as low as possible.

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SUMMARY

Contamination of poultry feed with mycotoxins is a worldwide problem. At present, acute mycotoxicosis caused by high doses is rare. Ingestion of low to moderate amounts of *Fusarium* mycotoxins is more common and generally does not result in obvious intoxication. However, these low to moderate contamination levels may impair intestinal health, immune function and/or pathogen fitness, resulting in altered host pathogen interactions and thus a different outcome of infections.

Mycotoxins are secondary fungal metabolites, which are not directly involved in the normal growth, development, or reproduction of the mould. Mycotoxins of importance for the poultry industry are mainly produced by fungi of the genera *Aspergillus*, *Fusarium* or *Penicillium*. The *Fusarium* mycotoxins deoxynivalenol (DON) and fumonisins (FBs) are the most frequently detected mycotoxins on a worldwide basis. A long-term global mycotoxin survey conducted during 2004-2011 indicated that the mycotoxins DON and FBs contaminated 56% and 54%, respectively, of samples of livestock feed and feed raw materials. Although high contamination levels were present, the majority of the feed samples was found to comply with European Union (EU) maximum guidance levels. The EU maximum guidance level for poultry feed is set at 5 mg DON/kg feed and 20 mg fumonisin B_1 (FB_1)+fumonisin B_2 (FB_2)/kg feed.

Worldwide, necrotic enteritis (NE) is one of the economically most important enteric diseases in poultry production. NE in broilers may arise when changes in the gut microbial homeostasis allow colonization of the virulent Gram-positive rod-shaped anaerobic *Clostridium perfringens* type A strains expressing the NetB toxin gene. The presence of predisposing factors is required in the pathogenesis of NE, allowing the proliferation of *C. perfringens* by either providing nutrients and/or favorable ecological niches. These predisposing factors mainly include type of diet, co-infection with other pathogens particularly coccidia, as well as environmental, nutritional and management factors.

Since both mycotoxin exposure and NE are important economical problems in poultry industry, the **general aim** of this thesis was to examine whether the *Fusarium* mycotoxins DON and FBs at concentrations in the feed approaching EU maximum guidance levels predispose for NE in broiler chickens, and to gain insights in the mechanisms responsible for this interaction.

In **Chapter 1** an experimental *C. perfringens* infection study was performed in order to evaluate the predisposing effect of DON exposure to the development of NE in broiler chickens. No coccidial challenge was included in the experimental infection model. Chickens were fed either a control diet or a DON contaminated diet (3-4 mg/kg feed) for three weeks. After *C. perfringens* challenge, DON significantly increased the percentage of birds developing subclinical NE compared to the control group (47 ± 3.0% *vs*. 20 ± 2.6%). The distribution of NE lesions in mainly duodenum and jejunum was similar as in previously described NE trials, where a coccidial challenge was included as predisposing factor. Hence, we showed that DON is able to replace the role of coccidiosis as predisposing factor for NE. In this study, DON negatively affected the proximal part of the intestinal tract, demonstrated by a significantly reduced villus height in the duodenum. Besides, *ex vivo* evaluation of the epithelial integrity by the Ussing chamber technique revealed a reduced transepithelial electrical resistance in the duodenal segment. These results clearly indicate a negative impact of DON on the intrinsic component of the intestinal barrier, which is composed of the epithelial cell layer and the tight junctions that interconnect these epithelial cells. Consequently, this intestinal damage may lead to an increased luminal protein content by leakage of plasma proteins or altered absorption of dietary proteins. Protein analysis of duodenal content indeed showed that DON contamination resulted in a significant increase in total protein concentration, which may stimulate growth and toxin production of *C. perfringens*. Additionally, DON had no effect on *in vitro* growth, alpha toxin production and *netB* toxin transcription of *C. perfringens*. In conclusion, this study clearly indicates that feeding a DON contaminated feed at contamination levels below the EU maximum guidance level, is a predisposing factor for de development of *C. perfringens* induced NE in broiler chickens due to the negative influence on the intestinal barrier, and to an increased intestinal nutrient availability for clostridial proliferation.

In **Chapter 2** the impact of feeding a FBs contaminated diet on the intestinal morphology and microbiota composition was studied. Broiler chickens were fed either a control diet or a FBs contaminated diet (18.6 mg FB_1+FB_2/kg feed) for 15 days. The negative effect of FBs on the sphingolipid biosynthesis was demonstrated by a significantly increased plasma sphinganine/sphingosine ratio in the FBs-treated group compared to the control group (0.21 ± 0.016 *vs*. 0.14 ± 0.014).

Furthermore, a negative impact of FBs on the intestinal morphology was demonstrated by a decreased total length of the small intestine and significantly reduced villus height and crypt depth in the ileum. Denaturing gradient gel electrophoresis showed a shift in the intestinal microbiota composition in the ileum in the FBs exposed group compared to the control group. A reduced presence of low-guanine-cytosine containing operational taxonomic units in ileal digesta of birds exposed to FBs was observed, and identified as a reduced abundance of *Candidatus* Savagella and *Lactobaccilus* spp. such as *L. johnsonii*. *Candidatus* Savagella belongs to the segmented filamentous bacteria, and is characterized by its attachment to the intestinal epithelium and its important role in modulating the host immune system. *L. johnsonii* has been extensively investigated for its probiotic activities including pathogen inhibition, epithelial cell attachment, and immunomodulation. Remarkably, similar shifts in the intestinal microbiota have been reported previously in cases of coccidiosis and when feeding fishmeal to broiler chickens, two well-known predisposing factors for NE. Furthermore, quantification of total *C. perfringens* in these ileal content samples by qPCR using *cpa* gene (alpha toxin) indicated an increase in *C. perfringens* numbers in chickens fed a FBs contaminated diet compared to the control diet. Subsequently, an experimental *C. perfringens* infection study was performed investigating the predisposing effect of FBs exposure to the development of NE in broiler chickens. After *C. perfringens* challenge, a significantly higher percentage of birds developed subclinical NE in the group fed a FBs contaminated diet compared to the control group (45 ± 2.2% *vs.* 30 ± 5.5%). Hence, feeding a FBs contaminated diet at contamination levels close to the EU maximum guidance level negatively affects the intestinal microbiota and intrinsic component of the intestinal barrier, predisposing broiler chickens to the development of *C. perfringens* induced NE.

In **Chapter 3**, the impact of DON and FBs on the intestinal barrier was further examined for selected functional and structural extrinsic components of the intestinal barrier, to gain insights in the associated consequences for intestinal colonization and nutrient availability for *C. perfringens*. In the first part (**Chapter 3.1**), the impact of feeding broiler chickens a DON and/or FBs contaminated diet on intestinal mucus layer and induction of oxidative stress was examined. Broiler chickens were divided into four groups, and were fed for 15 days either a control diet, a DON contaminated diet (4.6 mg DON/kg feed), a FBs contaminated diet (25.4 mg FB_1+FB_2/kg feed), or a DON and FBs contaminated diet (4.3 mg DON and 22.9 mg FB_1+FB_2/kg feed). DON and FBs affected the duodenal mucus layer by decreasing the *intestinal mucin 2* (*MUC2*) gene expression and altering the duodenal mucin monosaccharides

composition, i.e. N-acetyl-neuraminic acid, N-acetyl-galactosamine, and galactose. These changes in mucin monosaccharides composition may affect mucus layer integrity, and intestinal colonization and nutrient availability for microorganisms such as *C. perfringens*, and subsequently modulate the intestinal microbiota.

Both mycotoxins negatively affected cellular homeostasis by the induction of oxidative stress. Subsequently, the response of the intestinal epithelial cells was characterized by the induction and modulation of several anti-oxidative mechanisms. A compensatory down-regulation of the transcription of the gene encoding xanthine dehydrogenase in the jejunum of chickens fed a FBs or a FBs and DON contaminated diet, suggest a decreased production of reactive oxygen species (ROS) as byproduct of the enzymatic conversion of hypoxanthine to uric acid. Similarly, feeding a DON contaminated diet to broilers induced an up-regulation of the mRNA expression of the gene encoding heme-oxigenase, which could induce a decreased cellular content of pro-oxidants such as heme and iron, and an increased cellular content of the anti-oxidant biliverdin. Furthermore, a decreased gene expression of the intestinal basolateral zinc transporter-1 was observed in all mycotoxin challenged groups, which may indicate a protection mechanism of the intestinal epithelial cells to maintain the intracellular zinc level, which is important for preserving its critical anti-oxidant activity in case of mycotoxin induced oxidative stress. Finally, feeding a mycotoxin contaminated diet modulated the intestinal uptake of methionine, by affecting the expression of different intestinal brushborder and basolateral methionine transporter mechanisms. Methionine participates in the synthesis of cysteine and glutathione, both direct scavengers of ROS. In conclusion, this study clearly indicate that feeding a DON and/or FBs contaminated diet, at concentration close to the EU maximum guidance levels, affects the extrinsic part of the intestinal barrier by changing the intestinal mucus layer and the intestinal modulation of several anti-oxidative mechanisms.

Because the mycotoxins DON and FBs frequently co-occur, and taken into account that FBs have a low oral bioavailability in healthy chickens and DON impairs the intestinal barrier and decreases the total intestinal absorption surface area. Therefore, the last study (**Chapter 3.2**) aimed to investigate whether chronic exposure to DON could influence the intestinal absorption of FBs leading to an altered systemic exposure and increased toxic effects of the latter mycotoxin in broiler chickens. A toxicokinetic study was performed in broiler chickens, which were administered an oral bolus of 2.5 mg FBs/kg bodyweight after a three-weeks exposure to either control feed or feed contaminated with 3.12 mg DON/kg feed. No

significant differences in toxicokinetic parameters of FB_1 could be demonstrated between both groups. Also, no increased nor decreased body exposure to FB_1 was observed, since the relative oral bioavailability of FB_1 after chronic DON exposure was 92.2% compared to the control.

This doctoral thesis demonstrates for the first time that feeding a DON or FBs contaminated diet is a predisposing factor for the development of *C. perfringens* induced NE in broiler chickens. This coincides with negative effects on selected components of the intrinsic and extrinsic intestinal barrier of the chicken host, i.e. villus height, tight junctions, mucus, oxidative stress, and microbiota homeostasis. Consequently, exposure to DON and/or FBs at concentrations approaching the EU maximum guidance level in feed provides both nutrients and a favorable ecological niche for clostridial proliferation. Therefore, this doctoral thesis demonstrates that in addition to the best-known predisposing factors, coccidiosis and fishmeal, DON and/or FBs contaminated feed should be included as major risk factor for the development of *C. perfringens* induced NE in broiler chickens. Similarly to coccidiosis, contamination of poultry feed with mycotoxins, a.o. DON and FBs, is a worldwide frequently occurring problem in poultry industry. This dissertation shows that feeding a diet contaminated with mycotoxins at concentrations close to the EU maximum guidance levels is nevertheless responsible for more distinct negative effects of DON and FBs at the intestinal level in broiler chickens, and consequently respecting these maximum contamination levels does not protect against the predisposing effect of both mycotoxins on the development of NE in broilers. Accordingly, major preventive efforts should be performed to keep the animal exposure to mycotoxins as low as possible.

SAMENVATTING

De contaminatie van pluimveevoeder met mycotoxines vormt wereldwijd een belangrijk probleem. Acute mycotoxicosen, welke veroorzaakt worden door de opname van hoge gehaltes aan mycotoxines, zijn echter zeldzaam. De opname van lage tot matige hoeveelheden *Fusarium* mycotoxines daarentegen, komt wel zeer frequent voor. Deze lagere gehaltes van mycotoxines leiden niet tot duidelijke symptomen van intoxicatie, maar hebben wel een negatieve invloed op de darmgezondheid, het immuunsysteem en/of pathogeen fitness. Dit kan mogelijke gastheer-pathogeen interacties beïnvloeden, en dus ook het verloop van eventuele infecties.

Mycotoxines zijn secundaire metabolieten van schimmels, die niet rechtstreeks betrokken zijn bij de normale groei, ontwikkeling of voortplanting van de schimmel. De belangrijkste mycotoxines voor de pluimvee-industrie worden voornamelijk geproduceerd door schimmels van de genera *Aspergillus*, *Fusarium* en *Penicillium*. De *Fusarium* mycotoxines deoxynivalenol (DON) en fumonisines (FBs) zijn wereldwijd de meest voorkomende mycotoxines. De resultaten van een grootschalige screening van voeders en grondstoffen bestemd voor voeders voor landbouwhuisdieren in de periode van 2004-2011 tonen aan dat 56% van de stalen gecontamineerd waren met DON en 54% met FBs. Slechts enkele stalen waren gecontamineerd met zeer hoge gehaltes mycotoxines. Het contaminatieniveau van de meeste voederstalen bevond zich onder de maximaal aanbevolen richtwaarde voorgesteld door de Europese Unie. Voor pluimveevoeder is dit vastgelegd op 5 mg DON/kg voeder en 20 mg fumonisine B_1 (FB_1)+fumonisine B_2 (FB_2)/kg voeder.

Necrotische enteritis (NE) is wereldwijd een van de economisch meest belangrijke darmziekten in de pluimvee industrie. NE ontstaat wanneer veranderingen in het microbiële evenwicht in de darm aanleiding geven tot kolonisatie van de darm met virulente NetB-toxine producerende *Clostridium perfringens* type A stammen. *C. perfringens* is een Gram-positieve, staafvormige anaerobe bacterie. De aanwezigheid van predisponerende factoren zijn zeer belangrijk in de pathogenese van NE omdat ze de overgroei van *C. perfringens* stimuleren door het voorzien van de nodige nutriënten en een gunstige omgeving voor bacteriële proliferatie. Gekende predisponerende factoren zijn het type voeder, co-infectie met andere pathogenen (voornamelijk coccidia), en andere omgevings-, nutritionele, en managements-factoren.

Zowel mycotoxine-blootstelling als NE zijn belangrijke economische problemen in de pluimveesector. De **algemene doelstelling** van dit proefschrift was dan ook om te onderzoeken of contaminatieniveaus van de *Fusarium* mycotoxines DON en FBs die de Europese aanbevolen maximale richtwaarden benaderen in pluimveevoeder, predisponeren voor NE in vleeskippen, en om inzicht te verwerven in de mechanismen die hiervoor verantwoordelijk zijn.

Hoofdstuk 1 beschrijft een experimentele *C. perfringens* infectiestudie die het predisponerend effect van DON blootstelling op de ontwikkeling van NE in vleeskippen onderzoekt. In dit experimenteel infectiemodel werd er geen gebruik gemaakt van coccidiose als predisponerende factor voor NE. De kippen werden gedurende 3 weken gevoederd met ofwel een controle-voeder, ofwel een voeder gecontamineerd met DON (3-4 mg/kg voeder). Na inoculatie met *C. perfringens*, ontwikkelden significant meer dieren subklinische NE-letsels in de DON groep ten opzichte van de controle groep (47 ± 3.0% *vs*. 20 ± 2.6%). NE letsels werden voornamelijk waargenomen in het duodenum en jejunum, wat gelijkaardig was met voorheen beschreven NE infectiemodellen, waarbij een blootstelling aan coccidia werd gebruikt als predisponerende factor. Uit deze studie kon bijgevolg besloten worden dat DON in staat is om de rol van coccidiose als predisponerende factor voor NE te vervangen. DON had in deze studie voornamelijk een negatief effect op het proximale deel van de dunne darm, zo was bijvoorbeeld de lengte van de villi significant korter in het duodenum. Daarnaast werd ook de intestinale epitheliale integriteit geëvalueerd door gebruik te maken van de *ex vivo* Ussing kamer techniek met darmexplanten. De resultaten toonden aan dat DON ook leidde tot een significant verminderde transepitheliale elektrische weerstand in het duodenum. Deze resultaten wijzen duidelijk op een negatieve invloed van DON op de intrinsieke component van de darmbarrière, die bestaat uit een laag epitheelcellen en de tight junctions die deze cellen verbinden. De darmschade veroorzaakt door DON kan leiden tot een verhoogd eiwitgehalte in het darmlumen door lekkage van plasmaproteïnen, of door een veranderde opname van voedingseiwitten. Eiwitanalyse van de inhoud van het duodenum toonde inderdaad aan dat blootstelling aan DON een significante verhoging van de totale eiwitconcentratie in dit darmsegment tot gevolg had. Dit kan leiden tot een verhoogde groei en toxine-productie van *C. perfringens*. Daarnaast had DON geen effect op de *in vitro* groei, productie van alfa toxine en transcriptie van *netB* toxine door *C. perfringens*.

Tot slot toont deze studie aan dat de opname van voeder gecontamineerd met DON onder de EU aanbevolen maximale richtwaarde een predisponerende factor is in de ontwikkeling van door *C. perfringens* veroorzaakte NE in broilers. Dit effect is te wijten aan de negatieve invloed op de darmbarrière en aan een verhoogde beschikbaarheid van nutriënten in het darmlumen voor vermeerdering van clostridia.

In **Hoofdstuk 2** werd de invloed van de opname van een FBs gecontamineerd voeder op de morfologie van de darmwand en de samenstelling van de microbiota bestudeerd. Vleeskippen werden gedurende 15 dagen gevoederd met een controle voeder of een voeder gecontamineerd met FBs (18.6 mg FB_1+FB_2/kg voeder). Het negatief effect van FBs op de sphingolipide biosynthese werd aangetoond aan de hand van een significant gestegen sphinganine/sphingosine verhouding in het plasma van kippen uit de FBs groep in vergelijking met dieren uit de controle groep (0.21 ± 0.016 vs. 0.14 ± 0.014). Daarnaast hadden dieren die het FBs gecontamineerde voeder verstrekt kregen een significant kortere dunne darm en een significant verminderde villuslengte en diepte van de crypten in het ileum, wat de negatieve impact van FB op de morfologie van de darm aantoont.

Met behulp van denaturerende gradiënt gelelectroforese kon een shift in de samenstelling van de darmmicrobiota worden aangetoond onder invloed van FBs. In de groep die werd blootgesteld aan FBs werd een verminderde aanwezigheid van laag-guanine-cytosine houdende operationele taxonomische eenheden vastgesteld. Deze eenheden werden geïdentificeerd als een verminderde aanwezigheid van *Candidatus* Savagella en Lactobacillen zoals *L. johnsonii*. *Candidatus* Savagella is een gesegmenteerde filamenteuze bacterie, die wordt gekenmerkt door zijn vasthechting aan het darmepitheel en zijn belangrijke invloed op het immuunsysteem van de gastheer. *L. johnsonii* werd reeds uitgebreid onderzocht voor zijn probiotische effecten zoals inhibitie van pathogenen, vasthechting aan de epitheelcellen en zijn immunomodulerend effect. Het is opvallend dat gelijkaardige veranderingen werden gemeld bij coccidiose-infecties en bij het voederen van vismeel aan broilers, twee goed gekende predisponerende factoren voor NE.

Daarnaast werd ook de totale hoeveelheid *C. perfringens* in de inhoud van het ileum gekwantificeerd door middel van qPCR, gebruik makend van het *cpa* gen dat codeert voor alfa toxine. Hieruit bleek een significant verhoogd aantal *C. perfringens* aanwezig bij kippen die een FBs gecontamineerd voeder kregen, ten opzichte van dieren die met het controle voeder werden gevoederd.

Vervolgens werd een infectiestudie uitgevoerd waarbij het predisponerend effect van FBs op de ontwikkeling van NE bij vleeskippen werd onderzocht. Na inoculatie met *C. perfringens* ontwikkelden significant meer kippen subklinische NE-letsels in de groep die gevoederd werd met een FBs gecontamineerd voeder, ten opzichte van dieren die het controle voeder toegediend kregen (45 ± 2.2% *vs.* 30 ± 5.5%).

Ten slotte kan gesteld worden dat de opname van een voeder gecontamineerd met FBs, aan een gehalte dat de EU aanbevolen maximale richtwaarde benadert, een negatief effect heeft op de darmmicrobiota en op de intrinsieke component van de darmbarrière, en aldus bij vleeskippen predisponeert voor de ontwikkeling van *C. perfringens* geïnduceerde NE.

In **Hoofdstuk 3** werd de impact van DON en FBs op de specifieke functionele en structurele componenten van de extrinsieke darmbarrière onderzocht. Hierdoor werd inzicht verworven in de gevolgen met betrekking tot de intestinale kolonisatie en beschikbaarheid van nutriënten in het darmlumen voor *C. perfringens*. In het eerste deel (**Hoofdstuk 3.1**) werd de invloed van het verstrekken van een DON en/of FBs gecontamineerd voeder aan vleeskippen onderzocht op de intestinale mucuslaag en op de ontwikkeling van oxidatieve stress in intestinale epitheelcellen. Vleeskippen werden verdeeld in vier groepen en gedurende 15 dagen gevoerd met ofwel een controle voeder, een DON gecontamineerd voeder (4.6 mg DON/kg), een FBs gecontamineerd voeder (25.4 mg FB_1+FB_2/kg), of een DON en FBs gecontamineerd voeder (4.3 mg DON en 22.9 mg FB_1+FB_2/kg). DON en FBs tastten de mucuslaag aan in het duodenum door een significant verminderde expressie van het intestinale mucine 2 gen en door een significante invloed op de monosacchariden samenstelling van het duodenale mucine, met name op N-acetyl-neuraminezuur, N-acetyl-galactosamine en galactose. Deze veranderingen in de monosacchariden samenstelling kunnen een effect uitoefenen op de integriteit van de mucuslaag. Dit kan bijgevolg ook de kolonisatie en beschikbaarheid van nutriënten voor micro-organismen zoals *C. perfringens* en als dusdanig de darmmicrobiota beïnvloeden.

Beide mycotoxines hadden ook een negatief effect op de homeostase van het cellulaire milieu door de ontwikkeling van oxidatieve stress. Dit gaf aanleiding tot de inductie en modulatie van een aantal anti-oxidatieve mechanismen in intestinale epitheelcellen. Er werd een significante down-regulatie van de transcriptie van het gen dat codeert voor xanthine dehydrogenase vastgesteld in het jejunum van vleeskippen gevoederd met een FBs of DON en FBs gecontamineerd voeder. Dit kan wijzen op een verminderde productie van reactieve

zuurstofradicalen (ROS) als bijproduct van de enzymatische omzetting van hypoxanthine naar urinezuur. Daarnaast veroorzaakte DON een significante up-regulatie van het gen dat codeert voor heem-oxigenase, wat kan leiden tot een verlaagd intracellulair gehalte van pro-oxidanten zoals heem en ijzer, en een verhoogd gehalte van het anti-oxidant biliverdine. Daarenboven werd een significant verminderde genexpressie van de intestinale, basolaterale zink transporter-1 gezien, wat kan wijzen op een beschermingsmechanisme van de intestinale epitheelcellen om het intracellulaire zinkniveau te behouden. Dit is belangrijk voor het onderhouden van de kritische anti-oxidatieve activiteit in het geval van door DON en FBs geïnduceerde oxidatieve stress. De opname van een DON en FBs gecontamineerd voeder beïnvloedt tenslotte ook de intestinale opname van methionine door een invloed uit te oefenen op de expressie van verschillende intestinale apicale en basolaterale methionine transporter mechanismen. Methionine speelt een belangrijke rol bij de synthese van cysteïne en glutathione, die beide de cel beschermen tegen ROS. Deze studie toont dus aan dat het voederen van een DON en/of FBs gecontamineerd voeder, in gehaltes die de EU aanbevolen maximale richtwaarden benaderen, de extrinsieke component van de darmbarrière beïnvloedt, door de intestinale mucuslaag te veranderen en verschillende anti-oxidatieve mechanismen te moduleren.

DON en FBs-contaminatie van voeders komen vaak samen voor. Bij gezonde kippen hebben FBs een lage orale biologische beschikbaarheid. DON heeft echter een negatief effect op de darmbarrière en vermindert het totale absorptieoppervlak van de darm. Daarom was het doel van de laatste studie (**Hoofdstuk 3.2**) na te gaan of chronische blootstelling van vleeskippen aan DON kan leiden tot een verhoogde intestinale absorptie van FBs, met als gevolg een veranderde systemische blootstelling en verhoogde toxische effecten van FBs. Er werd een toxicokinetische studie uitgevoerd bij vleeskippen, waarbij een orale bolus van 2.5 mg FBs/kg lichaamsgewicht werd toegediend, na een blootstelling van 3 weken aan ofwel een controle voeder, ofwel een voeder gecontamineerd met 3.12 mg DON/kg voeder. Er werden geen significante verschillen in de toxicokinetische parameters van FB_1 waargenomen tussen de twee groepen. Ook kon er geen effect op de systemische blootstelling aan FB_1 worden gezien. De relatieve orale biologische beschikbaarheid van FB_1 na chronische blootstelling aan DON bedroeg 92.2%.

Deze doctoraatsthesis toont voor het eerst aan dat het verstrekken van voeder gecontamineerd met DON of FBs predisponeert voor de ontwikkeling van *C. perfringens* geïnduceerde NE bij vleeskippen. Dit gaat samen met negatieve effecten op geselecteerde componenten van de intrinsieke en extrinsieke darmbarrière van de kip, namelijk villus lengte, tight junctions, mucus, oxidatieve stress en homeostase van de microbiota. Hieruit volgt dat blootstelling aan DON en/of FBs aan gehaltes die het Europees aanbevolen maximum benaderen, zowel de nodige nutriëntenbeschikbaarheid als een gunstige ecologische niche creëren voor de proliferatie van *C. perfringens*. In dit proefschrift werd aangetoond dat in aanvulling op de meest belangrijke predisponerende factoren, namelijk coccidiose en vismeel, ook voedercontaminatie met DON en/of FBs moet worden opgenomen in de lijst van belangrijke risicofactoren voor de ontwikkeling van *C. perfringens* geïnduceerde NE bij vleeskippen. Net zoals coccidiose, is ook de contaminatie van pluimveevoeder met mycotoxines, waaronder DON en FBs, een wereldwijd zeer frequent voorkomend probleem in de pluimveesector. Dit proefschrift toont aan dat voedercontaminatie met DON en FBs, aan contaminatieniveaus die de EU maximale aanbevolen hoeveelheden benaderen, toch een nadelig effect heeft op de darmgezondheid. Het in acht nemen van deze Europese wetgeving beschermt bijgevolg niet tegen het predisponerend effect van de mycotoxines DON en FBs op NE bij vleeskuikens. Daarom is het belangrijk om de nodige preventieve maatregelen te nemen om de mycotoxine blootstelling van de kip zo laag mogelijk te houden.

CURRICULUM VITAE

Gunther Antonissen werd geboren op 19 maart 1987 te Ekeren (Antwerpen).

Na het behalen van het diploma hoger secundair onderwijs aan het Sint-Jozefinstituut te Essen, richting Wetenschappen-Wiskunde, startte hij in 2005 met de studies diergeneeskunde aan de Universiteit Gent. Hij behaalde in 2011 het diploma van Master in de Diergeneeskunde met onderscheiding (optie gezelschapsdieren).

In september 2011 vatte hij bij de Vakgroep Farmacologie, Toxicologie en Biochemie en de Vakgroep Pathologie, Bacteriologie en Pluimveeziekten zijn doctoraatsonderzoek aan, waarin hij de mogelijke interactie tussen de mycotoxines deoxynivalenol en fumonisines en de pathogenese van necrotische enteritis bij vleeskippen bestudeerde. Dit onderzoek werd gefinancierd door Biomin GmbH (Herzogenburg, Oostenrijk). Gedurende dit onderzoek verbleef hij drie weken aan het Department of Nutritional Physiology and Feeding (Agricultural University of Athens, Griekenland). Verder begeleidde hij als promotor verschillende studenten in het behalen van hun masterproef en vervolledigde hij in 2015 het trainingsprogramma van de Doctoral School of Life Sciences and Medicine van de Universiteit Gent.

Gunther Antonissen is auteur en co-auteur van meerdere wetenschappelijke publicaties in internationale tijdschriften, gaf verschillende presentaties op meerdere nationale en internationale congressen en is reviewer voor diverse tijdschriften in zijn vakgebied.

Gunther Antonissen was born on March 19, 1987 in Ekeren (Antwerp).

After he graduated from secondary school at the St. Joseph's Institute in Essen, he started in 2005 with the studies of Veterinary Medicine at Ghent University. He obtained in 2011 a Master's degree in Veterinary Medicine with distinction (main subject pet animals).

In September 2011, he started his doctoral study at the Department of Pharmacology, Toxicology and Biochemistry and the Department of Pathology, Bacteriology and Avian Diseases, investigating the possible interaction between the mycotoxins deoxynivalenol and fumonisins and the pathogenesis of necrotic enteritis in broiler chickens. His doctoral research was funded by Biomin GmbH (Herzogenburg, Austria). During his doctoral research he spent three weeks at the Department of Nutritional Physiology and Feeding (Agricultural University of Athens, Greece). He also supervised several students as a promoter in obtaining their master's thesis and completed in 2015 the Doctoral training program of the School of Life Sciences and Medicine of Ghent University.

Gunther Antonissen is author and co-author of several scientific papers published in international peer reviewed journals. He presented his work at different national and international conferences and he is a reviewer for several scientific journals in his research field.

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