Effects of *Fusarium* T-2 Toxin on Avian Aspergillosis

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List of Abbreviations

15-ADON	15-acetyldeoxynivalenol
3-ADON	3-acetyldeoxynivalenol
AF	aflatoxin
AFB1	aflatoxin B1
AFB2	aflatoxin B2
AFG1	aflatoxin G1
AFG2	aflatoxin G2
ALT	altenuene
AME	alternariol methylether
ANOVA	analysis of variance
AOH	alternariol
APC	antigen-presenting cell
ATA	alimentary toxic aleukia
b.w.	body weight
CCLi	chemokine (C-C motif) ligand
CEUAM	cytokine expression per unit amount of macrophages
CFU	colony forming unit
CLSI	Clinical and Laboratory Standard Institute
Ct	threshold cycle
CXCLi	chemokine (C-X-C motif) ligand
DAS	diacetoxyscirpenol
DMEM	Dulbecco's Modified Eagle Medium
DON	deoxynivalenol
EC	European Commission
EFSA	European Food Safety Authority
FA	fumonisin A
FB	fumonisin B
FB1	fumonisin B1
FB2	fumonisin B2
FB3	fumonisin B3
FC	fumonisin C
FP	fumonisin P
FUS-X	fusarenon-X
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
H&E	hematoxylin and eosin
HBSS	Hank's Buffered Salt Solution
HT-2	HT-2 toxin
IARC	International Agency for Research on Cancer
IFN	interferon
IL	interleukin
JNK	c-Jun N-terminal kinases
LC-MS/MS	liquid chromatography tandem mass spectrometry
LD50	lethal concentration, 50%
LOD	limit of detection
LOQ	limit of quantification
MCP	monocyte chemoattractant protein
MIC	minimal inhibitory concentration
MIP	macrophage inflammatory protein
mRNA	messenger ribonucleic acid
NEO	neosolaniol
NET	neutrophil extracellular trap
NIV	nivalenol

OD	optical density
OTA	ochratoxin A
p38 MAP kinases	p38 mitogen-activated protein kinases
PAS	periodic acid-Schiff
PCR	polymerase chain reaction
PRR	pattern recognition receptor
qRT-PCR	quantitative reverse transcriptase polymerase chain reaction
ROS	reactive oxygen species
rpm	round per minute
RPMI	Roswell Park Memorial Institute
Sa:So ratio	sphinganine to sphingosine ratio
SD	standard deviation
ST	sterigmatocystin
T-2	T-2 toxin
TCA	citric acid
TeA	tenuazonic acid
TGF	transforming growth factor
Th	T helper cell
TLR	toll like receptor
TNF	tumor necrosis factor
ZEA	zearalenone
ZEL	zearalenol

General Introduction

1. Introduction on mycotoxins

1.1. Definition and general background of mycotoxins

Mycotoxins are fungal secondary metabolites which are toxic to humans and other animals. Fungal secondary metabolites which are mainly toxic to bacteria (antibiotics) and plants (phytotoxins), which are toxic only at high concentrations (e.g. ethanol), and which are produced by macrofungi (mushroom poisons) are not considered mycotoxins (Bennett and Klich, 2003). Fungi can produce mycotoxins in a variety of natural and artificial environments (Elmholt, 2008; Hoerger et al., 2009; Mayer et al., 2008; Smoragiewicz et al., 1993; Tuomi et al., 2000), and typically in agricultural crops, food and feed stuffs (Monbaliu, 2011). The most prevalent mycotoxins include *Alternaria* toxins, fumonisins, ochratoxins, zearalenone, aflatoxins and trichothecenes, which are classified by the chemical structures and producers (Logrieco et al., 2003; Njumbe Ediage et al., 2011). Surveys on the yearly occurrence of mycotoxins in food and feed materials showed that mycotoxin levels are generally low (Table 1), but levels and prevalence can vary greatly every year due to the influence of weather conditions, so high concentrations can readily occur due to climatic aberrations. Constant monitoring and effort on prevention and minimization of mycotoxin contamination are therefore necessary (Garrido et al., 2012; Streit et al., 2013). Because of the safety risk caused by mycotoxins, the European Commission (EC) has regulated and recommended maximum levels of some mycotoxins in animal feeds (Table 2).

Table 1. Overall results of the mycotoxin survey from 2004 to 2011 (Streit et al., 2013).

	Aflatoxins	Zearalenone	Deoxynivalenol	Fumonisins	Ochratoxin A
Number of tested samples	10172	13578	15549	9682	6053
Number of positive samples (%)[a]	2757 (27)	4932 (36)	8608 (55)	5239 (54)	1502 (25)
Average (µg/kg)	16	101	535	914	4
Average positives (µg/kg)	58	277	967	1689	16
Median positives (µg/kg)	12	87	462	750	3
1st quartile positives (µg/kg)	4	46	250	332	1
3rd quartile positives (µg/kg)	43	215	960	1828	7
Maximum (µg/kg)	6105	26728	50289	77502	1589
Sample origin[b]	Vietnam	Australia	Central Europe	China	China
Sample type (year of analysis)[b,c]	Corn (2009)	Silage (2007)	Wheat (2007)	Finished feed (2011)	Finished feed (2011)

[a] Values in parentheses specify the percentage of samples testing positive for each mycotoxin.
[b] Referring to the samples containing the highest detected concentration of the respective mycotoxin.
[c] Values in parentheses specify the year of analysis.

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Table 2. Overview of the current European regulatory limits for mycotoxins in animal feeds

Mycotoxin	Products intended for animal feed	Max. level [d]
Aflatoxin B1[a]	All feed materials	0.02
	Complete feedingstuffs for cattle, sheep and goats with the exception for:	0.02
	- Dairy animals	0.005
	- Calves and lambs	0.01
	Complete feedingstuffs for pigs and poultry (except young animals)	0.02
	Other complete feedingstuffs	0.01
	Complementary feedingstuffs for cattle, sheep and goats (except for dairy animals, calves and lambs)	0.02
	Complementary feedingstuffs for pigs and poultry (except young animals)	0.02
	Other complementary feedingstuffs	0.005
Deoxynivalenol[b]	Cereals and cereal products with the exception of maize by-products	8
	Maize by-products	12
	Complementary and complete feedingstuffs with the exception for:	5
	- Pigs	0.9
	- Calves (<4 months), lambs and kids	2
Zearalenone[b]	Cereals and cereal products with the exception of maize by-products	2
	Maize by-products	3
	Complementary and complete feedingstuffs for:	
	- Piglets and gilts (young sows)	0.1
	- Sows and fattening pigs	0.25
	- Calves, dairy cattle, sheep (including lamb) and goats (including kids)	0.5
Ochratoxin A[b]	Cereals and cereal products	0.25
	Complementary and complete feedingstuffs for:	
	- Pigs	0.05
	- Poultry	0.1
Fumonisin B1 + B2[b]	Maize and maize products	60
	Complementary and complete feedingstuffs for:	
	- Pigs, horses (Equidae), rabbits and pet animals	5
	- Fish	10
	- Poultry, calves (< 4 months), lambs and kids	20
	- Adult ruminants (> 4 months) and mink	50
T-2 + HT-2 toxins[c]	Unprocessed barley (including malting barley) and maize	0.2
	Unprocessed oats (with husk)	1
	Unprocessed wheat, rye and other cereals	0.1
	Oat milling products (husks)	2
	Other cereal products	0.5
	Compound feed, with the exception of feed for cats	0.25

[a] Regulated by EC (2003); [b] Recommended by EC (2006); [c] Recommended by EC (2013); [d] mg/kg.

1.2. Types of mycotoxins

1.2.1. *Alternaria* toxins

Alternaria toxins, represented by alternariol (AOH), alternariol methylether (AME), tenuazonic acid (TeA) and altenuene (ALT), are produced by *Alternaria* spp. Their chemical structures are shown in Figure 1. These mycotoxins have been detected in raw agricultural products such as grains, sunflower seeds, apples, tomatoes, grapes as well as in processed

products like apple juice and tomato products. *Alternaria* spp. are characterized by their ability to grow at low temperatures, therefore food and feedstuffs are subject to contamination by *Alternaria* toxins even in refrigerated storage conditions (EFSA, 2011a). Vegetables, nuts and oilseeds are particularly vulnerable to *Alternaria* toxin contamination, and AOH concentration can reach up to 1200 μg/kg (EFSA, 2011a). *Alternaria* toxins show genotoxicity, cytotoxicity, reproductive and developmental toxicity (fetotoxicity and teratogenicity) (EFSA, 2011a). AOH is an inhibitor of DNA topoisomerases, which are essential in DNA replication, transcription, and repair (Fehr et al., 2009). TeA is thought to inhibit protein synthesis by suppressing the release of newly synthesized proteins from the ribosomes (Davis et al., 1977). The LD50 values for ALT, AOH and AME in mice are 50, 400 and 400 mg/kg b.w., respectively (Pero et al., 1973). The reported LD50 of TeA is 37.5 mg/kg b.w. for day-old chicks (Giambrone et al., 1978) and 548 μg per egg for chicken embryos (Griffin and Chu, 1983). Daily dosed AOH and AME at 100 to 200 mg/kg b.w. was reported to increase the fatality and malformation of rodent fetus (Pero et al., 1973; Pollock et al., 1982). Chicks fed a diet supplemented with AME, AOH and ALT (24, 39 and 10 μg/g respectively) or AME (100 mg/kg) did not show any toxicological effects, suggesting the low toxicity of these mycotoxins in poultry (Griffin and Chu, 1983; Sauer et al., 1978). Feeding a diet with 10 mg TeA/kg feed or daily oesophageal intubation of 1.25 or 2.5 mg TeA/kg b.w. to 3-week old broilers for 3 weeks resulted in decreased weight gain, lowered feed efficiency, enlarged and mottled spleen, haemorrhage in the intestinal lumen and in thigh muscle, but no mortality or morbidity was noted (Giambrone et al., 1978).
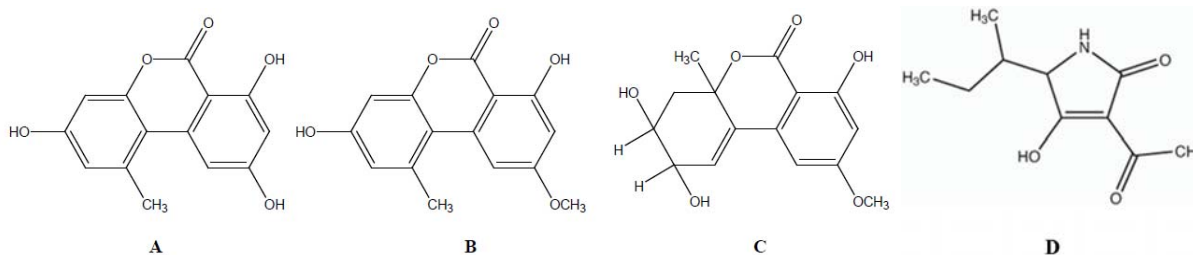
Figure 1. Chemical structures of AOH (A), AME (B), ALT (C) and TeA (D) (Monbaliu, 2011).

1.2.2. Aflatoxins and sterigmatocystin

Aflatoxins (AFs) are a group of mycotoxins produced by *Aspergillus* spp., primarily *A. flavus* and *A. parasiticus*. Among the approximately 20 currently known AFs, aflatoxin B1 (AFB1), aflatoxin B2 (AFB2), aflatoxin G1 (AFG1) and aflatoxin G2 (AFG2) are the most common, and sterigmatocystin (ST) is a biogenic precursor of AFB1 (Figure 2). AFs contamination mainly occurs in agricultural products in tropical and subtropical regions. AFs

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have been detected in pistachio, almond, hazelnut, Brazil nut, peanut, spice, fig, cashew, maize, fruits, sunflower seed, and other cereals (EFSA, 2007; EC, multiple years). The highest AFs concentration detected was 3337 μg/kg in Brazil nuts. AFB1 took the majority of total aflatoxins in all samples. AFB1 is converted by cytochrome P450 enzymes to the toxic 8,9-epoxide form, which can bind to the guanine residues of nucleic acids, inducing genotoxicity, and to proteins inducing cytotoxicity (Diaz et al., 2010a; Diaz et al., 2010b; Doi et al., 2002). The main toxic effects of AFs include carcinogenicity, hepatotoxicity, and immunotoxicity (EFSA, 2007). AFs are classified in Group 1 (carcinogenic to humans) by the International Agency for Research on Cancer (IARC), and their carcinogenicity targets particularly the liver. ST has similar toxicological effects but is less potent than AFs so it is classified in Group 2B (possibly carcinogenic to humans) (Monbaliu, 2011). The most susceptible species are rabbits and ducks, while chickens and rats are more tolerant. Studies in poultry, pigs, and rats showed that exposure to AFs suppresses cell-mediated immune response (EFSA, 2007).
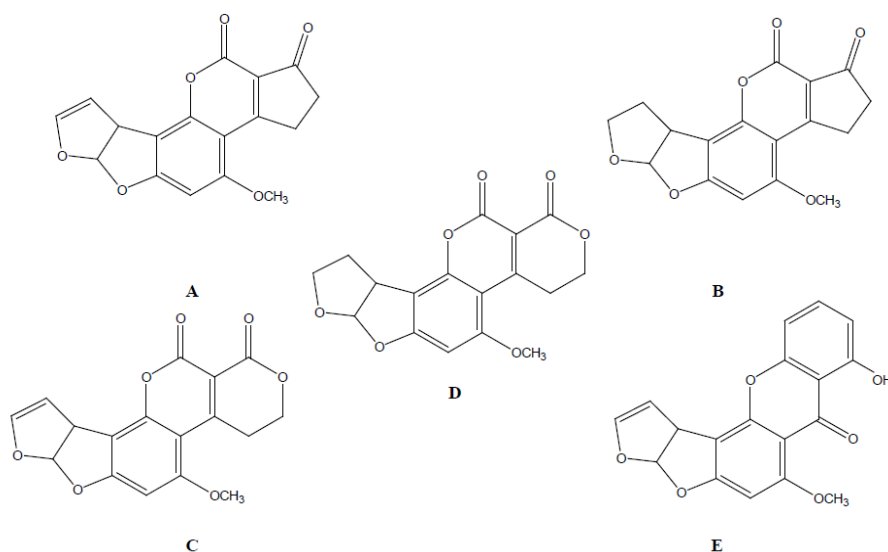
Figure 2. Chemical structures of AFB1 (A), AFB2 (B), AFG1 (C), AFG2 (D) and ST (E) (Monbaliu, 2011).

1.2.3. Ochratoxin A

Ochratoxin A (OTA) is a mycotoxin produced by several species of *Aspergillus* and *Penicillium*, including *A. ochraceus*, *A. niger*, *A. sulphureus*, *A. carbonarius*, *P. verrucosum* (formerly named to as *P. viridicatum II*), and *P. nordicum* (formerly named *P. viridicatum III*). The chemical structure of OTA is represented in Figure 3. Agricultural crops such as maize, wheat, barley, oats, triticale, rye, soybean and sunflower are all subject to contamination by OTA, and the levels of OTA can occasionally exceed 1850 μg/kg. The biochemical mode of

action of OTA is not well illustrated but it is thought to inhibit the enzymes associated with the synthesis of phenylalanine tRNA-complex (Bennett and Klich, 2003). Nephrotoxicity, immunotoxicity, reproductive and developmental toxicity (teratogenicity) have been reported in poultry, pigs, dogs, and ruminants. Chickens are considered less sensitive to OTA than pigs. Broiler chickens given a diet containing OTA at 2.5 mg/kg diet showed reduced weight gain and increased kidney weight, and egg production decreased dose-dependently when laying hens were given feed with OTA at 1.3 to 5.2 mg/kg in feed (EFSA, 2004b).
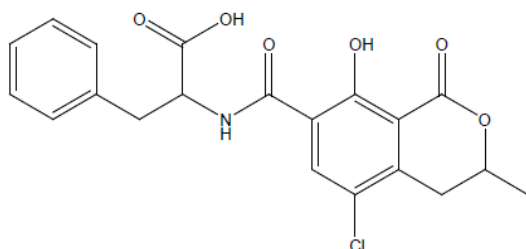
Figure 3. Chemical structure of OTA (Monbaliu, 2011).

1.2.4. Fumonisins

Fumonisins are produced by *Fusarium* spp., particularly by *F. verticillioides* (formerly named *F. moniliforme*). In total 28 different fumonisins have been identified and classified into 4 main groups, i.e. fumonisins A (FA), B (FB), C (FC) and P (FP) (Rheeder et al., 2002), among which FB is the most prevalent group and can be further divided into fumonisin B1 (FB1), fumonisin B2 (FB2) and fumonisin B3 (FB3) (Figure 4), with FB1 as the most important representative of fumonisins (Arranz et al., 2004). Fumonisins occur mainly in maize (Bullerman and Tsai, 1994), and incidentally in wheat, asparagus, tea and cowpea (Trucksess, 2003). The level of FB1 can reach up to 87 mg/kg in maize, but is mostly lower than 10 mg/kg (EFSA, 2005). Fumonisins show nephrotoxicity, hepatotoxicity, immunotoxicity, foetal toxicity and cardiovascular toxicity (EFSA, 2005). Fumonisins disrupt the ceramide and sphingolipid metabolism by inhibiting the ceramide synthase, resulting in an increase of free sphinganine (Sa) level and sphingosine (So) level (to a lesser extent), and a decrease of complex sphingolipids formation (Devreese, 2013). The increased Sa:So ratio in body fluids and tissues serves as a sensitive biomarker of fumonisin intoxication. Toxic effects of FB1, which included reduced weight gain and feed conversion, increased weight of organs (liver, kidney, proventriculus and gizzard), immunosuppression, and histological lesions in liver, were mostly observed in chickens, turkeys, and Pekin ducklings when its level exceeded 100 mg FB1/kg feed (EFSA, 2005).

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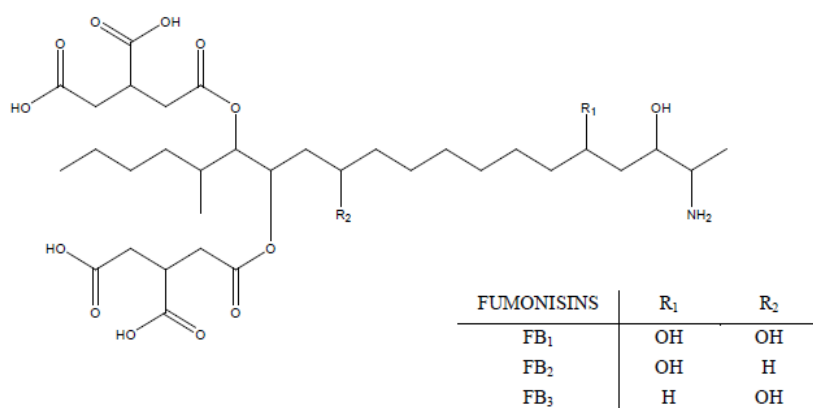
Figure 4. Chemical structures of fumonisins (Monbaliu, 2011).

1.2.5. Zearalenone

Zearalenone (ZEA) is a mycotoxin produced by *Fusarium* spp., particularly *F. graminearum* (formerly known as *F. roseum*) and also *F. culmorum*, *F. equiseti* and *F. verticillioides*. The chemical structure of ZEA is represented in Figure 5. It is commonly found in maize but can be found also in other crops such as wheat, oat, rice, barley, sorghum and rye, most frequently in moist cool conditions. The level of ZEA in unprocessed wheat was reported to reach up to 2969 µg/kg (EFSA, 2011c). Studies have shown that ZEA binds to estrogen receptors α and β, inducing oestrogenic-like effects by activating gene transcription via oestrogen-responsive elements (Metzler et al., 2010). ZEA also shows genotoxicity, apoptogenicity, immunotoxicity, reproductive and developmental toxicity. Signs of ZEA intoxication include haematological changes, hepatic disturbances and oestrogenic effects in orally dosed rodents. Pigs are considered the most sensitive animal species to ZEA, but poultry is more tolerant to ZEA. It was reported that chickens dieted with 50 mg ZEA/kg b.w. per day for 7 days showed no signs of intoxication (EFSA, 2011c).
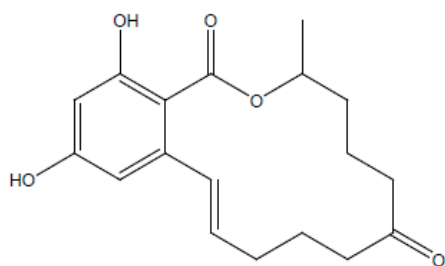
Figure 5. Chemical structure of ZEA (Monbaliu, 2011).

1.2.6. Trichothecenes

Trichothecenes are structurally related mycotoxins mainly produced by *Fusarium* spp., and are classified into type-A, type-B, type-C and type-D based on their molecular structures (Figure 6). Type-A and type-B trichothecenes are the dominant types in cereals and feeds

(Krska et al., 2001; Monbaliu, 2011). Type-A trichothecenes are represented by T-2 toxin (T-2), HT-2 toxin (HT-2), diacetoxyscirpenol (DAS) and neosolaniol (NEO), while Type-B trichothecenes are represented by nivalenol (NIV), deoxynivalenol (DON), 3-acetyldeoxynivalenol (3-ADON), 15-acetyldeoxynivalenol (15-ADON) and fusarenon-X (FUS-X) (Monbaliu, 2011). Trichothecenes occur in a variety of agricultural commodities such as wheat, maize, oat, barley, rye, rice, and other processed products, and the detected levels of T-2, HT-2, DON, and NIV can reportedly reach 6 mg/kg, 24 mg/kg, 927 mg/kg, and 7 mg/kg respectively (EFSA, 2011b; Placinta et al., 1999). DON is the most frequently occurring trichothecene, and T-2 is considered to have the most potent acute toxicity (Krska et al., 2001). The primary biochemical modes of action of trichothecenes are inhibition of protein synthesis and apoptogenesis, and the inhibition of DNA and RNA synthesis is possibly a secondary effect (Eriksen and Pettersson, 2004). Besides, trichothecenes also show cytotoxicity, haematotoxicity/myelotoxicity, immunotoxicity, and developmental and reproductive toxicity. DON stimulates pro-inflammatory response of immunocytes at low concentrations by upregulating the production of pro-inflammatory cytokines. Pigs exposed to DON showed decreased feed consumption and weight gain, lesions in the digestive tract and kidneys, but cattle, sheep and poultry are thought to be less sensitive to DON than pigs. Feed refusal and reduced weight gain were found in chickens only when DON concentrations exceeded 16 mg/kg in feed, and DON up to 83 mg/kg in feed did not affect the egg production in laying hens (EFSA, 2004a).

TYPE-A	R_1	R_2
NEO	OAc	OH
DAS	OAc	H
T-2	OAc	$OCOCH_2CH(CH_3)_2$
HT-2	OH	$OCOCH_2CH(CH_3)_2$

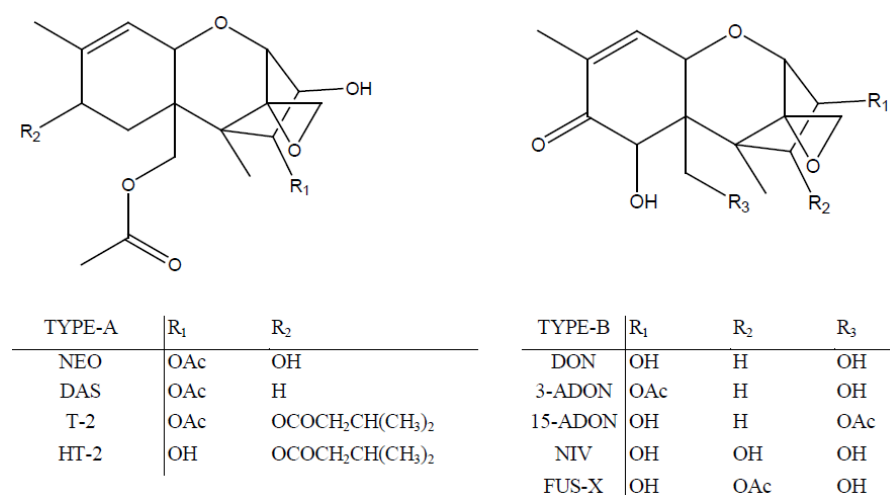| TYPE-B	R_1	R_2	R_3
DON	OH	H	OH
3-ADON	OAc	H	OH
15-ADON	OH	H	OAc
NIV	OH	OH	OH
FUS-X	OH	OAc	OH

Figure 6. Chemical structures of trichothecenes (Monbaliu, 2011).

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1.3. T-2 toxin

1.3.1. Occurrence of T-2 toxin

T-2 toxin is a type-A trichothecene mycotoxin mostly produced by *F. langsethiae*, *F. poae* and *F. sporotrichioides*, and it is typically detected in agricultural products such as rice, maize, wheat, barley, oat (EFSA, 2011b). As an environmental mycotoxin, T-2 and its derivatives have also been found in the dust of indoor ventilation systems (Smoragiewicz et al., 1993), crude building materials (Tuomi et al., 2000), and grain dust (Nordby et al., 2004). Levels of T-2 can reach 1200 µg/kg in grain dust (Nordby et al., 2004), and thousands of µg/kg in agricultural products. Table 3 summarizes the analytical results of T-2 concentrations across different feed groups (EFSA, 2011b).

Table 3. Concentrations (µg/kg) of T-2 toxin across feed groups (EFSA, 2011b).

Feed group	N[a]	LC (%)	LB/UB	Concentration (µg/kg)				
				Mean	P50	P75	P95	Max.
Undefined cereal grains, their products and by-products	34	71	LB	40	0	38	351	559
			UB	51	20	38	351	559
Oats	164	62	LB	24	0	37	107	268
			UB	40	30	37	107	268
Oat middlings	220	3.20	LB	114	51	145	470	825
			UB	114	51	145	470	825
Barley	242	91	LB	4.2	0	0	25	221
			UB	23	20	30	30	221
Sorghum	3	100	LB	0	NC	NC	NC	0
			UB	15	NC	NC	NC	20[c]
Wheat	237	100	LB	0.1	NC	NC	NC	14
			UB	14	NC	NC	NC	35[c]
Wheat middlings	8	100	LB	0	NC	NC	NC	0
			UB	4	NC	NC	NC	4.0[c]
Wheat bran	9	89	LB	2.4	NC	NC	NC	22
			UB	10	NC	NC	NC	22
Wheat gluten	72	5.60	LB	85	72	115	184	199
			UB	86	72	115	184	199
Triticale	41	95	LB	1.4	0	0	0	38
			UB	6.9	5	5	19	38
Maize	231	79	LB	13	0	0	72	415
			UB	18	4	13	75	415
Maize middlings	33	82	LB	15	0	0	120	171
			UB	18	4	4	120	171
Maize gluten feed	18	61	LB	13	NC	NC	NC	109
			UB	15	NC	NC	NC	109
Soya (bean), toasted	15	100	LB	0	NC	NC	NC	0
			UB	4.4	NC	NC	NC	10[c]
Sunflower seed	20	100	LB	0	NC	NC	NC	0
			UB	5.3	NC	NC	NC	10[c]
Lucerne meal	3	100	LB	0	NC	NC	NC	0
			UB	4	NC	NC	NC	4[c]
Grass meal	3	100	LB	0	NC	NC	NC	0

	N	LC	Bound					
			UB	9.3	NC	NC	NC	20[c]
Maize silage[b]	129	99	LB	1.8	0	0	0	206
			UB	20	20	20	20	206
Compound feedingstuffs (undefined)	895	85	LB	3.3	0	0	20	321
			UB	7	4	4	20	321
Compound feedingstuffs for calves	10	100	LB	0	NC	NC	NC	0
			UB	19	NC	NC	NC	20[c]
Compound feedingstuffs for cattle	42	64	LB	9.3	0	25	25	38
			UB	21	20	25	25	38
Compound feedingstuffs for piglets	36	100	LB	0	0	0	0	0
			UB	17	20	20	20	20[c]
Compound feedingstuffs for pigs	33	88	LB	3	0	0	25	25
			UB	20	20	20	25	25
Compound feedingstuffs for sows	17	100	LB	0	NC	NC	NC	0
			UB	18	NC	NC	NC	20[c]
Compound feedingstuffs for poultry	18	78	LB	5.6	NC	NC	NC	25
			UB	18	NC	NC	NC	25
Other feed	180	90	LB	9.1	0	0	69	352
			UB	24	20	20	69	352

N, number of samples; LC, left censored data (values below the limit of detection or limit of quantification); LB, lowerbound; UB, upper-bound; P50, 50th percentile; P75, 75th percentile; P95, 95th percentile; NC, not calculated; [a] If N < 60 then the calculated P95 should be considered as an indicative value only due to the limited number of data; [b] concentration reported as μg/kg 88 % dry matter; [c] value represents the left-censoring limit.

1.3.2. Absorption, distribution, metabolism and excretion of T-2 toxin in animal body

Toxicokinetic studies in broiler chickens showed that an oral bolus of T-2 at 0.02 mg/kg b.w. has an unquantifiably low bioavailability. An intravenous administration of the same dose showed that T-2 has a very short elimination half-life of 3.9 min, a volume of distribution of 0.14 L/kg, and a total body clearance of 0.03 L/min/kg (Osselaere et al., 2013). T-2 orally administered in chickens and ducks is distributed widely and quickly to muscle, liver, and kidney (Giroir et al., 1991) (Table 4). T-2 toxin is rapidly metabolized by hydroxylations, deacetylation, acetylation, de-epoxidation and glucuronide conjugations (Figure 7), yielding more than 20 metabolites such as HT-2 toxin, T-2 triol, T-2 tetraol, neosolaniol, 3'-hydroxy HT-2 toxin, 3'-hydroxy T-2 toxin, 3'-hydroxy T-2 triol, dihydroxy HT-2 toxin, de-epoxy-3'-hydroxy T-2 toxin and de-epoxy-3'-hydroxy HT-2 toxin. The de-epoxidation of T-2 toxin and its metabolites is considered an important detoxification mechanism (Wu et al., 2010). T-2 toxin is eliminated rapidly mainly through feces, and about 80% of T-2 orally administered by broiler chickens was metabolized and eliminated in the excreta within 48 hours (Yoshizawa et al., 1980).
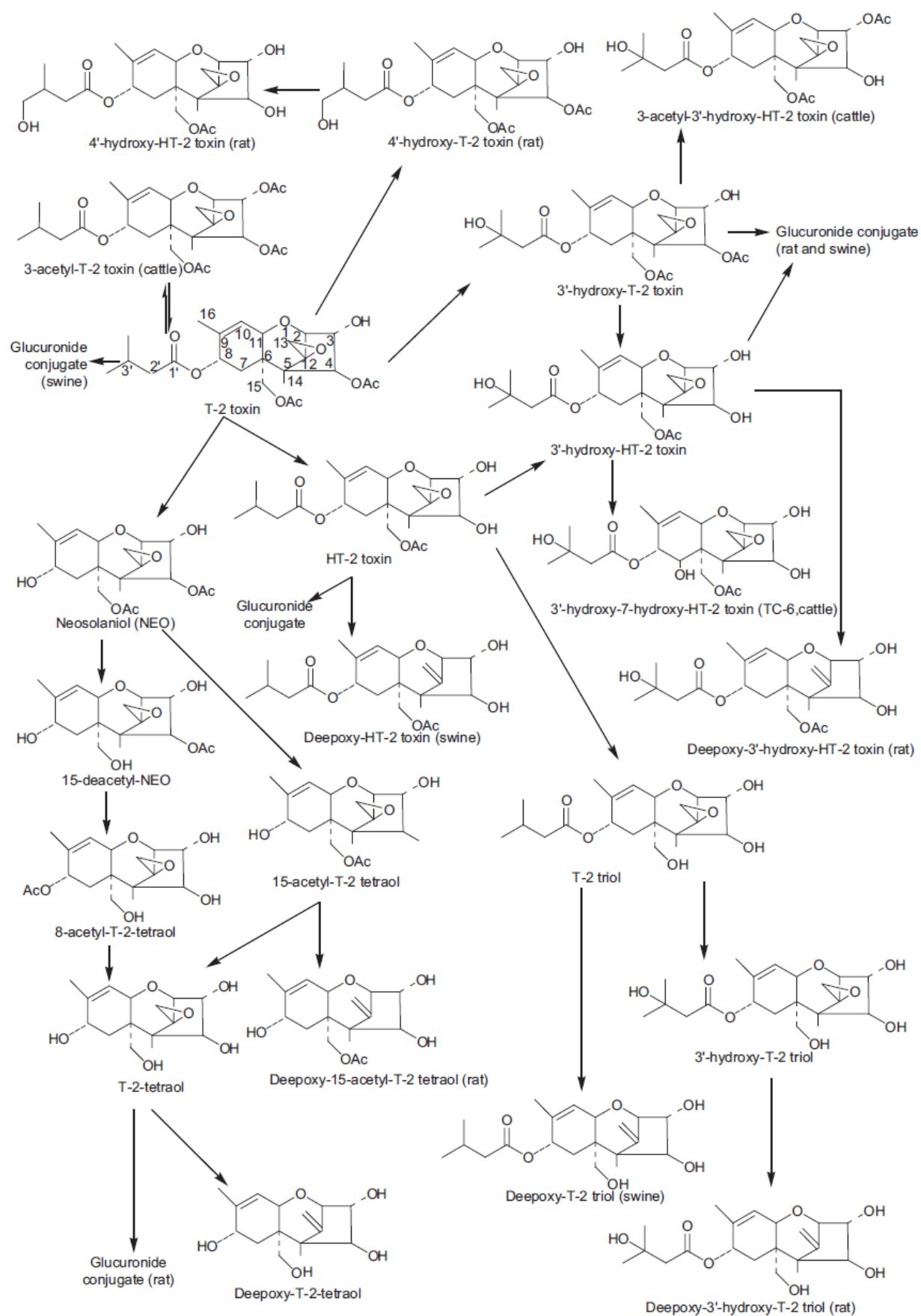
Figure 7. Proposed metabolic pathways of T-2 in animals (Wu et al., 2010).

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Table 4. T-2 toxin and its metabolites expressed as T-2 equivalents after single oral administration of 0.5 mg/kg b.w. of T-2 in chickens and ducks (Giroir et al., 1991).

Organ or tissue	T-2 equivalents (µg/kg)							
	6 hours		12 hours		24 hours		48 hours	
	Chicken	Duck	Chicken	Duck	Chicken	Duck	Chicken	Duck
Kidney	30	30	20	20	<10	<10	<10	<10
Liver	130	90	30	40	10	<10	<10	<10
Muscle								
Breast	30	50	40	40	<10	10	<10	<10
Thigh	30	30	20	10	<10	<10	<10	<10
Gizzard	40	40	30	20	<10	<10	<10	<10

1.3.3. T-2 toxicosis in animals

The 12,13-epoxide ring of T-2 is responsible for its toxic activity and de-epoxidation results in the loss of toxicity. In poultry, the toxic effects of T-2 can be classified as genotoxic and cytotoxic, immunomodulatory effects, effects on the digestive system and liver, effects on the nervous system and skin, and impairment of poultry performance (Sokolovic et al., 2008). The oral LD50 of T-2 for broiler chickens and quails is 4.97 and 14.7 mg/kg b.w., respectively (Sokolovic et al., 2008; Grizzle et al., 2004).

1.3.3.1. Genotoxicity and cytotoxicity

Inhibition of protein synthesis is the primary effect of T-2 in eukaryotic cells. It also inhibits DNA and RNA synthesis, affects the cell cycle, and induces apoptosis (Sokolovic et al., 2008; EFSA, 2011b). T-2 interacts with peptidyl transferase of the 60S ribosomal subunit, thus inhibiting the transpeptidation of peptide-bond formation. This results in the inhibition of prolongation and termination of protein synthesis (Jaradat, 2005; Liao et al., 1976). T-2 and other trichothecenes are widely reported to be ribotoxic in different tissues (Rocha et al., 2005). Inhibition of nucleic acid synthesis by T-2 has been reported in cells of the spleen, thymus, and bone marrow of treated mice, and in lymphocytes and thymus cells of humans by an unclear mechanism (Cooray, 1984; Munsch and Mueller, 1980; Rosenstein and Lafarge-Frayssinet, 1983). Dietary T-2 was found to significantly induce DNA damage in chickens (Frankic et al., 2006; Rezar et al., 2007; Sokolovic et al., 2007). Toxic effects of T-2 on cellular membrane have been demonstrated in a variety of cell lines. As an amphophilic molecule, T-2 is taken up into the cellular bilayer membrane and damages it by producing reactive oxygen species (free radicals) and inducing lipid peroxidation (EFSA, 2011b). Apoptogenicity of T-2 was demonstrated by the apoptototic cell death in the thymus of broiler chickens dieted with 1 mg T-2/kg feed (Venkatesh et al., 2005). T-2 also induced apoptosis in lymphoid organs, haematopoietic tissues, intestinal crypt, brain, skin, and cell types such as

HL60, Jurkat, U937, Vero cells and hepatoma cells in humans and other animals (Bouaziz et al., 2006; Bouaziz et al., 2008; Grizzle et al., 2004; Huang et al., 2007; SCF, 2001; Sehata et al., 2004). T-2 toxin induces cell apoptosis by the activation of c-Jun N-terminal kinases (JNKs) and p38 mitogen-activated protein kinases (p38 MAP kinases), but the precise mechanism has not yet been well elucidated (Sokolovic et al., 2008).

1.3.3.2. Immunomodulatory activity

The immune system is one of the main targets of T-2, and the immunomodulatory activity of T-2 is dose-dependent, e.g. it stimulates the immune response at low doses, and suppresses immune responses at high doses in poultry. It is thought that immunosuppression at high doses is due to the damage of bone marrow, lymph nodes, spleen, thymus, intestinal mucosa, leucopenia and the inhibition of the functions of immune cells, while immunostimulation at low doses is thought to be caused by rapid and transient activation of the genes responsible for immune responses such as serum antibodies and inflammation. Unfortunately, only a limited number of studies have reported the immunostimulatory effects of T-2, and possible mechanisms have never been elucidated (EFSA, 2011b; Sokolovic et al., 2008). Lesions, atrophy, and weight change of immune organs such as thymus, bursa of Fabricius, and spleen were found in turkeys, Pekin ducks, and chickens exposed to T-2 (Nataraja et al., 2003; Rafai et al. 2000; Rajeev et al., 2003; Richard et al., 1978). T-2 and HT-2 intoxication can induce alimentary toxic aleukia (ATA) in humans and cats (EFSA, 2011b; Lutsky et al., 1978). Exposure of chickens to T-2 caused higher mortality due to *Salmonella* infection and lower antibody titres against Newcastle disease and infectious bursal disease (Sokolovic et al., 2008). Exposure to T-2 also altered the production of cytokines in rodent macrophages (Ahmadi and Riazipour, 2008; Dugyala and Sharma, 1997), suppressed the phagocytic activity of rodent macrophages against *Staphylococcus aureus* (Sorenson et al., 1986) and *Pseudomonas aeruginosa* (Vidal and Mavet, 1989) and promoted the susceptibility of porcine macrophages to *Salmonella* Typhimurium invasion (Verbrugghe et al., 2012). The immunomodulatory activity of T-2 is also time-dependent. Enhanced resistance to *Listeria* in rodents was observed after short-term preinoculation with T-2, whereas postinoculation resulted in immunosuppression (Bondy and Petska, 2000).

1.3.3.3. Toxicity on digestive system and liver

Digestive disorders in poultry are characterized by diarrhoea, feed and water refusal, decreased body weight, increased feed conversion ratio, lesions of the digestive tracts, and

impaired resorption of nutrients (EFSA, 2011b; Sokolovic et al., 2008). Lesions in oral cavity, tongue, proventriculus, gizzard, intestine and liver, were frequently observed in chickens, Pekin ducks and turkeys exposed to dietary T-2 (EFSA, 2011b; Sokolovic et al., 2008) (Figure 8). The liver is thought to be the main target of the toxic effects of T-2. Inhibition of protein synthesis reduces the activity of the enzymes associated to the metabolism of toxic substances, induces lipid peroxidation, and increases the activity of glutathione reductase (Sokolovic et al., 2008).
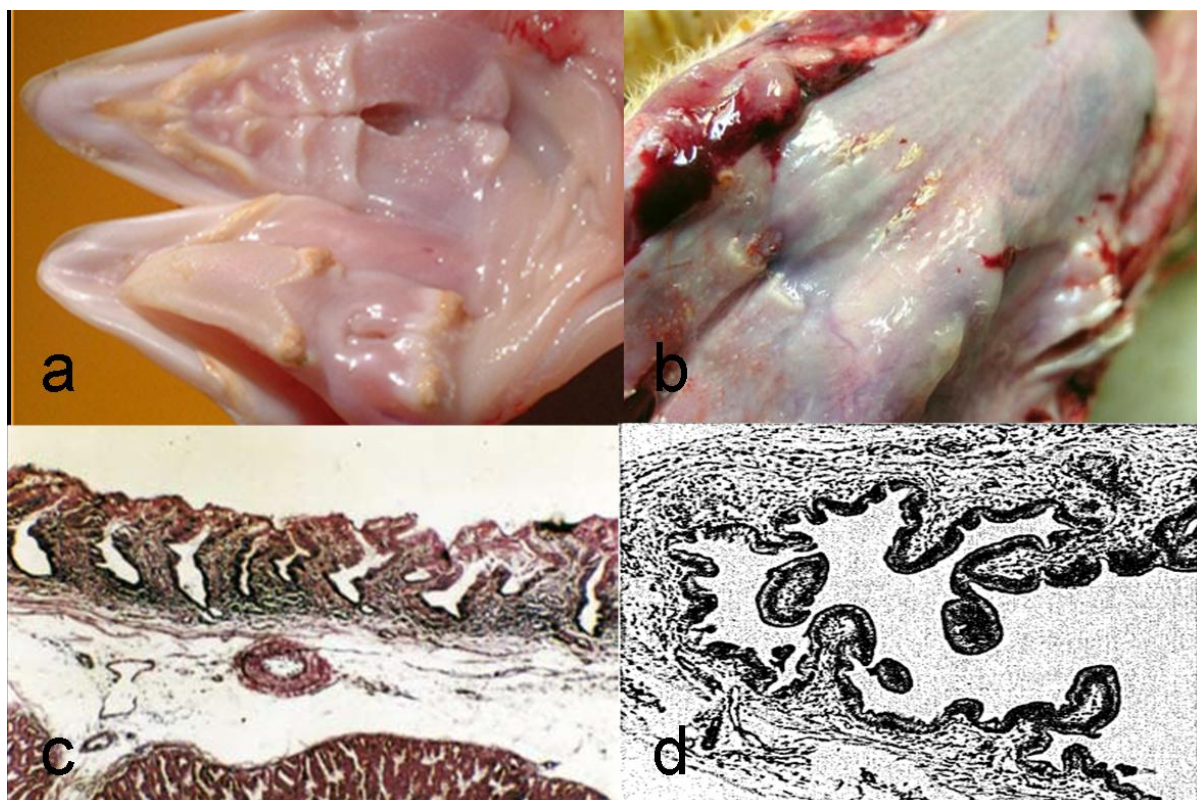

Figure 8. Some T-2-induced lesions in the digestive system of poultry. (a) Oral lesions in a 7-day-old turkey poult given feed containing 1 mg T-2/kg for 10 days (Shlosberg, 2001); (b) Erosions in the proximal esophagus caused by exposure to T-2 (Ruiz, year unknown); (c) Shortening of villi and mononuclear cell infiltration in the proventriculus of a 4-week-old broiler chicken with T-2 toxicosis (H&E, ×320) (Krishnamoorthy et al., 2007); (d) Oedema and congestion of the gallbladder of a chicken given two times daily 2.5 mg/kg doses of T-2 (H&E) (Hoerr et al., 1982).

1.3.3.4. Other toxic effects of T-2

Developmental toxicity, reproductive toxicity, neurotoxicity, and carcinogenicity of T-2 have also been reported, but are not considered critical effects of T-2 (EFSA, 2011b). Delay of puberty, decreased egg fertility and hatchability, and thinner egg shell were found in laying hens, laying geese, and quails exposed to dietary T-2 (Chi et al., 1977; Diaz et al., 1994; Grizzle et al., 2005; Vanyi et al., 1994; Wyatt et al., 1975). T-2 also showed effects on neurotransmitter levels in chickens and rats, and the neurotoxic effects in rats were seen at

levels as low as 0.1 mg/kg b.w. per day (EFSA, 2011b). It was also reported that incidence of pulmonary and hepatic adenomas increased in male mice when fed a T-2 containing diet (Schiefer et al., 1987).

1.4. Effects of mycotoxins on microorganisms

Secondary metabolites are not directly essential for fungal growth (Betina, 1994), but many studies agree that fungi produce mycotoxins and other secondary metabolites to create or maintain a competitive advantage over other organisms, especially under environmental stresses (Magan and Aldred, 2007). Besides their toxicity to animals, many mycotoxins are toxic to microorganisms as well (Bennett and Klich, 2003). ZEA and its derivatives exerted a toxic effect on fungi *Sordaria fimicola*, *Epicoccum purpurascens*, *Cladosporium herbarum*, and *Alternaria alternata*, with the effect declining in the order ZEA>α-ZEL>β-ZEL (Utermark and Karlovsky, 2007). The antagonism of *Penicillium* spp. towards the soil fungus *Trichoderma* sp. and the phytogenic fungus *Rhizoctonia solani* was thought to be associated with the production of the mycotoxin patulin by *Penicillium* spp. for competition for nutrient resources (Nicoletti et al. 2004; Norstadt and McCalla 1969). AFB1 significantly reduced the populations of viable bacteria and fungi in agar medium and soil, and showed mutagenicity on the bacterium *Rhizobium japonicum* (Angle and Wagner 1981). The bacterium *Bacillus brevis* was sensitive to AFB1, OTA, citrinin, patulin, penicillic acid, cyclopiazonic acid, penitrem A, and ZEA, but not to trichothecenes, e.g. T-2, HT-2, DAS and DON, while the yeast *Kluyveromyces marxianus* was inhibited by all these four trichothecenes but not by the other mycotoxins (Madhyastha et al., 1994). DON exhibited weak, but T-2 and DAS showed stronger antimicrobial activity against the fungi *Penicillium digitatum*, *Mucor ramannianus*, and *Saccharomyces bayanus*, but these 3 trichothecenes showed no antimicrobial activity against the gram-positive, gram-negative, or acid-fast bacteria (Vesonder et al., 1981). T-2 exhibited inhibitory effect on the fungi *Penicillium digitatum*, *Mucor ramannianus*, *Rhodotorula rubra*, *R. glutinus*, *Saccharomyces carlsbergensis* and *S. pastorianus*, but not on *Candida krusei*, *C. albicans*, *Cryptococcus albidus*, *Aureobasidium pullulans*, *Tremella mesenterica*, or any of the 54 tested bacterial strains (Burmeister and Hesseltine, 1970). T-2 did not affect the growth of *Salmonella* Typhimurium, but reduced its flagella gene expression and motility (Verbrugghe et al., 2012). Although intake of *Fusarium* mycotoxins such as DON, T-2 and FB1 have been widely reported to alter the susceptibility of animals to pathogens (e.g. *Salmonella* Typhimurium, *Escherichia coli*, *Clostridium perfringens*, *Edwardsiella ictaluri*) (Antonissen et al., 2014), studies have been focused on the effects of

mycotoxins on the hosts, while possible effects of mycotoxins on the pathogens have largely been neglected and therefore deserve further studying.

General Introduction

2. Introduction on avian aspergillosis

2.1. *Aspergillus fumigatus*

2.1.1. Etiology

Aspergillus fumigatus is the primary pathogen of aspergillosis, an opportunistic infectious noncontagious disease that occurs in human and animals. The taxonomic classification of *A. fumigatus* is described in Table 5. *A. fumigatus* is a ubiquitous saprophytic fungus living on organic debris. The conidia of this fungus are produced on the conidial heads and released into the air by environmental disturbance and air currents, which makes *A. fumigatus* the most prevalent airborne fungal pathogen (Latgé, 1999). Although other *Aspergillus* species, such as *A. flavus*, *A. niger*, *A. glaucus*, *A. nidulans* can also cause aspergillosis, *A. fumigatus* is the predominant species of airborne fungal infections because of the small size of its conidia (2-3 μm in diameter). Because of their small size, *A. fumigatus* conidia are more likely to escape the mucociliary clearance by the upper respiratory tract, and can reach the lungs and air sacs more easily (Richard et al., 1981; Richard and Thurston, 1983).

Table 5. The taxonomic classification of *A. fumigatus* (Taylor, 2006).

Kingdom	Phylum	Class	Order	Family	Genus	Species
Fungi	Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	*Aspergillus*	*fumigatus*

2.1.2. Culture and morphological characteristics of *A. fumigatus*

A. fumigatus is a rapidly growing thermophilic fungus. It can grow at temperatures up to 55°C, and survive at up to 70°C (Latgé, 1999). A young *A. fumigatus* colony is white but turns green to dark blue-green after a few days of growth due to sporulation (Figure 9). A colony consists of a dense felt of conidiophores, arising from aerial hyphae (de Hoog et al., 2000; Oglesbee, 1997). Hyphae are the main mode of vegetative growth and are collectively called a mycelium (Klich, 2002). The mycelium cell wall mainly consists of polysaccharides such as galactomannan, chitin, α(1,3)-glucans and β(1,3)-glucans (Bernard and Latgé, 2001). Conidiophores are specialized hyphae with a swollen end known as vesicle (20-30 μm in diameter), from which the green phialides (5-9 by 2-3 μm) directly arise. A chain of green smooth-walled conidia (2-3 μm in diameter) emerges from each phialide (Klich, 2002). The conidium cell wall is composed of a dense pigmented outer layer and a translucent inner layer (Bernard and Latgé, 2001). The vesicle, phialides and the attached conidial chains compose a

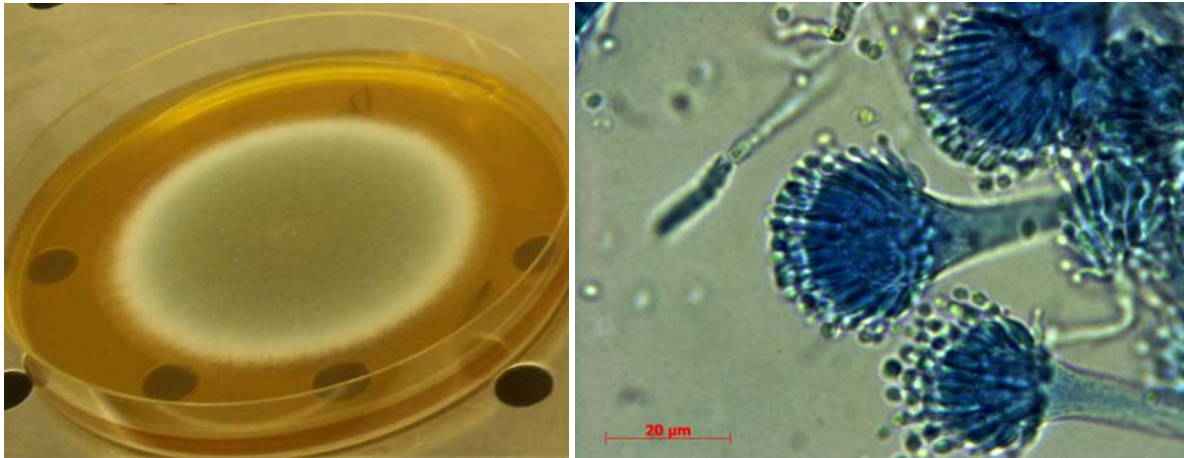conidial head (asexual fruiting body), with a "holy water sprinkler" appearance (Oglesbee, 1997) (Figure 9).

Figure 9. (left) *A. fumigatus* **culture grown on Sabouraud dextrose agar plate. (right)** *A. fumigatus* **conidiophores, vesicles, phialides and conidia (lactophenol blue staining).**

2.1.3. Life cycle of *A. fumigatus*

A. fumigatus has both an asexual and sexual life cycle (O'Gorman et al., 2009) (Figure 10).

2.1.3.1. Asexual life cycle

The asexual cycle is the primary means of replication for *A. fumigatus* and protects the fungal genome in unfavourable conditions. The *A. fumigatus* genome remains haploid throughout the whole asexual life cycle. Sporulation produces conidia (haploid, uninucleated asexual spores) (Ward et al., 2006). Conidiogenesis of *A. fumigatus* occurs in an enteroblastic phialidic manner by which chains are formed by a single element which is pushed out of the phialide opening and differentiates into conidia (Reiss et al., 2011) (Figure 11). Vegetative growth is initiated by germination of a conidium, with formation of tubular hyphae, growing in a polar fashion by apical extension and branching to form a network of mycelium, which acquires nutrients from the environment (Ward et al., 2006).

General Introduction
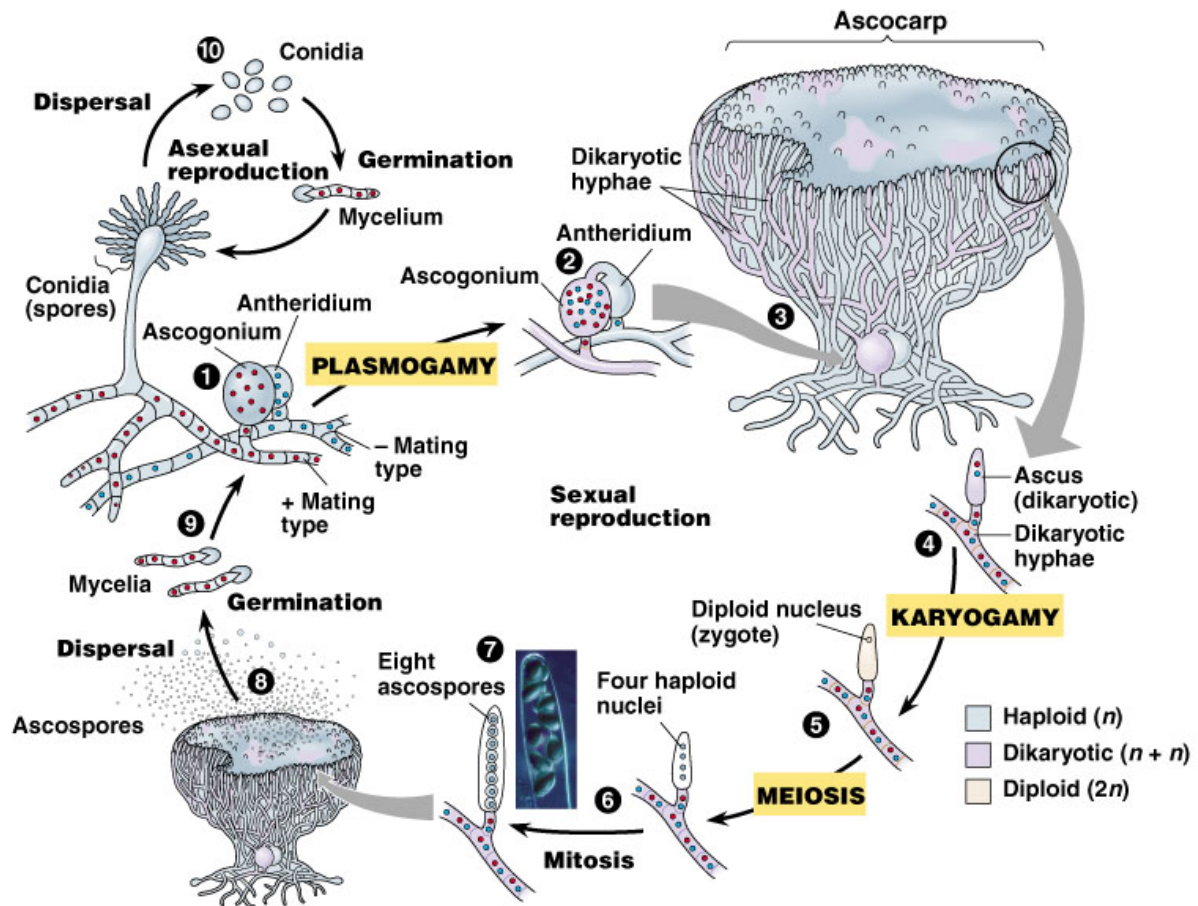
Figure 10. The typical life cycle of an ascomycete (Yen and Shen, year unknown). 1. Two parental haploid hyphae become intertwined and form an ascogonium (female) and an antheridium (male); 2. The ascogonium accepts nuclei from the antheridium through plasmogamy and forms a dikaryon; 3. The dikaryotic ascogenous hyphae extend and develop into an ascocarp (a globose and closed type of ascocarp (cleistothecia) in case of *A. fumigatus*); 4. Asci (dikaryotic) are formed in the dikaryotic ascogenous hyphae in the ascocarp; 5. Diploid ascus nucleus is formed by karyogamy; 6. The resultant diploid ascus nucleus is divided into four genetically distinct haploid nuclei via meiosis; 7. An additional round of mitosis yields eight haploid nuclei in the ascus, which then develop into eight haploid ascospores (irregularly arranged in case of *A. fumigatus*); 8. The ascospores are dispersed out of the ascocarp; 9. The ascospores germinate and grow into new haploid mycelia, which can start new asexual and sexual cycles; 10. Asexual life cycle. Conidiogenesis occurs in the haploid mycelium and produces asexual spores (conidia), which germinate and develop into new mecylia.
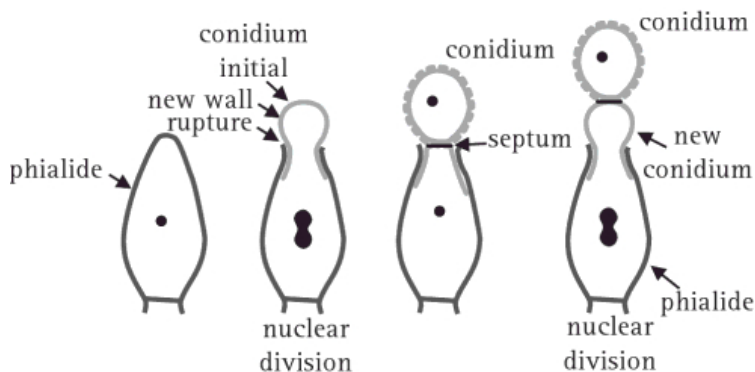

Figure 11. Enteroblastic phialidic conidiogenesis (Moore et al., 2011)

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2.1.3.2. Sexual life cycle

The sexual reproduction of *A. fumigatus* (teleomorph named *Neosartorya fumigate*) occurs only in strictly manipulated growth conditions (O'Gorman et al., 2009). The sexual cycle (Figure 10) is initiated when two haploid parental *A. fumigatus* isolates grow into contact. Plasmogamy gives rise to dikaryotic ascogenous hyphae which will form diploid asci in cleistothecia, a globose and closed type of ascocarp (sexual fruiting bodies). An ascus produces 8 haploid ascospores (sexual spores) by one meiosis and then one mitosis (O'Gorman et al., 2009; Ward et al., 2006) (Figure 10). Sexual reproduction allows genetic recombination between two parental fungal isolates, and yields progeny genotypes which are unique and different from those of the parents (O'Gorman et al., 2009). This increases the fitness of this next generation, augmenting adaptation to the changing environment and improving their chances for long-term survival (Dyer and O'Gorman, 2012).

2.1.4. Genome and proteome of *A. fumigatus*

A study on a virulent clinical *A. fumigatus* isolate Af293 revealed its haploid genome of 29.4 million base pairs, which consists of 8 chromosomes containing 9,926 genes (Nierman et al., 2005). Many of these genes exhibit extensive similarity with genes from the yeast *Saccharomyces cerevisiae* (Ward et al., 2006) and the most abundant encoded proteins are mentioned in Table 6 (Teutschbein et al., 2010).

Table 6. 40 most abundant proteins in *A. fumigatus* conidial proteome (Teutschbein et al., 2010).

Protein	Protein function [a]
Translation elongation factor EF-1 alpha subunit, putative	C, M, N
Spore-specific catalase CatA	D, N
Enolase/allergen Asp F 22	I, K, N
Conidial hydrophobin Hyp1/RodA	S
Peptidyl-prolyl cis-trans isomerase/cyclophilin, putative	C, D, F, L, M, N, P, Q
Hypothetical protein AFUA_6G12000	S
Asp hemolysin-like protein	S
Oxidoreductase, zinc-binding dehydrogenase family	A, D, K, N
Mitochondrial aconitate hydratase, putative	I, K, N
Phosphoglycerate kinase PgkA, putative	I, K, N
Mitochondrial Hsp70 chaperone (Ssc70), putative	B, K, L, N
Glyceraldehyde 3-phosphate dehydrogenase GpdA	I, J, K, N
Mannitol-1-phosphate dehydrogenase	G, K
Mitochondrial F1 ATPase subunit alpha, putative	F, I, J, N
NAD-dependent formate dehydrogenase AciA/Fdh	I, K
6-phosphogluconate dehydrogenase Gnd1, putative	I, K, N
Conserved hypothetical protein	S
Citrate synthase (Cit1), putative	A, I, K
Actin Act1	A, B, C, D, E, G, J, L, N, Q
Aldehyde reductase (AKR1), putative	D, I, J, K, N

Transaldolase	I, K, N
Glycerol dehydrogenase (GldB), putative	D, I, J, K, N
Aldehyde dehydrogenase AldA, putative	A, D, G, I, K, N
Mitochondrial processing peptidase beta subunit, putative	G, I, L, N
Glucose-6-phosphate 1-dehydrogenase	D, I, K, N
Zinc-binding oxidoreductase, putative	G, I, K, N
Malate dehydrogenase, NAD-dependent	A, G, I, K, N
Cu,Zn superoxide dismutase SOD1	C, D, F, J, N, O
Transketolase TktA	I, K, N
Triosephosphate isomerase	A, I, K
Hypothetical protein AFUA_4G00740	S
Zinc-containing alcohol dehydrogenase, putative	I, K
Phosphoglycerate mutase, 2,3-bisphosphoglycerateindependent	I, K, N
Bifunctional catalase-peroxidase Cat2	D
G-protein complex beta subunit CpcB	F, I, K, L, M
Actin Act1	A, B, C, D, E, G, J, L, N, Q
Hypothetical protein AFUA_7G04920	S
Translation elongation factor EF-2 subunit, putative	K, M, N, Q
Malate synthase AcuE	I, K
ThiJ/PfpI family protein	D, K, N

[a] Protein function: A, biogenesis of cellular components; B, cell cycle and DNA processing; C, cell fate; D, cell rescue, defense and virulence; E, cell type differentiation; F, cellular communication/signal transduction mechanism; G, cellular transport, transport facilitation and transport routes; H, development (systemic); I, energy; J, interaction with the environment; K, metabolism; L, protein fate (folding, modification, destination); M, protein synthesis; N, protein with binding function or cofactor requirement (structural or catalytic); O, regulation of metabolism and protein function; P, systemic interaction with the environment; Q, transcription; R, transposable elements, viral and plasmid proteins; S, unknown.

2.2. Avian aspergillosis

2.2.1. Virulence factors of *A. fumigatus*

The genes and molecules involved in the pathogenesis of *A. fumigatus* infections are functionally classified by Abad et al. (2010) into groups involved in thermotolerance, cell wall composition, resistance to the immune response, toxins, nutrient uptake, allergen, signaling and metabolism regulation (Figure 12). Gliotoxin, phospholipase, protease, and elastase were found to be associated with the pathogenesis of aspergillosis in humans (Gardiner et al., 2005; Rementeria et al., 2005; Sugui et al., 2007). Gliotoxin is the major and the most potent toxin of *A. fumigatus* (Kwon-Chung and Sugui, 2009) and is thought to be involved in the pathogenesis of aspergillosis in turkeys (Richard et al., 1994; Richard et al., 1996). Gliotoxin modulates the immune response by targeting primarily the activity of neutrophils or phagocytes, and induces apoptosis and necrosis in cells and tissues (Scharf et al., 2012; Tell, 2005). Different *A. fumigatus* isolates were found to be remarkably different in pathogenicity in turkeys (Peden and Phoades, 1992), suggesting differences in expression of virulence factors.
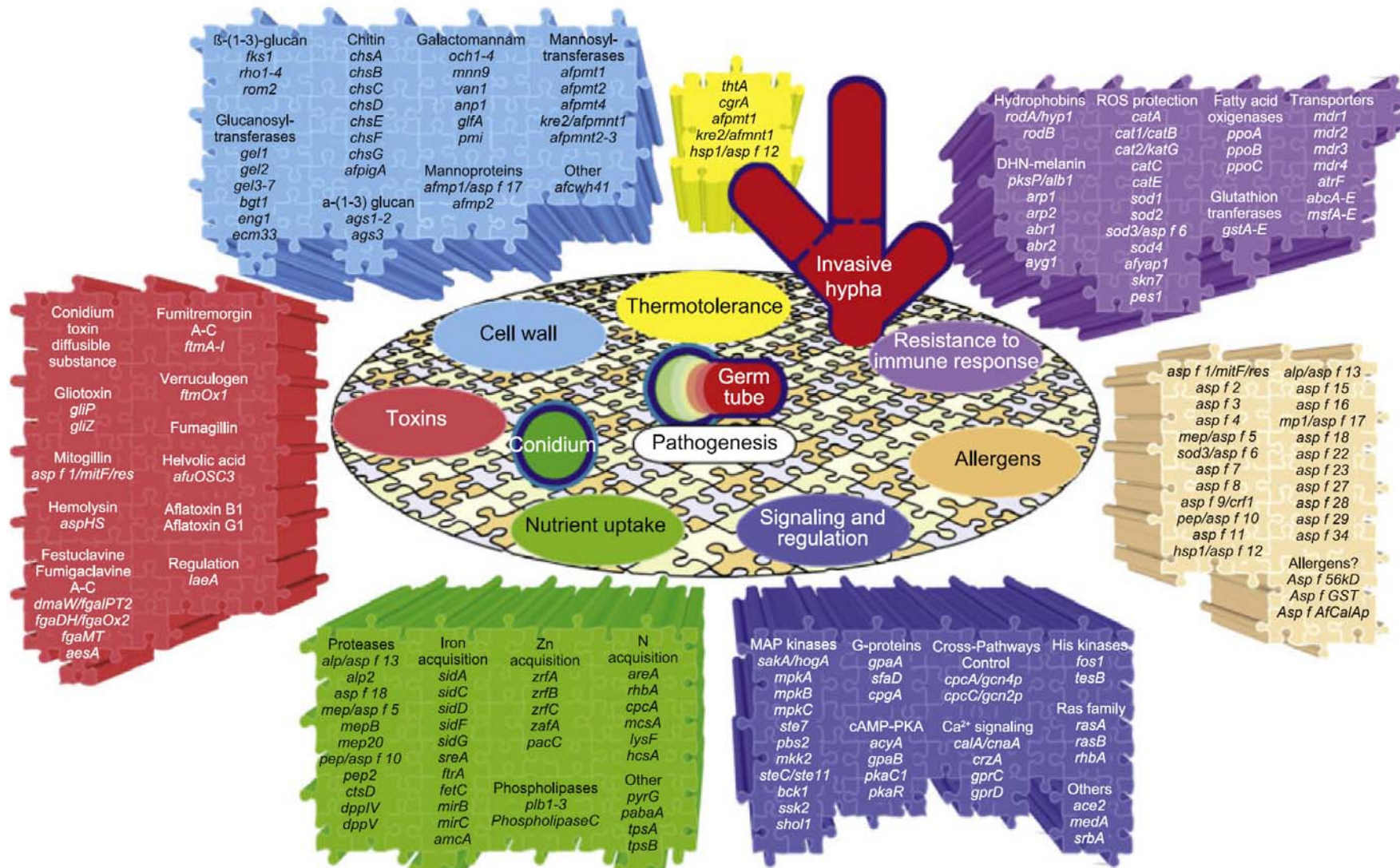
Figure 12. Summary of genes and molecules associated with the virulence of *A. fumigatus* (Abad et al., 2010).

2.2.2. Risk factors for birds

The major risk factors for an *Aspergillus* infection are an overwhelming number of conidia and immunosuppression of the host. An overwhelming amount of spores can rapidly develop in a warm humid environment with poor ventilation and poor sanitation (Oglesbee, 1997; Phalen, 2000; Tell, 2005). Besides, improperly stored feeds can be a source of fungal pathogens (*A. fumigatus*, *A. flavus*, *A. glaucus* and *A. niger*) (Khosravi et al., 2008; Simpson and Euden, 1991). In those feeds not only the fungi, but also immunosuppressive mycotoxins, e.g. zearalenone, trichothecenes, aflatoxins and/or fumonisins can be present (EFSA, 2004a; EFSA, 2005; EFSA, 2007; EFSA, 2011b; EFSA, 2011c; EFSA, 2013). Intensive production strategies, severe genetic manipulation, inadequate management and husbandry practices of domestic birds may also weaken the immunological defense (Maina, 2002; Nganpiep and Maina, 2002). Other immunosuppressive factors that can predispose birds to aspergillosis include administration of tetracyclines, vaccination, metabolic bone disease, overcrowding, shipping, quarantine or capture of wild birds, starvation, thermal discomfort, migration, inbreeding, circovirus infection and lymphoproliferative disorders, toxicosis, traumatic injuries and reproductive activity (Beernaert et al., 2010).

2.2.3. Avian susceptibility to aspergillosis

Birds are considered to be particularly susceptible to aspergillosis (Van Waeyenberghe et al., 2012), probably because of the anatomical and physiological characteristics of the avian respiratory system as compared to mammals and humans. These characteristics include the high average body temperature (38-45°C) favorable for the thermophilic fungus (Tell, 2005), the absence of an epiglottis, which otherwise prevents particles from reaching the lower respiratory tract, the lack of a diaphragm disabling a strong cough reflex, the limited distribution of ciliated epithelium through the respiratory tract (Tell, 2005), a greater respiratory surface area and a thinner air-blood capillary barrier (Maina, 2002), and the presence of the air sac system, which widely extends throughout most of the body. These warm and oxygenated air sacs provide a favorable condition for the vegetative growth and even sporulation of *Aspergillus* (Tell, 2005). In addition, the unidirectional airflow in the lungs and the bidirectional airflow in the air sacs hinder the elimination of inhaled particles (Toth, 2000) (Figure 13). The paucity of free respiratory macrophages in the avian respiratory system (Ficken et al., 1986; Holt, 1979; Maina and Cowley, 1998; Nganpicp and Maina, 2002) is also assumed to obstruct the respiratory immunity against respiratory pathogens (Toth and Siegel, 1986), but this might be compensated by the phagocytic epithelial cells in the atria and

infundibula, the pulmonary intravascular macrophages, and subepithelial macrophages (Maina, 2002; Nganpiep and Maina, 2002; Reese et al., 2006), which can be efficiently translocated to the epithelial surface (Kiama et al.. 2008; Mutua et al., 2011).
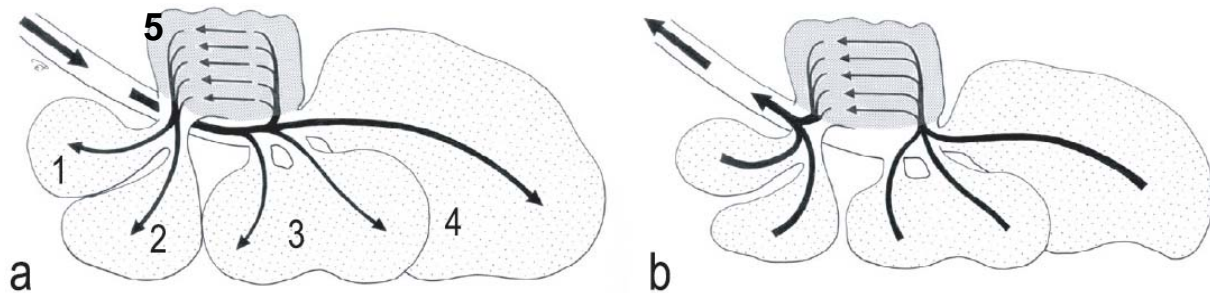

Figure 13. The pathway of air through the avian respiratory system during inspiration (a) and expiration (b). 1: Clavicular air sac, 2: Cranial thoracic air sac, 3: Caudal thoracic air sac, 4: Abdominal air sac, 5: Lung (Reese et al., 2006).

2.2.4. Pathogenesis of aspergillosis in birds
2.2.4.1. Infection route and colonization

Due to the ubiquitous existence and small size of the airborne *A. fumigatus* conidia (Latgé, 1999), aspergillosis strikes primarily in the respiratory system by inhalation of these conidia in humans, mammals and birds (Kousha et al., 2011; Oglesbee, 1997; Tell, 2005), although *A. fumigatus* is occasionally able to infect other body sites, such as the eye or skin (Beckman et al., 1994; Hoppes et al., 2000; Suedmeyer et al., 2002; Tsai et al., 1992). Some inhaled *A. fumigatus* conidia are not trapped in the nasal cavity and trachea, and are therefore able to colonize the lungs and air sacs (Fedde, 1998), which makes the lungs and air sacs the primary sites of *Aspergillus* infection (Figure 13). The tracheal bifurcation can also be infected due to the conidia deposition in the narrow lumen (Xavier, 2008). The consequences of colonization of *A. fumigatus* conidia ultimately depend on the interaction between the host immune system and the fungus (Ben-Ami et al., 2010).

2.2.4.2. Tissue invasion in the lung and respiratory tract

The *A. fumigatus* conidia which colonize the lung get embedded in the atria and parts of the infundibula in the parabronchus, and are first attacked by the phagocytic epithelial cells, subepithelial macrophages, and intravascular macrophages (Fedde, 1998; Maina, 2002; Nganpiep and Maina, 2002; Reese et al., 2006). If the conidia overwhelm the immune defense and reach a favorable environment, they break dormancy and start germinating by mitotic divisions (Momany and Taylor, 2000; Oglesbee, 1997). The high body temperature and the avian lung-air sacs system provide a warm, oxygen and nutrient rich environment ideal for

fungal growth (Tell, 2005). Germination switches the fungal morphotype from unicellular conidia to multicellular hyphae, which extend and enable tissue invasion (Ben-Ami et al., 2010). As the hyphae invade, tissues necrotize and plaques are formed in the lung and respiratory tract and obstruct the trachea or bronchi or fill an air sac (Oglesbee, 1997). Occasionally, sporulation occurs in the lungs and air sacs (Cacciuttolo et al., 2009; Nardoni et al., 2006).

2.2.4.3. Dissemination of infection

Dissemination of infection can occur in blood vessels and other organs. Hyphae are tissue- and angio-invasive (Dahlhausen et al., 2004), and mycosis can be disseminated by hyphal extension through the air sacs and lung tissues, causing serositis and superficial necrosis in the liver, kidney, intestine and ovary (Tsai et al., 1992). Vascular invasion also occurs by hematogenous spreading (Dahlhausen et al., 2004). Conidia can get attached to erythrocytes (Richard and Thurston, 1983) or ingested by respiratory macrophages and then carried by the blood and lymphatic stream to other organs (Richard and Thurston, 1983; Thompson and Patterson. 2008; van Veen, 1999).

2.3. Avian immune response to aspergillosis

2.3.1. Tissue response against *A. fumigatus*

Tissue reactions against *A. fumigatus* infections in birds can be granulomatous and/or infiltrative. The granulomatous form is characterized by a necrotic center containing hyphae and/or heterophils surrounded by abundant inflammatory cells including giant cells, macrophages and lymphocytes, and encapsulated by an outer layer of fibrous connective tissue. Neither exudative inflammation nor vascular lesions are seen in the neighboring tissues (Beernaert et al., 2010). The infiltrative type induces an exudative cellular inflammation with giant cells, macrophages, heterophils and lymphocytes. In this type, the fungus frequently invades blood vessels and forms aggregates of radiating hyphae containing a large number of conidiophores and conidia without forming structured granuloma (Beernaert et al., 2010).

2.3.2. Cellular and humoral immunity against *A. fumigatus*

Both cellular and humoral immunity are involved in the avian immune response against aspergillosis. Macrophages and heterophils play the primary role in phagocytosing the invading *A. fumigatus* conidia and hyphae (Arné et al., 2011), followed by antibody reactions for adaptive immunity (Richard et al., 1981).

2.3.2.1. Avian respiratory macrophages

The avian respiratory macrophages form the early immune defense against *A. fumigatus* infection in birds (Arné et al., 2011). Birds lack free respiratory macrophages in the respiratory system (Ficken et al., 1986; Holt, 1979; Maina and Cowley, 1998; Nganpiep and Maina, 2002). Instead, avian respiratory macrophages are present in the epithelia and the subepithelial interatrial septa of the atria and infundibula (Figure 14), and can be reinforced by the pulmonary intravascular macrophages (Klika et al., 1996; Maina, 2002; Maina and Cowley, 1998; Reese et al., 2006). Avian macrophages can transmigrate from the epithelia and the interatrial areas or the vascular system into the air surface (Kiama et al., 2008; Maina, 2002; Mutua et al., 2011; Nganpiep and Maina, 2002), and play an important role in the removal of particles or pathogens from the air (Nganpiep and Maina, 2002; Reese et al., 2006).
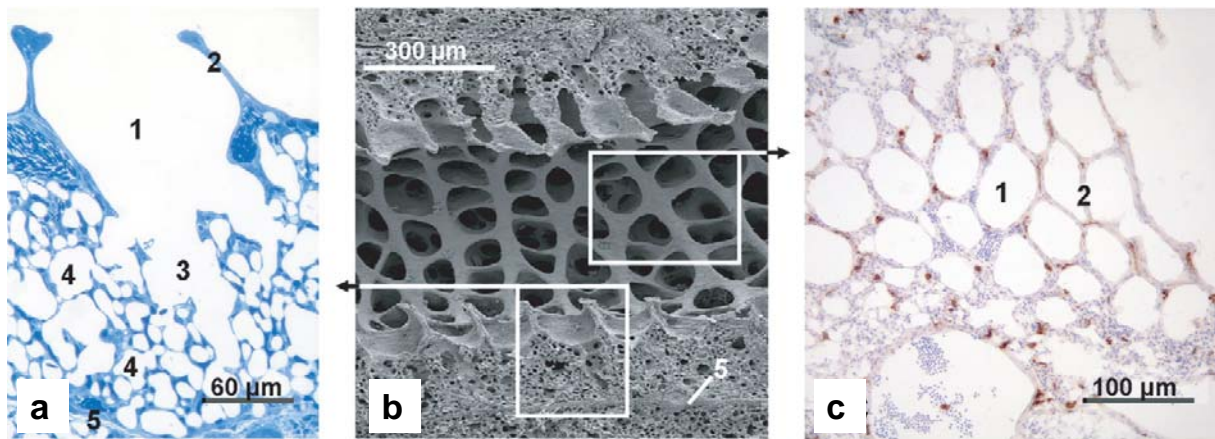

Figure 14. Avian respiratory macrophages. (a) Longitudinal section (methylene blue staining) of the parabronchus wall of a chicken. 1: Atrium; 2: Interatrial septum; 3: Infundibulum; 4: Air capillaries; 5: Interparabronchial septum. (b) Scanning electron micrograph of a parabronchus of a chicken cut in longitudinal section. (c) Horizontal section of the atria demonstrating macrophages in the interatrial septa by immunohistochemistry (stained with mab Kul01) (Reese et al., 2006).

2.3.2.2. Pathogen recognition and phagocytosis

As known in humans and other mammals, *A. fumigatus* conidia are recognized by immune cells via two kinds of pattern recognition receptors (PRRs), namely the soluble PRRs (e.g., lung surfactant proteins A and D, pentraxin-3, mannan-binding lectin) and cell-bound PRRs (e.g., dectin-l receptor and toll like receptors (TLRs)) (Ben-Ami et al., 2010; Park and Mehrad, 2009). Soluble PRRs work as opsonins for many microorganisms, and opsonization of the *A. fumigatus* conidia helps their binding to and phagocytosis by alveolar macrophages, neutrophils and dendritic cells (Park and Mehrad, 2009). The cell-bound PRRs are found on polymorphonuclear leucocytes, alveolar macrophages and dendritic cells (Brown and Gordon, 2001), and are believed to bind the polysaccharide components (e.g., β-glucan, mannan, chitin

and galactomannan) of the cell walls of swollen conidia (Hohl et al., 2005; Latgé et al., 2005), and facilitate recognition, binding and phagocytosis of these conidia (Ben-Ami et al., 2010; Park and Mehrad, 2009). Mannan-binding lectin, lung surfactant proteins, and different TLRs have been found in birds (Hughes, 2007; Juul-Madsen et al., 2008; Zeng et al., 1998), but their exact role in recognition of *Aspergillus* conidia is still to be studied. During phagocytosis by avian macrophages, particles are ingested to form phagosomes, which then fuse with lysosomes to form a phagolysosome. The antimicrobial enzymes in the lysosomes, such as acid phosphatase and β-glucuronidase, are responsible for the digestion and destruction of the ingested particles (Kaspers et al., 2008). Reactive oxygen and nitrogen intermediates are important microbiocidal mechanisms of avian macrophages (Kaspers et al., 2008).

2.3.2.3. Recruitment of leukocytes to the infection site

The avian immune response is regulated by cytokines, which can be produced by virtually every cell type, and chemokines are a group of cytokines which regulate leukocyte traffic. The uptake and destruction of fungal pathogens by phagocytes are accompanied by the secretion of signaling molecules, such as cytokines and chemokines, to activate additional arms of the immune system (Kaspers et al., 2008). During aspergillosis in humans and mice, release of IL-10, IL-15, TGF-β1, TNF-α, IFN-γ, IL-18, IL-1β, IL-12, MIP-1α, MIP-2, and MCP-1 was induced (Brieland et al., 2001; Sambatakou et al., 2006; Schelenz et al., 1999). Recruitment of leukocytes (e.g. macrophages, heterophils, and dendritic cells) to the infection site is primarily mediated by the interaction between the circulating leukocytes and the chemokines released from the infection site (Kaiser and Stäheli, 2008). In mammals, neutrophils are not only responsible for hyphal killing, but also conidial killing (Bonnet et al., 2006; Levitz and Farrel, 1990; Kozel et al., 1989; Zarember et al.. 2007). Neutrophils kill fungi using reactive oxidative intermediary agents (Latgé, 1999), and neutrophil extracellular traps (NETs) may form to handle larger fungal amounts, including tissue-invading hyphae (Bruns et al., 2010). The heterophil is the avian equivalent to the mammal neutrophil. Instead of the oxidative mechanisms used in neutrophils, heterophils use cationic proteins, hydrolases, and lysosymes to kill fungal hyphae (Tell, 2005), but more research is needed to elucidate the fungal killing mechanisms in avian heterophils.

2.3.2.4. Adaptive immunity activation

Antigen presentation in birds is primarily carried out by dendritic cells, but also by macrophages and other immune cells (Kaspers et al., 2008). In mammals, after the antigen-

presenting cells (APCs) have phagocytosed and processed the pathogens, they are drained to the lymph nodes, where they interact with and activate T helper cells. In birds, they migrate to the spleen instead (Gallego et al., 1997; Kaspers et al., 2008). In the Th1/Th2 paradigm for mammals, APCs present the processed antigen fragment to T helper cells, which then differentiate into T helper cells Type 1 (Th1) or Type 2 (Th2). Th1 in return promotes cellular immunity by maximizing the killing efficacy of the macrophages, while Th2 activates the development of B cells into plasma cells, which produce antibodies for the original antigens (humoral immunity). However, the avian adaptive immune response against aspergillosis is poorly known (Kaspers et al., 2008). A study on the humoral response of pigeons to *A. fumigatus* antigens showed an early rise of IgM and a later rise of IgG upon injection of *A. fumigatus* culture filtrate (Martinez-Quesada et al., 1993), and serum *Aspergillus* antibody levels can be used to assist the diagnosis of aspergillosis in birds (Brown and Redig, 1994; Davidow et al., 1997; Jones and Orosz, 2000; Redig, 1994).

2.4. Clinical symptoms of aspergillosis in birds

Clinical manifestations of avian aspergillosis depend on the infection dose, the pathogen distribution, pre-existing disease, and the immune response of the host (Dahlhausen et al., 2004). Avian aspergillosis is distinguished into the acute form and chronic form (Beernaert et al., 2010). The acute form is thought to be caused by exposure to an overwhelming number of *Aspergillus* conidia (Vanderheyden, 1993). The acute signs include dyspnea, anorexia, tail bobbing, open mouth breathing and gasping. Potential general signs are acute depression, inappetence, vomiting, crop stasis, ascites, polydipsia, polyuria and cyanosis. Death usually occurs within 7 days (Jenkins, 1991; McMillan and Petrak, 1989; Oglesbee, 1997; Vanderheyden, 1993). The chronic form is generally associated with immune suppression as a localized or disseminated disease (McMillan and Petrak, 1989). The chronic signs include decreased appetite, lethargy, weight loss, change or loss of voice, cough, open beak breathing, cyanosis, polyuria, depression and vomiting (Jenkins, 1991; McMillan and Petrak, 1989; Vanderheyden, 1993). Lesions frequently observed in lungs include pulmonary hypertension, congestion and fibrosis (Hofle et al., 2001; Julian and Goryo, 1990). Dissemination of aspergillosis can cause lesions in other organs such as liver and kidney, as well as neurological abnormalities (Dahlhausen, 2006; Jensen et al., 1997). Slow reaction, unilateral wing drooping, paralysis, ataxia, weakness or general disinclination to move, unsteady gait, falling on the side or back, torticollis and tremors are potential neurological signs caused by *Aspergillus* infection (Forbes, 1991; Forbes, 1992; Jensen et al., 1997).

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Scientific Aims

Mycotoxins are toxic secondary metabolites produced by fungal genera such as *Alternaria*, *Penicillium*, *Aspergillus* and *Fusarium*. As feed contaminants, dietary mycotoxins are known to impair animal health and predispose animals to infectious diseases. One of the most prevalent avian respiratory diseases, notably in psittacine birds but also in poultry, is aspergillosis, which is mainly caused by *Aspergillus fumigatus*. On the one hand, susceptibility to aspergillosis may be promoted by mycotoxin contamination of the avian diet through suppression of avian defence mechanisms. On the other hand, environmental mycotoxins may also suppress the fitness of *A. fumigatus* and thus reduce its impact on avian health. Exposure of birds and/or *A. fumigatus* to mycotoxins may thus alter their susceptibility to aspergillosis.

The general aim of this dissertation was to examine the interactions of mycotoxins in avian feed with *A. fumigatus* infections in birds.

Because information with regard to mycotoxin contamination of psittacine feeds is largely lacking, a first specific aim was to examine the occurrence of mycotoxins in commercial pet parrot feeds, and their potential pathological effects using cockatiels as model birds.

The second specific aim was to study the impact of mycotoxins on the antifungal activity of the avian macrophage, an important first line of defense against aspergillosis. T-2 toxin was selected as a model mycotoxin and chickens as model birds. T-2 toxin is a potent trichothecene, which has previously been shown to influence the pathogenesis of bacterial infections such as *Salmonella* infections in pigs.

The third specific aim was to assess the influence of environmental T-2 toxin on the virulence of *A. fumigatus* and the influence of dietary T-2 toxin on the susceptibility of birds to *A. fumigatus*, using the chicken as an animal model.

Experimental Studies

Experimental Study 1:

Occurrence and Pathology of Mycotoxins in Commercial Parrot Feeds

Experimental Study 2:

T-2 Toxin Impairs Antifungal Activities of Chicken Macrophages against *Aspergillus fumigatus* Conidia but Promotes the Pro-Inflammatory Responses

Experimental Study 3:

Exposure of *Aspergillus fumigatus* to T-2 Toxin Results in a Stress Response Associated with Exacerbation of Aspergillosis in Poultry

Experimental Study 1

Occurrence and Pathology of Mycotoxins in Commercial Parrot Feeds

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Experimental Study 1

Abstract

Mycotoxins are toxic secondary metabolites of fungi. Animal feeds can be easily infected by fungi during production and storage, resulting in mycotoxin contamination. This study was performed to evaluate the possible health risks of mycotoxin-contaminated feed for cockatiels. The occurrence of mycotoxins in commercial parrot feeds (5 seed mixes and 5 pelleted feeds) was investigated by liquid chromatography tandem mass spectrometry. The following 12 mycotoxins were detected: zearalenone, deoxynivalenol, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, fusarenon-X, aflatoxin B1, sterigmatocystin, alternariol, alternariol methylether, fumonisin B1, fumonisin B3, and ochratoxin A. Zearalenone was the most prevalent. Pathological effects after 21 days feeding mycotoxin-contaminated diets were examined in an *in vivo* trial with 3 groups of 5 cockatiels: Group 1 (control) was fed a non-contaminated extruded feed; Group 2 was fed an extruded feed containing zearalenone, deoxynivalenol, 15-acetyldeoxynivalenol, and fumonisins; and Group 3 was fed an extruded feed containing fumonisins. Average body weight gain and relative organ weight were not significantly different between the treatment groups and the control group. Apoptosis of renal tubular cells, diarrhoea, reduced appetite, enlargement of liver, kidney and proventriculus were occasionally observed in the birds from Groups 2 and 3. In summary, contamination with mycotoxins is common in parrot feeds. The mycotoxin levels did not reach toxic levels, but might pose a potential threat to some sensitive cockatiels.

1. Introduction

Mycotoxins are toxic secondary metabolites produced by fungi. They pose potential threats to human and animal health through the ingestion of contaminated agricultural products such as wheat, maize, nut, and rye. Mycotoxins can be oestrogenic, carcinogenic, neurotoxic, immunosuppressive or can exhibit other toxic effects on organs such as liver and kidney (Turner et al., 2009). Trichothecenes, aflatoxins, fumonisins, ochratoxins, *Alternaria* toxins, and zearalenone (ZEA) are the most well-known mycotoxins; they are widely investigated due to their frequent occurrence and severe effects on animal and human health (Logrieco et al., 2003; Njumbe Ediage et al., 2011). For animal feed, maximum concentrations of aflatoxin B1 are officially regulated (EC, 2003), and those of deoxynivalenol (DON), ZEA, ochratoxin A (OTA), and fumonisins are recommended (EC, 2006) in the European Union. Several studies on mycotoxins in bird feeds only focused on aflatoxins, OTA, fumonisins and DON (Henke et al., 2001; Maia and Pereira Bastos de Siqueira, 2002; Martins et al., 2003; Scudamore et al., 1997) and, therefore, a more comprehensive study on mycotoxins in pet bird feeds and their pathological effects is necessary.

This study includes an investigation on the occurrence of different mycotoxins, i.e. trichothecenes, aflatoxins, fumonisins, ochratoxins, *Alternaria* toxins, and zearalenone, in commercial parrot feeds and a subsequent *in vivo* trial to determine pathological effects on pet cockatiels fed extruded feed either contaminated with mycotoxins or uncontaminated.

2. Materials and methods

2.1. Parrot feeds

10 commercial parrot feeds, either pelleted feed or seed mixes, were bought from local stores in Flanders, Belgium.

2.2. Mycotoxin analysis by LC-MS/MS

The occurrence of the following 21 mycotoxins in the parrot feeds was analysed by liquid chromatography tandem mass spectrometry (LC-MS/MS) as previously described (Monbaliu et al., 2010): ZEA, DON, nivalenol, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol (15-ADON), T-2 and HT-2 toxins, fusarenon-X, neosolaniol, diacetoxyscirpenol, aflatoxins B1, B2, G1 and G2, sterigmatocystin, OTA, fumonisins B1 and B3, alternariol, alternariol methylether, and altenuene. Each time an analysis was performed, a

sample spiked at the cut-off value or the third point of the calibration curve was (re)injected at the end of the analytical run to control the performance (sensitivity) of the LC-MS/MS instrumentation. Matrix-matched calibration plots were constructed by applying the least-squares method and by plotting the response (peak area of toxin/peak area of internal standard) against the spiked concentration of the sample. The limit of detection (LOD) and the limit of quantification (LOQ) were calculated as 3 and 6 times, respectively, the standard error of the intercept divided by the slope of the calibration curve.

2.3. *In vivo* trial with cockatiels

An *in vivo* trial was performed to examine the possible toxic effects of the contaminated feeds on the health of cockatiels (*Nymphicus hollandicus*). This trial was carried out following the guidelines of the Belgian animal welfare regulations regarding animal experiments (ethical committee of the Faculty of Veterinary Medicine, Ghent University). Due to the limited possibility of using these exotic birds, 15 pet cockatiels were selected. These cockatiels, 5- to 6-weeks-old and clinically healthy, were purchased from a local store and randomly divided into 3 groups, with 5 birds in each group and each bird individually housed. Group 1 was fed an uncontaminated extruded feed and served as a control, whereas Groups 2 and 3 were fed extruded feed contaminated with different mycotoxins. The average initial body weights of Groups 1, 2, and 3 were 82, 85 and 80 g, respectively. Feed and water were given *ad libitum*. Feed intake was monitored and each bird was weighed weekly.

2.4. Histological examination

After 21 days of feeding, all birds were euthanized and necropsied, and the liver, gizzard, spleen, heart, kidney, lung, proventriculus, pancreas and bursa of Fabricius of each bird were weighed. Organ samples were fixed in 10% neutral buffered formalin and embedded in paraffin. Five μm sections of the paraffined organ samples were stained with haematoxylin and eosin (H & E) and then examined microscopically.

2.5. Statistical analysis

Differences in body weight gain and relative organ weight among the groups were assessed by performing ANOVA after determination of normality and variance homogeneity. The significance level was set at $P<0.05$.

3. Results and discussion

The analytical results of mycotoxins in parrot feeds are summarised in Table 1. Nine feeds were found to contain mycotoxins, but none of the detected mycotoxins exceeded the maximum levels regulated or recommended by the European Commission (EC, 2003; EC 2006). ZEA was present in nine out of ten feeds. ZEA is one of the most frequently occurring mycotoxins in cereal grains (De Smet, 2011). DON and 15-ADON were detected in 4 feeds. Interestingly, 15-ADON was detected in two feeds (Feeds 1 and 5), in which DON was not found. With some exceptions, DON mostly occurs at a higher level than acetyldeoxynivalenol (Monbaliu et al., 2010). OTA, aflatoxins and fumonisins were present in one, one and three feeds, respectively, in the present study. Scudamore et al. (1997) have found these mycotoxins in one, five and two out of thirty bird feed samples, respectively. In addition, aflatoxins were detected in 17% of seed samples for wild birds (Henke et al., 2001) and in 27% of pet bird feed samples (Maia and Pereira Bastos de Siqueira, 2002). *Alternaria* toxins were detected in four feeds, but mostly unquantifiable. *Alternaria* toxins have been detected in grains, sunflower seeds, and other raw and processed feed products (Monbaliu, 2011), but their specific occurrence in bird feed has not been reported before.

Feed 1 did not contain any mycotoxins at quantifiable concentrations. Feed 2 contained DON, 15-ADON, fumonisins and ZEA, while Feed 3 was mainly contaminated with fumonisins (Table 1). These three pelleted feeds, which were all extruded diets and had similar energy contents (as described by the manufacturers), were used in the *in vivo* trial.

In the *in vivo* trial, body weight gain as percentage of initial weight (Table 2) and relative organ weight as percentage of body weight (Table 3) of Groups 2 and 3 that had received mycotoxin contaminated feed (Feeds 2 and 3, respectively) did not differ significantly from the control group. The mycotoxins in Feed 2 and 3 did not seem to reach any toxic levels; the concentrations of fumonisins and DON were lower than the reported toxic levels for turkey and chickens (Ledoux et al., 1996; Weibking et al., 1993). Pathological changes were occasionally observed in cockatiels exposed to mycotoxin contaminated feeds, e.g. anisokaryosis in hepatocytes in the enlarged liver of one bird in Group 2, apoptosis of renal tubular cells, mild urate and mucin retention in the enlarged kidney, and severe secretion retention in the glands of the enlarged proventriculus of one bird in Group 3. One bird in Group 2 and three birds in Group 3 showed apoptosis of renal tubular cells (Table 3), which was previously reported to be induced by higher doses of dietary fumonisin B_1 in rats (Tolleson et al., 1996). Diarrhoea was observed in one bird in Group 3. One bird in Group 3

Experimental Study 1

was found dead during the trial with little feed in its crop found by necropsy, which might suggest a reduced appetite.

Trichothecenes, fumonisins and ZEA have been reported to cause organ enlargement, cell apoptosis, diarrhoea, and reduced appetite in other avian species (EFSA, 2004; EFSA, 2005; Ledoux et al., 1992; Ribeiro et al., 2010; Zhu et al., 2011). Due to the lack of significant differences from the control in the present study, it is difficult to conclude whether the rather low levels of mycotoxins in the feeds are responsible for the occasionally observed pathological changes.

Table 1. Occurrence of mycotoxins (µg/kg) in 10 commercial parrot feeds.

Mycotoxins[1]	LOD[2]	LOQ[2]	Pelleted feed					Mixed seeds					Occurrence frequency (n out of 10)
			1	2	3	4	5	6	7	8	9	10	
ZEA	35.99	65.31	NQ[3]	105	NQ	ND	80	NQ	202	127	462	105	9
15-ADON	5.29	11.24	NQ	23	NQ	ND	30	ND	ND	ND	ND	ND	4
FB1	64.34	116.5	NQ	708	1187	ND	ND	ND	ND	ND	ND	ND	3
AOH	23.23	43.84	ND[4]	ND	ND	ND	ND	ND	NQ	ND	NQ	161	3
DON	128.29	221.7	ND	326	NQ	ND	ND	ND	ND	ND	ND	ND	2
FB3	42.67	84.81	ND	88	183	ND	ND	ND	ND	ND	ND	ND	2
AME	39.00	64.96	ND	NQ	ND	ND	ND	ND	ND	ND	ND	NQ	2
Fus-X	34.62	60.68	ND	ND	ND	ND	ND	ND	ND	ND	NQ	NQ	2
OTA	8.93	12.6	ND	ND	ND	ND	ND	ND	ND	ND	ND	13	1
AFB1	3.20	6.41	ND	ND	NQ	ND	ND	ND	ND	ND	ND	ND	1
ST	8.99	17.38	NQ	ND	ND	ND	ND	ND	ND	ND	ND	ND	1
3-ADON	9.80	17.91	ND	ND	ND	ND	NQ	ND	ND	ND	ND	ND	1
DAS	1.36	2.45	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0
NIV	71.41	132.5	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0
Neo	17.53	31.45	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0
AFG2	4.79	8.74	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0
AFG1	4.19	7.07	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0
AFB2	2.57	5.59	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0
ALT	8.89	16.63	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0
HT-2	19.58	33.79	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0
T-2	19.47	34.31	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	0

[1] ZEA=zearalenone; 15-ADON=15-acetyldeoxynivalenol; FB1=fumonisin B1; AOH= alternariol; DON=deoxynivalenol; FB3=fumonisin B3; AME=alternariol methylether; Fus-X= fusarenon-X; OTA=ochratoxin A; AFB1=aflatoxin B1; ST=sterigmatocystin; 3-ADON=3-acetyldeoxynivalenol; DAS=diacetoxyscirpenol; NIV=nivalenol; Neo=neosolaniol; AFG2=aflatoxin G2; AFG1=aflatoxin G1; aflatoxin B2=aflatoxin B2; ALT=altenuene; HT-2 =HT-2 toxin; T-2=T-2 toxin. [2] LOD=limit of detection (µg/kg); LOQ = limit of quantification (µg/kg). [3] NQ=not quantifiable (<LOQ). [4] ND=not detected (<LOD).

Table 2. Mean body weight gain of cockatiels fed a commercial diet containing DON, 15-ADON, ZEA and fumonisins (Feed 2) or a diet containing fumonisins (Feed 3).

	Group 1 (Feed 1; control)	Group 2 (Feed 2)		Group 3 (Feed 3)	
	Weight gain[1]	Weight gain[1]	P-value	Weight gain[1]	P-value
Day 8	12.46±7.31	14.02±10.56	0.79	-0.04±18.34	0.19
Day 15	29.03±8.28	32.49±12.87	0.64	21.35±18.17	0.42
Day 21	35.02±5.69	31.53±16.09	0.66	21.94±23.25	0.26

[1] % of initial weight ± standard deviation.

Table 3. Mean relative organ weight of cockatiels fed a commercial diet containing DON, 15-ADON, ZEA and fumonisins (Feed 2) or a diet containing fumonisins (Feed 3).

	Group 1 (Feed 1; control)	Group 2 (Feed 2)		Group 3 (Feed 3)	
	Weight[1]	Weight[1]	P-value	Weight[1]	P-value
Liver	2.12±0.27	2.07±0.60	0.87	2.08±0.21	0.81
Gizzard	1.29±0.32	1.01±0.15	0.12	1.61±0.42	0.22
Spleen	0.05±0.02	0.07±0.03	0.18	0.06±0.02	0.64
Heart	1.46±0.12	1.56±0.22	0.38	1.61±0.25	0.27
Kidney[2]	0.73±0.08 (0/5)	0.66±0.03 (1/5)	0.08	1.03±0.86 (3/5)	0.47
Lung	1.59±0.14	1.60±0.17	0.94	1.50±0.26	0.51
Proventriculus	0.39±0.04	0.37±0.09	0.74	0.43±0.20	0.68
Pancreas	0.27±0.03	0.25±0.06	0.49	0.29±0.05	0.56
Bursa	0.06±0.02	0.06±0.02	0.88	0.05±0.01	0.46

[1] % of body weight ± standard deviation.
[2] Number of birds with apoptosis of renal tubules shown between brackets.

4. Conclusions

Contamination with low levels of mycotoxins is common in commercial parrot feeds. Although these mycotoxins did not reach toxic levels, they might pose a potential threat to the health of some sensitive cockatiels.

Experimental Study 1

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Experimental Study 2

T-2 Toxin Impairs Antifungal Activities of Chicken Macrophages against *Aspergillus fumigatus* Conidia but Promotes the Pro-inflammatory Responses

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Experimental Study 2

Abstract

Aspergillosis is the most common fungal disease of the avian respiratory tract and is caused primarily by *Aspergillus fumigatus*. The respiratory macrophages provide important defense against aspergillosis. T-2 toxin (T-2), a trichothecene mycotoxin produced by *Fusarium* spp. in the field and in improperly stored agricultural products, has immunomodulatory effects. We studied the impact of T-2 on the antifungal response of the chicken macrophage cell line HD-11 against *A. fumigatus* infection. The macrophages were first exposed to 0.5 to 10 ng/ml T-2 for 24 h, and then their viability, antifungal activity, and cytokine expression in response to *A. fumigatus* conidial infection were determined. The viability of macrophages decreased when exposed to T-2 at concentrations higher than 1 ng/ml. One hour after conidial infection, phagocytosed conidia were observed in 30% of the non-T-2-exposed macrophages, but in only 5% of the macrophages exposed to 5 ng/ml T-2. Seven hour after infection, 24% of the conidia associated with non-T-2-exposed macrophages germinated, in contrast to 75% of those with macrophages exposed to 5 ng/ml T-2. *A. fumigatus* infection induced upregulation of interleukin (IL)-1β, CXCLi1, CXCLi2 and IL-12β, and downregulation of TGF-β4 in macrophages. Exposure of *A. fumigatus*-infected macrophages to T-2 at 1 to 5 ng/ml further upregulated the expression of IL-1β, IL-6, CCLi2, CXCLi1, CXCLi2, IL-18 (at 1 and 2 ng/ml) and IL-12β, and further downregulated that of transforming growth factor-β4 (at 5 ng/ml). In conclusion, T-2 impaired the antifungal activities of chicken macrophages against *A. fumigatus* conidia, but might stimulate immune response by upregulating the expression of pro-inflammatory cytokines, chemokines and T-helper 1 cytokines.

1. Introduction

Aspergillosis is the most common fungal disease of the avian respiratory tract and is caused primarily by *Aspergillus fumigatus* (Beernaert et al., 2009). *A. fumigatus* produces airborne conidia in huge amounts into the environment, and these conidia are easily inhaled by a bird and then colonize the lower respiratory tract (Bain et al., 2007; Tell, 2005). As the first line of defense against the inhaled conidia, the respiratory macrophages are responsible for phagocytosing and killing the inhaled fungal conidia (Luther et al., 2008; Dagenais and Keller, 2009; Van Waeyenberghe et al. 2012). The development of aspergillosis depends on the number of conidia inhaled and the host immune status (Alley et al., 1999; Beernaert et al., 2010).

T-2 toxin (T-2) is a trichothecene mycotoxin produced by *Fusarium* spp. and is usually detected in the field and in improperly stored agricultural products such as maize, wheat, barley, oat and rye, and the detected concentrations can reach up to thousands of µg/kg (EFSA, 2011). This mycotoxin is reported to influence the immune system with a time- and dose- dependent mode of action. Specifically, T-2 induces immunosuppression at high doses and immunostimulation at low doses in poultry (Sokolović et al., 2008), despite a short half-life of elimination in broiler chicken plasma (Osselaere et al., 2013). The physiological concentrations of T-2 in the respiratory tract of chickens have never been reported in literatures.

Treatments of mammalian macrophages with T-2 ranging from 0.47 to 46.7 ng/ml in earlier studies showed suppressed phagocytosis of bacterial and fungal pathogens by mammalian macrophages (Gerberick et al., 1984; Vidal and Mavet, 1989). On the other hand, treatments with T-2 up to 10 ng/ml did not affect the viability or microscopic morphology of *A. fumigatus* K24 (unpublished data). However, there is no information about the effect of T-2 on the antifungal activities of avian macrophages against *A. fumigatus* conidia. We hypothesize that T-2 modulates the interaction of avian macrophages with *A. fumigatus* conidia, thus influencing the course of infection.

The inflammatory response against pathogens involves the regulation of multiple cytokines. Transcription levels of pro-inflammatory cytokines interleukin (IL)-1β, IL-6, CCLi2, CXCLi1, CXCLi2, IL-12β, IL-18, as well as anti-inflammatory cytokine transforming growth factor (TGF)-β4 in chicken were found to be altered in bacterial and mycoplasmal infections (Hong et al., 2006; Mohammed et al., 2007; Beeckman et al., 2010),

but the regulation of these cytokines in *A. fumigatus* infection, whether or not exposed to T-2, has never been studied before.

The chicken HD-11 cell line is a macrophage-like cell line transformed with avian myelocytomatosis virus (MC29). The HD-11 cell line demonstrates the properties of primary macrophage, such as phagocytic capacity, cell surface antigens, and Fc receptor expression (Beug et al., 1979), despite its nitric oxide production being reported to be different from that of chicken primary peripheral blood leukocyte-derived macrophages upon stimulation (Lillehoj and Li, 2004). Chicken HD-11 cell line has been used extensively as an *in vitro* model to study pathogenic interactions with chicken macrophages, including those in the respiratory system (Beeckman et al., 2010; Hartley et al., 2012; Lyon and Hinshaw, 1991).

The objective of this study was to evaluate the effects of T-2 on the antifungal responses of HD-11 cells against *A. fumigatus* conidial infection. For this purpose, we examined the effect of exposure of HD-11 cells to T-2 on their antifungal activity and cytokine expression in response to *A. fumigatus* conidial infection.

2. Material and methods

2.1. *Aspergillus fumigatus* conidia

The *A. fumigatus* isolate, K24, used in this study was obtained from a racing pigeon, which died from pulmonary aspergillosis (Beernaert et al., 2008). Five day old cultures of this isolate on Sabouraud dextrose agar (CM0041, Oxoid Ltd., Basingstoke, England) were washed with 5 ml of 0.01% Tween 20 in Dulbecco's Modified Eagle Medium (DMEM) to harvest *A. fumigatus* conidia. The conidia were washed three times in DMEM containing 0.01% Tween 20 and the suspension was adjusted by haemocytometer count to the desired concentrations in DMEM supplemented with 10% fetal bovine serum, 1% glutamine and 1% pyruvate (DMEM+).

2.2. Macrophage cell line

The avian MC29 virus-transformed macrophage cell line from chicken, HD-11 (Beug *et al*, 1979) was maintained in DMEM containing 10% fetal bovine serum, 1% L-glutamine, 1% penicillin/streptomycin, and 1% kanamycin and was incubated at 37°C, 5% CO_2, with passage every 3 days. For the experiments, 3 days old HD-11 cells were adjusted to the desired concentrations in DMEM+ by haemocytometer count.

2.3. Cytotoxicity of T-2 on HD-11 macrophages

The effect of T-2 on the viability of HD-11 macrophages was determined using the neutral red (3-amino-7-dimethylamino-2-methyl-phenazine hydrochloride) uptake assay as previously described (Borenfreund and Puernen, 1985). Two hundred µl of a HD-11 cell suspension of 5×10^5 cells/ml was seeded per well in a 96 well plate. After incubation at 37 °C, 5% CO_2 for 4 h, the cells in each well were exposed to 200 µl T-2 solution (Sigma-Aldrich, Steinheim, Germany) at different final concentrations (0, 0.5, 1, 2, 5 or 10 ng/ml) in DMEM+, with cells exposed to 0 ng/ml T-2 serving as negative control. After 24 h of incubation at 37°C, 5% CO_2, the medium was removed and the wells were gently rinsed with Hank's Buffered Salt Solution + Ca^{2+} and Mg^{2+} (HBSS+). The cells in each well were incubated with 200 µl of a 33 µg/ml neutral red solution for 2 h at 37°C, 5% CO_2. Subsequently, the cells were gently rinsed with HBSS+ and the neutral red dye was eluted with 200 µl elution mixture (acetic acid/ethanol/water, 1/50/49 v/v/v) by shaking at 550 rpm in a MTS 2/4 digital microtiter shaker (IKA, Germany) for 10 min. One hundred and fifty µl of the eluate from each well was transferred into a new 96 well plate (IWAKI, Japan) and absorbance at 540 nm was read on an ELISA reader. This test was performed in sextuplicate. Viability of HD-11 cells was calculated using the following formula:

$$Viability= (a-b)/(c-b) \times100\%$$

In this formula a = OD_{540} derived from a well incubated with T-2, b = OD_{540} derived from a blank well without cells, c = OD_{540} derived from a negative control well.

2.4. Phagocytosis of *A. fumigatus* conidia by T-2-exposed HD-11 macrophages

Phagocytic ability of HD-11 macrophages either or not exposed to T-2 was assessed by fluorescence microscopy as previously described (Van Waeyenberghe et al. 2012). One ml of a HD-11 cell suspension of 10^5 cells/ml was seeded per well on a glass coverslip in a 24 well plate and exposed to 1 ml T-2 solution at a final concentration of 0, 0.5, 1, 2, or 5 ng/ml respectively for 24 h as described above. The HD-11 cells in each well were then exposed to 2×10^5 K24 conidia in DMEM+ with the same concentration of T-2, and conidial exposure was synchronized by centrifugation at 1500 rpm at 37 °C for 10 min. Subsequently, the cells were allowed to phagocytose the conidia for 1 h at 37 °C, 5% CO_2. Medium containing the free conidia was removed, and wells were rinsed using HBSS+. Each well was then incubated with 1 ml of 25 µM Calcofluor White M2R (Life Technologies Europe BV, Ghent, Belgium) for 30 min at 37 °C to stain the extracellular conidia, but not the intracellular conidia. After washing with HBSS+ and fixing with 4% paraformaldehyde, the coverslip was mounted on a

microscopic slide with DABCO glycerol. The numbers of macrophages with intracellular and extracellular conidia in 100 randomly observed cells from each treatment were counted with a fluorescence microscope. This test was performed in quadruplicate.

2.5. Germination rate of *A. fumigatus* conidia associated with T-2-exposed HD-11 macrophages

One ml of a HD-11 macrophage suspension of 10^5 cells/ml was seeded per well in a 24 well plate and exposed to 1 ml T-2 at a final concentration of 0, 0.5, 1, 2 or 5 ng/ml respectively for 24 h as described above. The HD-11 cells in each well were then exposed to 2×10^5 K24 conidia in DMEM+ with the same concentration of T-2, and the conidial exposure was synchronized by centrifugation at 1500 rpm at 37 °C for 10 min. After 1 h of incubation, the free conidia were removed by washing with HBSS+, and the HD-11 cells with conidia were incubated in DMEM+ containing T-2 at the same concentrations for another 6 h. After washing with HBSS+ and fixed with 4% paraformaldehyde, the cells were covered by a glass coverslip with DABCO glycerol. One hundred macrophages with conidia in each treatment were randomly observed with a light microscope, and the germination rate of conidia was calculated by dividing the number of cell-associated conidia which had germinated by the total number of cell-associated conidia. This test was performed in quadruplicate.

2.6. Cytokine mRNA expression in HD-11 macrophages in response to *A. fumigatus* conidial infection

Cytokine mRNA expression was examined by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR), and the fold change in Cytokine Expression per Unit Amount of Macrophages (CEUAM) between a treatment and a control was then calculated by $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). First, kinetic change of cytokines expression in HD-11 cells was established during 20 h post *A. fumigatus* conidial infection. Then, to evaluate the effect of T-2 on cytokine expression in *A. fumigatus*-infected macrophages, cytokine expression was examined in *A. fumigatus*-infected HD-11 cells either or not exposed to T-2.

To establish the kinetic cytokine expression, 1 ml of a HD-11 cell suspension of 5×10^5 cells/ml was seeded per well in a 24 well plate as described above. The macrophages were then exposed to 10^6 K24 conidia in 1 ml DMEM+, and the conidial exposure was synchronized by centrifugation at 1500 rpm at 37 °C for 10 min. macrophages not exposed to conidia served as negative control. At 1, 4, 6, 12 and 20 h post conidial exposure, total RNA

from the macrophages was isolated using RNeasy Mini Kit (Qiagen), and reversely transcribed using iScript cDNA Synthesis kit (Bio-Rad Laboratories) according to the manufacturers' instructions. Information of the analyzed genes and their primers for qRT-PCR analysis is given in Table 1. GAPDH and β-actin were used as reference genes. qRT-PCR reactions were carried out using SensiMix SYBR No-ROX Kit (Bioline) in a C1000 Thermal Cycler coupled with a CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories). The cycle profile was as follows: one cycle of 95 °C for 10 min and 45 cycles of 95 °C for 10 sec and 60 °C for 30 sec. The threshold cycle values (Ct) were first normalized to the geometric means of reference mRNAs and the fold changes in CEUAM between infected macrophages and uninfected macrophages (control) were calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Table 1. Genes and sequences of the primers used for qRT-PCR analysis

Cytokine		Primer sequence (5' to 3')	Amplicon size (base pairs)	Accession number	Reference
β-actin	forward	CAACACAGTGCTGTCTGGTGG	205	X00182	Lu et al. (2009)
	reverse	ATCGTACTCCTGCTTGCTGAT			
GAPDH	forward	ATTCTACACACGGACACTTCA	153	K01458	Mohammed et al. (2007)
	reverse	CACCAGTGGACTCCACAACATA			
IL-1β	forward	TGGGCATCAAGGGCTACA	244	Y15006	Hong et al. (2006)
	reverse	TCGGGTTGGTTGGTGATG			
IL-6	forward	GCTCGCCGGCTTCGA	71	AJ309540	Beeckman et al. (2010)
	reverse	GGTAGGTCTGAAAGGCGAACAG			
TGF-β4	forward	AGGATCTGCAGTGGAAGTGGAT	137	M31160	Beeckman et al. (2010)
	reverse	CCCCGGGTTGTGTTGGT			
CCLi2	forward	GGCAGACTACTACGAGACCAACAG	70	AJ243034	Beeckman et al. (2010)
	reverse	ACGGCCCTTCCTGGTGAT			
CXCLi1	forward	TGGCTCTTCTCCTGATCTCAATG	527	AF277660	Beeckman et al. (2010)
	reverse	GCACTGGCATCGGAGTTCA			
CXCLi2	forward	GCCCTCCTCCTGGTTTCAG	923	AJ009800	Beeckman et al. (2010)
	reverse	TGGCACCGCAGCTCATT			
IL-12β	forward	TGAAGGAGTTCCCAGATGC	152	AY262752	Mohammed et al. (2007)
	reverse	CGTCTTGCTTGGCTCTTTATAG			
IL-18	forward	AGGTGAAATCTGGCAGTGGAAT	94	AJ276026	Beeckman et al. (2010)
	reverse	ACCTGGACGCTGAATGCAA			

To compare cytokine mRNA expression in response to conidial infection between HD-11 macrophages either or not exposed to T-2, the same assay was used, but the macrophages were exposed to T-2 at a final concentration of 0, 1, 2 or 5 ng/ml during 24 h prior to conidial exposure as described above. Then, the cells were inoculated with K24 conidia as described above and incubated in medium containing the respective concentration of T-2. Total mRNA was isolated at 6 h after conidial exposure. By $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001),

the fold changes in CEUAM between T-2-exposed macrophages and non-T-2-exposed macrophages (control) in response to conidial infection were calculated. Both tests were performed in triplicate.

2.7. Data analysis

The differences in cell viability, phagocytic activity, conidial germination and cytokine expression among different treatments were assessed by performing one-way ANOVA after determination of normality and variance homogeneity. Significance level was set at 0.05.

3. Results

3.1. Cytotoxicity of T-2 on HD-11 macrophages

The viability of the HD-11 macrophages significantly decreased when exposed to T-2 at concentrations higher than 1 ng/ml, but the cell viability was not affected by T-2 at 0.5 and 1 ng/ml (Figure 1). No morphological changes were noticed at 0.5 and 1 ng/ml, but the cells at T-2 higher than 5 ng/ml seemed to have shrunk somewhat.
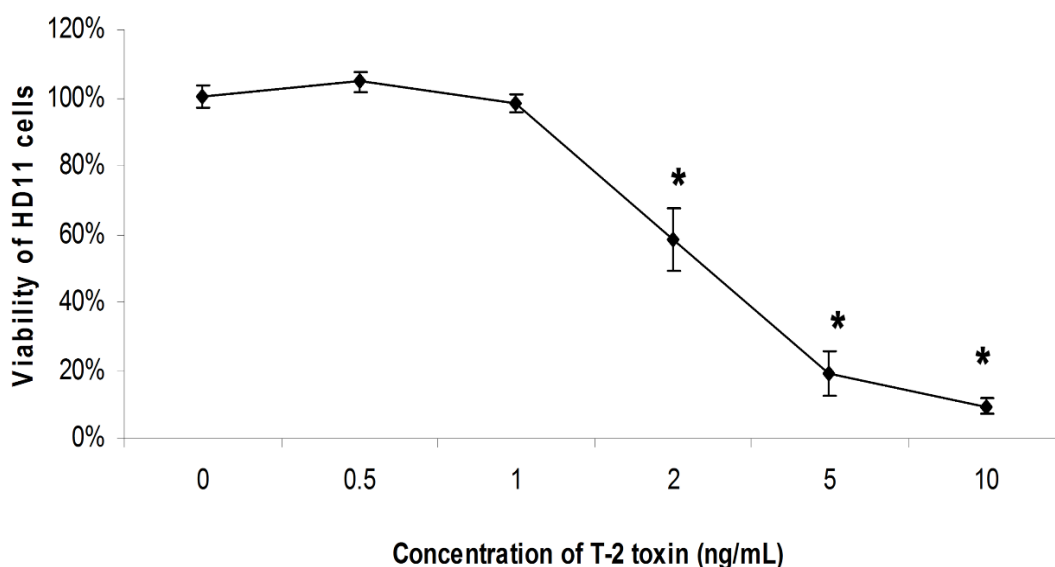
Figure 1. Viability of chicken macrophages 24 h after exposure to 0 to 10 ng/ml T-2. Results are expressed as mean ± standard deviation of 6 replicates. * Significant difference compared with the control (0 ng/ml).

3.2. Phagocytosis and germination of *A. fumigatus* in T-2-exposed HD-11 macrophages

In both phagocytosis assay and germination assay, the same number (100) of macrophages was counted in different treatments (0, 1, 2, and 5 ng/ml) to calculate the phagocytosis and germination rates of conidia. One hour after conidial exposure, phagocytosed (intracellular) and cell-associated (intracellular + extracellular) conidia were

observed in 30% and 59% of the non-T-2-exposed macrophages (0 ng/ml), respectively, while in macrophages exposed to 5 ng/ml T-2, these percentages decreased to 5% and 26% respectively (Figure 2). Six hour later, 24% of the conidia associated with non-T-2-exposed macrophages germinated, in contrast to 75% in macrophages exposed to 5 ng/ml T-2 (Figure 3). Phagocytosis and germination rate at high T-2 concentrations (2 and 5 ng/ml) were significantly lower and higher than the controls, respectively.

Figure 2. Phagocytosis of *A. fumigatus* conidia by chicken macrophages exposed to 0 to 5 ng/ml T-2 determined 1 h post conidial infection. Results are expressed as mean ± standard deviation of four replicates. * Significant difference compared with the control (0 ng/ml).
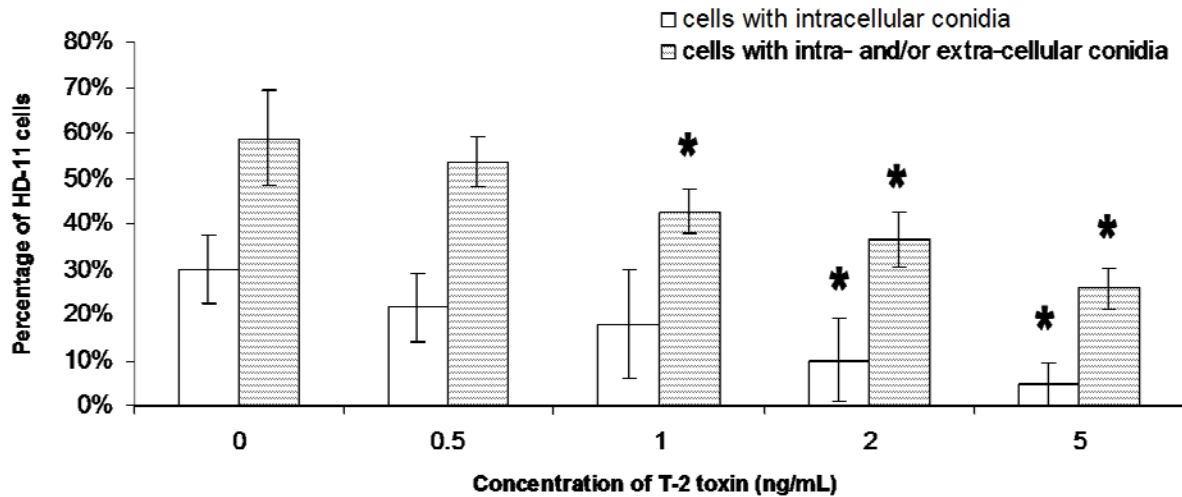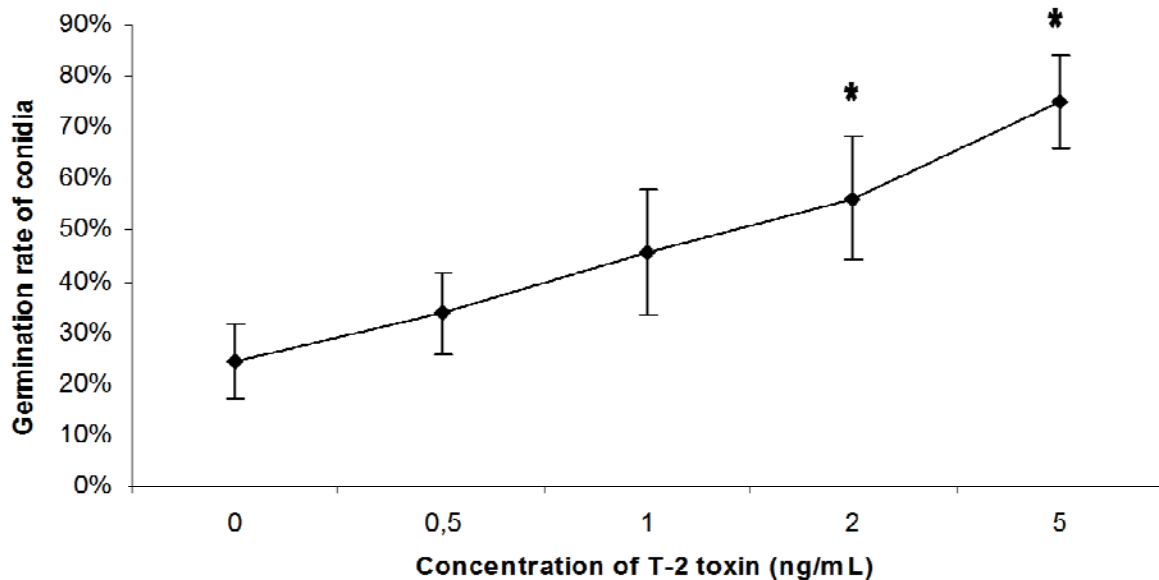

Figure 3. Germination rate of *A. fumigatus* conidia associated with chicken macrophages exposed to 0 to 5 ng/ml T-2 determined 7 h post conidial infection. Results are expressed as mean ± standard deviation of four replicates. * Significant difference compared with the control (0 ng/ml).

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Experimental Study 2

3.3. Kinetic cytokine mRNA expression in HD-11 macrophages in response to *A. fumigatus* conidial infection

Cytokine expression in *A. fumigatus*-infected HD-11 macrophages was compared to that in uninfected controls at 1, 4, 6, 12 and 20 h post infection (p.i.). Significant upregulation of IL-1β, CXCLi1, CXCLi2 and IL-12β was noticed from 6, 4, 4 and 12 h p.i., and finally increased to 248, 727, 371 and 387 folds of that in uninfected controls at 20 h p.i., respectively. Significant downregulation of TGF-β4 was noticed from 12 h p.i., and finally decreased to 0.4 fold of that in uninfected controls at 20 h p.i.. *A. fumigatus* conidial infection did not induce any significant change in IL-6 and IL-18 expression throughout the 20 h incubation period (Figure 4).
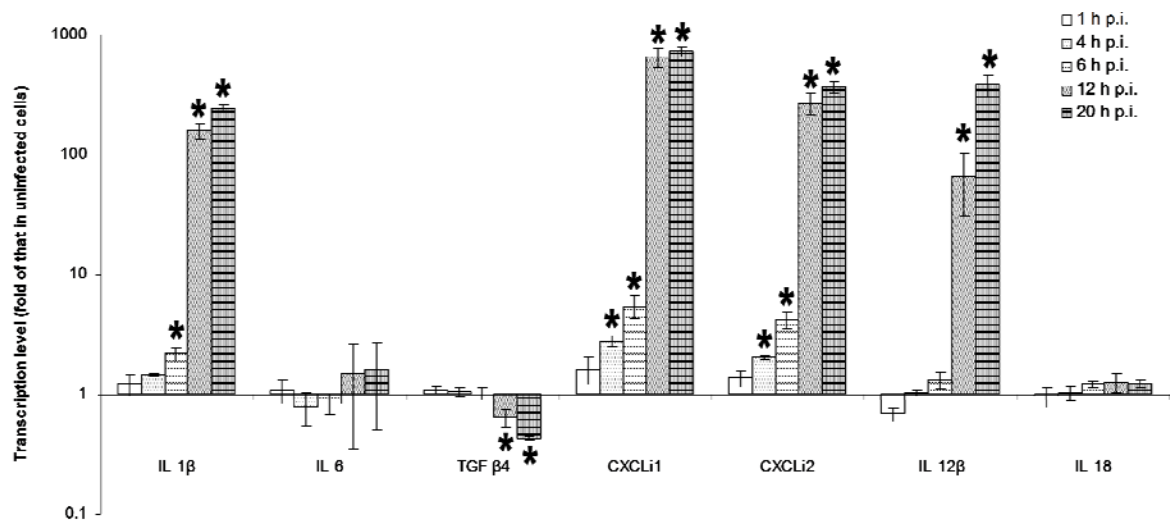
Figure 4. Transcription level of cytokine mRNA in chicken macrophages during 20 h after infection with *A. fumigatus* conidia. Data represent the normalized transcription level of a cytokine in the infected macrophages relative to that in the uninfected macrophages (control), which is considered one. Results expressed as mean ± standard deviation of three replicates. * Significant difference compared with the control.

3.4. Cytokine mRNA expression in T-2-exposed HD-11 macrophages in response to *A. fumigatus* conidial infection

Compared with the non-T-2-exposed controls, the macrophages exposed to 1 to 5 ng/ml T-2 showed significantly upregulated expression of IL-1β, IL-6, CCLi2, CXCLi1, CXCLi2, IL-12β and IL-18 (at 1 and 2 ng/ml), and significantly downregulated expression of TGF-β4 (at 5 ng/ml) 6 h after exposure to *A. fumigatus* conidia. Expression patterns were dose-dependent from 1 to 5 ng/ml except for IL-18 (Figure 5).
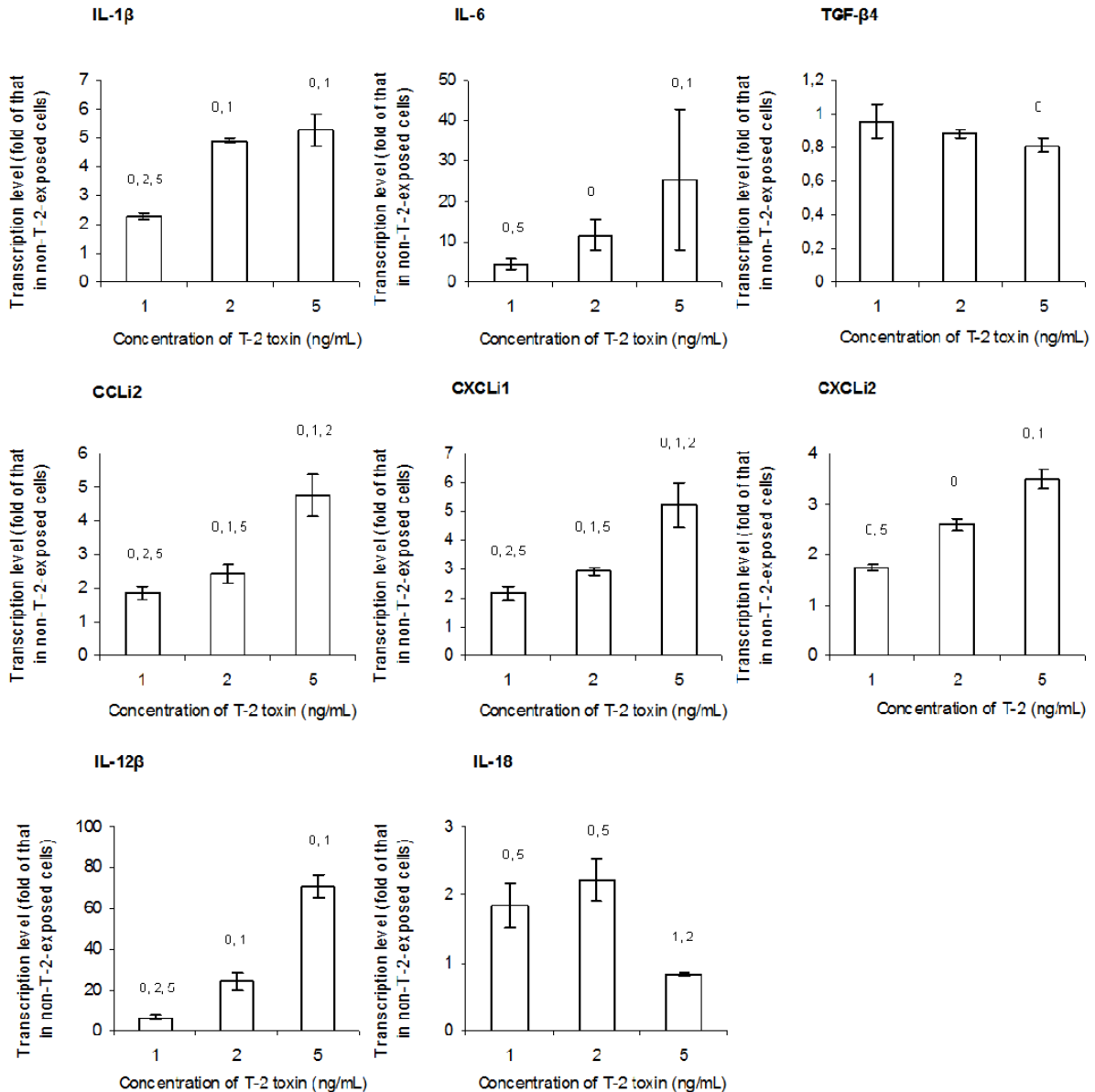
Figure 5. Transcription level of cytokine mRNA in chicken macrophages exposed to T-2 (at 1, 2 and 5 ng/ml) determined 6 h after infection with *A. fumigatus* conidia. Data represent the normalized transcription level of a cytokine in the T-2-exposed macrophages relative to that in the non-T-2-exposed macrophages (control), which is considered one. Results expressed as mean ± standard deviation of three replicates. Columns marked 0, 1, 2 and 5 indicate a significant difference from control, macrophages exposed to 1 ng/ml, 2 ng/ml, and 5 ng/ml T-2, respectively.

4. Discussion

T-2 was shown to be highly toxic at very low concentrations (> 1 ng/ml) for chicken macrophages. A similar result was observed in a study by Jaradat et al. (2006), where T-2 inhibited mitogen-stimulated chicken lymphocyte proliferation *in vitro* at concentrations of 1 ng/ml or higher, and proliferation was completely abolished at 10 ng/ml. In a study by Kidd et al. (1997), 1 h exposure of chicken macrophages to 10 μg/ml and 160 μg/ml T-2 tetraol, a T-2 derivative resulted in respectively 83% and 63% viability. The half inhibition

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concentration (IC50) in this study was approximately 2 ng/ml, while that of human macrophages was demonstrated at 10 ng/ml (Hymery et al., 2009). Exposure to similar levels of T-2 *in vivo* may thus be expected to negatively affect macrophage viability and as a consequence the first line defense mechanism against aspergillosis.

Further evidence for impaired macrophage function was the decreased phagocytic capacity of the macrophages after exposure to T-2. This result is in agreement with those obtained in mammalian macrophages. The phagocytosis of *Pseudomonas* sp. by mouse peritoneal macrophages exposed to 0.47 ng/ml to 46.7 ng/ml T-2 decreased dose-dependently (Vidal and Mavet, 1989). T-2 at 4.7 ng/ml and 23.4 ng/ml decreased the phagocytic capacity of rat alveolar macrophages for yeast cells to respectively 76.9% and 16.8% of the controls (Gerberick et al., 1984), and serum from rabbits treated with 0.5 mg of T-2 per kg of body weight per day suppressed the phagocytosis of *A. fumigatus* conidia by rabbit alveolar macrophages (Niyo et al., 1988). Impaired macrophage function by T-2 was further supported by the increased germination rate of macrophage-associated *A. fumigatus* conidia in this study. This result is compatible with a report by Verbrugghe et al. (2012) that T-2 at 1 ng/ml increased the susceptibility of porcine macrophages to *Salmonella* Typhimurium invasion. Phagocytosis and germination of *A. fumigatus* conidia in chicken HD-11 cells were similar to those in primary respiratory macrophages of pigeons (Van Waeyenberghe et al., 2012), but the current study is the first to address the effect of T-2 on the antifungal response of chicken macrophages. In conclusion, pronounced cytotoxicity of T-2 for avian macrophages coincided with impaired antifungal activity, which would thus facilitate conidial infection in the avian respiratory tract.

In response to *A. fumigatus* infection, the expression of pro-inflammatory cytokines IL-1β, IL-12β (T-helper (Th) 1 cytokine), CXCLi1 (chemokine) and CXCLi2 (chemokine) in HD-11 macrophages was upregulated, and that of TGF-β4 (anti-inflammatory cytokine) was downregulated. These results suggest that *A. fumigatus* infection promotes inflammatory response in chicken macrophages, induces migration of other immunocytes to the infection sites, and stimulates Th1 immune responses (Beeckman et al., 2010). The upregulated expression of IL-1 and IL-12 in *A. fumigatus*-infected HD11 cells was compatible with the *A. fumigatus*-induced production of these cytokines in mouse alveolar and peritoneal macrophage (Taramelli et al., 1996), equine alveolar macrophages (Laan et al., 2005), and mouse bronchoalveolar lavage fluid (Cenci et al., 1998). Apergillosis also induced upregulation of IL-6 and IL-18 in dog mucosal tissue (Day, 2009) and mouse bronchoalveolar lavage fluid and lung tissue (Brieland et al., 2001), but upregulation of IL-6 and IL-18 did not

happen to the chicken HD-11 cells upon conidial infection. Significant changes in cytokine expression occurred from around 6 h after exposure to *A. fumigatus* conidia, which corresponded to the time of conidial germination. Therefore the effect of T-2 exposure on cytokine expression in *A. fumigatus*-infected macrophages was subsequently determined at 6 h p.i.

In response to *A. fumigatus* infection, the expression of pro-inflammatory cytokines IL-1β, IL-6, CCLi2 (chemokine), CXCLi1, CXCLi2, IL-12β and IL-18 (Th1 cytokine) was upregulated, and that of TGF-β4 was downregulated in T-2-exposed HD-11 macrophages as compared with the non-T-2-exposed macrophages (control). This result suggests that exposure of chicken macrophages to T-2 further promotes the pro-inflammatory response, immunocyte migration, and Th1 immune response induced solely by *A. fumigatus* conidia. It is interesting that *A. fumigatus* infection did not induce the expressions of IL-6 and IL-18 when the HD-11 cells were not exposed to T-2, but both the expression of IL-6 and IL-18 were upregulated upon *A. fumigatus* infection when the HD-11 cells were exposed to T-2. These findings support the notion that T-2 may enhance the immune response by increasing the production of pro-inflammatory cytokines (Kankkunen et al., 2009). No previous study has been performed on how T-2 affects cytokine expression in fungus-infected macrophages. Production of IL-1β, IL-6 and IL-12 in mammalian macrophages was reduced by T-2 exposure when the macrophages were uninfected (T-2 at ≤ 1 ng/ml) (Ahmadi and Riazipour, 2008a) or co-stimulated with bacterial lipopolysaccharide (Dugyala and Sharma, 1997), lingzhi (*Ganoderma lucidum*) extract (Ahmadi and Riazipour, 2008b), or TLR-agonists (Seeboth et al., 2012), but upregulation of IL-1β, IL-6 and IL-18 was also reported in macrophages stimulated solely with T-2 (Kankkunen et al., 2009), or co-stimulated with bacterial lipopolysaccharide (Wang et al., 2012). With these diverse effects of T-2, the co-effects of T-2 and different pathogens or antigens on macrophages have to be examined specifically. T-2 has been reported to have both suppressive and stimulatory effects on immunity, and the enhanced immune response was suggested to be associated with the increased production of pro-inflammatory cytokines and migration of macrophages (EFSA, 2011; Kankkunen et al., 2009; Sokolović et al., 2008). The changes in cytokine expression in T-2-exposed HD-11 cells seemed to be the result of the immunostimulatory effect of T-2.

Another study of our group showed that K24 conidia did not secrete any proteins or mycotoxins in the non-germination condition (Li et al., unpublished data). The assays on phagocytosis, conidial germination, and cytokine expression involving T-2 were performed in the non-germination condition or during the early germination stage to avoid potential

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interferences of other K24 metabolites with the effects of T-2. In conclusion, T-2 impaired the antifungal activity of HD-11 cells against *A. fumigatus* conidial infection, but promoted a pro-inflammatory response in infected macrophages, which might compensate for the observed macrophage functional impairment.

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Exposure of *Aspergillus fumigatus* to T-2 Toxin Results in a Stress Response Associated with Exacerbation of Aspergillosis in Poultry

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Abstract

Aspergillus fumigatus is a ubiquitous airborne pathogen. Saprophytic growth in the presence of environmental mycotoxins might affect its fitness and virulence. T-2 toxin (T-2) is a trichothecene mycotoxin produced by *Fusarium* spp. in various substrates. This study aimed to evaluate the effects of T-2 on the fitness of *A. fumigatus in vitro* and its virulence in experimentally inoculated chickens. We cultured *A. fumigatus* on agar media containing T-2, and examined the changes in viability, morphology, growth rate, proteome expression, and susceptibility to antimycotics and oxidative stress of this fungus. Results showed that exposure to 1000 ng/ml T-2 did not reduce the viability of *A. fumigatus*, but its growth was inhibited, with wrinkling and depigmentation of the colonies. Proteomic analysis revealed 21 upregulated proteins and 33 downregulated proteins, including those involved in stress response, pathogenesis, metabolism, transcription and so on. The proteome seems to have shifted to enhance the glycolysis, catabolism of lipids, and amino acid conversion. Assays on fungal susceptibility to antimycotics and oxidative stress showed that T-2 exposure did not affect the minimal inhibitory concentrations of amphotericin B, itraconazole, voriconazole and terbinafine against *A. fumigatus*, but increased the susceptibility of *A. fumigatus* to H_2O_2 and menadione. Experimental inoculation of chickens with *A. fumigatus* showed that exposure of *A. fumigatus* to T-2 significantly exacerbated aspergillosis in chickens exposed to dietary T-2. In conclusion, *A. fumigatus* is capable of surviving and growing on substrates containing levels of T-2 up to 1000 ng/ml. Growth in the presence of T-2 induces a stress response in *A. fumigatus*, which is associated with exacerbation of aspergillosis *in vivo*.

1. Introduction

The ubiquitous saprophytic fungus *Aspergillus fumigatus* is an opportunistic human and animal pathogen that releases abundant small conidia (2 to 3 μm in diameter) into the air, which makes it the most prevalent airborne fungal pathogen (Latgé, 1999; Beernaert et al., 2010). The respiratory system is the primary target of aspergillosis, and birds are considered to be particularly susceptible to aspergillosis (Van Waeyenberghe et al., 2012b). After inhalation of conidia, respiratory macrophages form the first line of defence against this fungal pathogen by phagocytosis and production of reactive oxygen species (ROS) (Juul-Madsen et al., 2008; Van Waeyenberghe et al., 2012a). Although *A. fumigatus* is regarded as an opportunistic pathogen, it can be fatal for immunocompromised hosts (Latgé, 1999).

Mycotoxins are fungal secondary metabolites which are toxic to humans and other animals, and they have been detected in samples from a variety of environments (Elmholt, 2008; Hoerger et al., 2009; Mayer et al., 2008; Smoragiewicz et al., 1993; Tuomi et al., 2000). It is very likely that *A. fumigatus*, as a ubiquitous fungus, lives and grows on mycotoxin-contaminated substrates in environments such as grain storehouses, livestock farms, mushroom farms, sawmills, wood pulp mills, waste treatment plants, and even offices, libraries, and museums. Apart from their toxicity to animals, mycotoxins such as T-2 toxin (T-2), HT-2 toxin, diacetoxyscirpenol, deoxynivalenol, zearalenone, patulin, aflatoxin B1, ochratoxin A, citrinin, penicillic acid, cyclopiazonic acid and penitrem A are known to have antimicrobial activity on different fungi and/or bacteria (Elmholt, 2008), but the effects of mycotoxins in the substrates on the growth and virulence of *A. fumigatus* are not known. T-2 is a trichothecene mycotoxin produced by *Fusarium* spp. (EFSA, 2011). T-2 and its derivatives have been found in the dust of indoor ventilation systems (Smoragiewicz et al., 1993), crude building materials (Tuomi et al., 2000), and more typically in agricultural products (EFSA, 2011) and grain dust (Nordby et al., 2004). Exposure of mice, pigs and chickens to T-2 affected these animals' susceptibility to bacterial infections (Corrier and Ziprin, 1987; Verbrugghe et al., 2012; Ziprin and Elissalde, 1990; Kubena et al., 2001), and T-2 has been shown to influence the function of chicken macrophages against *A. fumigatus* conidia (Li et al., 2013b). If exposure to T-2 during *A. fumigatus* growth also alters the fungus' fitness and virulence, this may affect the development of aspergillosis in chickens.

This study aimed to evaluate the potential effects of T-2 on the fitness of *A. fumigatus* and its virulence in experimentally inoculated chickens. Therefore, we examined the impact of exposure to T-2 on the viability, morphology, growth rate and proteome expression of *A.*

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fumigatus and on its susceptibility to antimycotics and oxidative stress. An *in vivo* experiment was then performed to examine the virulence of T-2-exposed *A. fumigatus* in chickens.

2. Material and methods

2.1. *Aspergillus fumigatus* strain

The *A. fumigatus* isolate, K24, used in this study was obtained from a racing pigeon which died from pulmonary aspergillosis (Beernaert et al., 2008), and stored in Microbank[TM] Bacterial and Fungal Preservation System (Pro-lab Diagnostics, Round Rock, TX, USA) at -75 °C until use.

2.2. Effect of T-2 on the viability of *A. fumigatus*

A 5 days old culture of *A. fumigatus* K24 on Sabouraud dextrose agar (Oxoid, Basingstoke, England) was washed with Hank's Buffered Salt Solution (HBSS) supplemented with 0.01% Tween 20 to harvest the conidia. The conidia were washed three times in the same buffer and a serial dilution of the conidial suspension was titrated on Sabouraud dextrose agar supplemented with 0 (control), 10, 100, or 1000 ng/ml T-2 (Sigma-Aldrich, St. Louis, MO, USA). Colony forming units (CFU) were counted after 20 h of growth at 37 °C. The viability of conidia was calculated by dividing the CFU recovered from each treatment by that from the control. This test was performed in 6 replicates. Differences in viability between the control and T-2-exposed conidia were statistically analyzed by t-test, and the significance level was set at $P < 0.05$.

2.3. Effect of T-2 on the growth phenotype of *A. fumigatus*

To observe the morphological growth of *A. fumigatus*, a sample taken from the *A. fumigatus* K24 conidia stock using a 1 μl Ansa Microloop (Biosigma, Cona, Italy) was seeded on Sabouraud dextrose agar supplemented with 0 (control), 10, 100, or 1000 ng/ml T-2 and cultured at 37°C. Every day, the diameters of the fungal colonies were measured, and the morphological characteristics of the colonies were examined macroscopically. To examine the microscopic morphological characteristics of the colonies, a small piece of each colony was streaked on a microscopic slide, stained with lactophenol cotton blue and observed by light microscopy every day. This test was performed in triplicate. Difference in diameter between the control and a T-2-exposed culture was statistically analyzed by t-test, and the significance level was set at $P < 0.05$.

2.4. Effect of T-2 on conidial proteome *A. fumigatus*

A proteomic analysis was performed to investigate how T-2 exposure might affect the conidial protein expression of *A. fumigatus*. Therefore, *A. fumigatus* K24 was cultured on Sabouraud dextrose agar supplemented with 0 (control), 10, 100 or 1000 ng/ml T-2 at 37°C for 5 days. The conidia were collected in HBSS with 0.05% Tween 20 and washed 3 times by centrifugation and resuspension. The conidial protein sample was prepared as described by Asif et al. (2006) with some modifications. In brief, the conidial suspension was first adjusted to 10^{10} conidia/ml in HBSS containing protease inhibitors and nuclease (One Complete Mini protease inhibitor cocktail tablet (Roche Diagnostics, Mannheim, Germany) and 10 μl Benzonase nuclease (Sigma-Aldrich, St. Louis, MO, USA) were dissolved in 40 ml HBSS beforehand). One ml of this conidial suspension was mixed with 0.4 μl silicon beads (0.1 mm in diameter) in a 2 ml PP microtube. Conidia were disrupted by 25 cycles of 20 s oscillation at the highest speed in a MagNa lyser Instrument (Roche Diagnostics, Rotkreuz, Switzerland) with a brief cooling step on ice between every two cycles. After centrifugation at 10,000×g for 10 min, the supernatant containing proteins was collected and filtrated through a Whatman^{TM} filter with 0.2 μm pore size (GE Healthcare, Buckinghamshire, UK) to remove conidial debris. Samples of each treatment were prepared in triplicate (Samples 1, 2 and 3). The protein concentration was then determined by Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA) according to the manufacturer's instructions.

Each protein sample was then subjected to reduction, cysteine blocking, digestion, and labeling using iTRAQ Reagents (AB Sciex, Foster City, CA, USA) according to the manufacturer's guidelines. Analysis was run in triplicate. In each run, 4 samples from different treatments were analyzed, with each sample labeled with one of the four-plex labels. The labeling of the samples was as follows:

Run 1 (Sample 1 of control: 114, Sample 1 of 10 ng/ml treatment: 115, Sample 1 of 100 ng/ml treatment: 116, Sample 1 of 1000 ng/ml treatment: 117); Run 2 (Sample 2 of control: 115, Sample 2 of 10 ng/ml treatment: 116, Sample 2 of 100 ng/ml treatment: 117, Sample 2 of 1000 ng/ml treatment: 114); Run 3 (Sample 3 of control: 116, Sample 3 of 10 ng/ml treatment: 117, Sample 3 of 100 ng/ml treatment: 114, Sample 3 of 1000 ng/ml treatment: 115).

After labeling, 6 μl of a 5% (v/v) hydroxylamine solution was added to hydrolyze unreacted labels and after incubation at room temperature for 5 min, the samples were pooled, dried and resuspended in 5 mM KH_2PO_4 (15% (v/v) acetonitrile) (pH 2.7). The combined set

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of samples was first purified on ICAT SCX (strong cation exchange) cartridges, desalted on a C18 trap column and finally fractionated using SCX chromatography. Each fraction was analyzed by nano LC-MS/MS as described by Bijttebier et al. (2009).

With no full *A. fumigatus* protein database available, different search parameters and databases, both EST and protein, were validated to obtain maximum spectrum annotation. Best results were obtained when searching UniProtKB/Swiss-Prot database. Significantly differentially expressed proteins in each run were isolated using Rover software (Z-score < -1.96 for downregulated and > 1.96 for upregulated proteins) (Colaert et al., 2011). Only proteins that were significantly differentially expressed in at least two samples at the same or different T-2 concentrations were retained. In parallel, dose-dependent changes of protein expression were analyzed using the software program Qlucore Omics Explorer by means of a ranked regression analysis. The functions of proteins were found by searching UniProtKB/Swiss-Prot database, and functionally unannotated proteins were subjected to BLAST to assess their potential biological function. The proteins were then functionally categorized according to Functional Catalogue (Funcat) with minor modifications. To find possible changes in the metabolic pathways, homologues of these proteins in the yeast *Saccharomyces cerevisiae*, which is a well studied model of fungus, were found by BLAST and then mapped in pathways documented in Reactome database.

2.5. Effect of T-2 on the susceptibility of *A. fumigatus* to antimycotics

Since exposure of *A. fumigatus* to T-2 had a profound effect on conidial protein expression, this might alter susceptibility of *A. fumigatus* conidia to antimycotics. *A. fumigatus* K24 was cultured on Sabouraud dextrose agar supplemented with 0 (control conidia) or 1000 ng/ml T-2 (T-2-exposed conidia) at 37°C for 3 days. Conidia were collected from these media and suspended in RPMI 1640 or RPMI 1640 supplemented with 1000 ng/ml T-2. The minimal inhibitory concentrations (MICs) of amphotericin B, itraconazole, voriconazole and terbinafine were determined and compared between the control conidia and T-2-exposed conidia using the broth microdilution method recommended by the Clinical and Laboratory Standard Institute (CLSI) document M38-A2. All antimycotics were purchased from Sigma-Aldrich, St. Louis, MO, USA, and stored at 1600 μg/ml in DMSO at -75°C. The concentration which gave little or no visible growth as compared to the control was considered the MIC. This test was performed in both RPMI-1640 and RPMI-1640 with 1000 ng/ml T-2, and was performed in triplicate.

2.6. Effect of T-2 on susceptibility of *A. fumigatus* to oxidative agents

To examine possible effects of T-2 in the substrate on the susceptibility of *A. fumigatus* to oxidative stress, fungal susceptibility to 3 oxidative agents was assayed as described by Qiao et al. (2010) with some modifications. Briefly, *A. fumigatus* K24 was cultured on Sabouraud dextrose agar supplemented with 0 (control conidia) or 1000 ng/ml T-2 (T-2-exposed conidia) at 37°C for 3 days, and the conidia were harvested and suspended in HBSS with 0.05% Tween 20. After allowing the heavy particles to settle for 5 min, the upper homogenous suspension was taken and adjusted to 6×10^8 conidia/ml. One µl of this conidial suspension was inoculated on Sabouraud dextrose agar supplemented with $1 - 8$ mM H_2O_2 (Merck, Hohenbrunn, Germany), $80 - 160$ µM menadione (Sigma-Aldrich, St. Louis, MO, USA), or $2 - 8$ mM diamide (Sigma-Aldrich, St. Louis, MO, USA), and blank agar medium without any oxidative agent served as negative control. The 1 µl inocula were air-dried immediately. The plates were incubated at 37°C for 48 h, and the diameter of growth was measured. A diameter was recorded as 0 cm if no growth was observed. This assay was performed in 6 replicates. Difference in diameter between a colony formed by T-2-exposed conidia and that by control conidia was statistically analyzed by t-test, and the significance level was set at $P < 0.05$.

2.7. Effect of T-2 exposure on the experimental infection of chickens with *A. fumigatus*

To examine the effect of exposure to T-2 on avian aspergillosis, chickens (Ross 308) which were fed either a blank feed or feed supplemented with T-2, were exposed to *A. fumigatus* that was grown on Sabouraud dextrose agar either or not containing T-2. Ninety one day old broiler chickens were divided into 6 groups of 15 birds. Three groups (Groups 1, 2, 3) were given blank feed (Bro Plus, Versele-Laga, Deinze, Belgium), and the other 3 groups (Groups 4, 5, 6) were given feed supplemented with 927 µg/kg T-2 (Fermentek, Jerusalem, Israel) for 29 days. The feed was spiked with T-2 beforehand based on the occurrence levels in previous studies (Binder et al.; 2007; Osselaere et al., 2013), and the final T-2 concentration in each feed was analyzed by LC-MS/MS as previously described (Li et al., 2013a). Feed and water were given *ad libitum*. *A. fumigatus* strain K24 was cultured on agar media with (T-2-exposed conidia) or without (control conidia) 1000 ng/ml T-2 as described above. Conidia were collected and the experimental inocula were prepared at 10^8 conidia/ml in HBSS immediately before inoculation. On Day 15, Groups 2 and 5 were inoculated intratracheally with 0.2 ml suspension of control conidia, Groups 3 and 6 were inoculated with the same dose of T-2-exposed conidia, and Group 1 (negative control) and Group 4 (T-2

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control) were sham-inoculated with 0.2 ml HBSS. Clinical signs (ruffled feathers, apathy, tracheal reflex, breathing noise, tachypnea, and/or dyspnea) were observed daily. The difference between the body weight immediately before inoculation and that at necropsy was considered the post-inoculation weight gain. Dead chickens were necropsied immediately, and all remaining chickens were euthanized and necropsied on Day 30. Swabs from the lungs and airsacs were cultured on Sabouraud dextrose agar to retrieve *A. fumigatus*. Lungs were cut and stored in 10% neutral buffered formalin, and the histological sections were stained with Periodic acid-Schiff (PAS) and examined microscopically. Severity of aspergillosis was scored for each bird as the sum of the following parameters:

Presence of clinical sign = 1; Post-inoculation weight gain < (average weight gain of the negative control group – 2×SD) = 1; Presence of macroscopic lesions of the respiratory tract at necropsy = 1; Presence of histological lesions in the lungs = 1; Retrieval of *A. fumigatus* isolate from lungs and/or airsacs = 1; Death = 1.

Mann–Whitney *U* test was performed to compare the aspergillosis scores between different groups, and the significance level was set at $P < 0.05$. This animal experiment was approved by the ethics committee of the Faculty of Veterinary Medicine, Ghent University (EC2013_101).

3. Results

3.1. Effect of T-2 on the viability and growth of *A. fumigatus*

The viability of the *A. fumigatus* conidia on media containing 0 to 1000 ng/ml T-2 ranged between 98.5% and 100%, without any significant difference between T-2-exposed conidia and the control (0 ng/ml T-2). However, the growth rates of the colonies were significantly lower on media containing 100 and 1000 ng/ml T-2 than those of the control, as indicated by the smaller colonial diameters (Table 1). Deep radial wrinkles and lighter pigmentation were observed on the colonies exposed to 1000 ng/ml T-2, and the colonies exposed to 100 ng/ml T-2 were also slightly wrinkled in the centre (Figure 1). Average conidial diameter from 3 days old cultures ranged from 2.5 to 2.6 μm in all control and T-2-exposed cultures. No morphological differences in phialide, conidial head, conidiophore, or hypha were noticed among different treatments when examined by light microscopy.

Table 1. Diameter of *A. fumigatus* colony grown on agar plates containing 0 (control), 10, 100 and 1000 ng/ml T-2 at 37 °C.

Culture time		Diameter of colony $\pm$ SD (cm)			
	T-2 concentration	0 ng/ml (control)	10 ng/ml	100 ng/ml	1000 ng/ml
1 day		1.03 ± 0.10	0.98 ± 0.05	0.83 ± 0.10	* 0.63 ± 0.10
2 days		3.90 ± 0.12	3.78 ± 0.05	* 3.58 ± 0.10	* 3.15 ± 0.06
3 days		6.73 ± 0.10	6.68 ± 0.05	* 6.28 ± 0.05	* 5.48 ± 0.05

The asterisk (*) indicates a significant difference from the control at the same day.
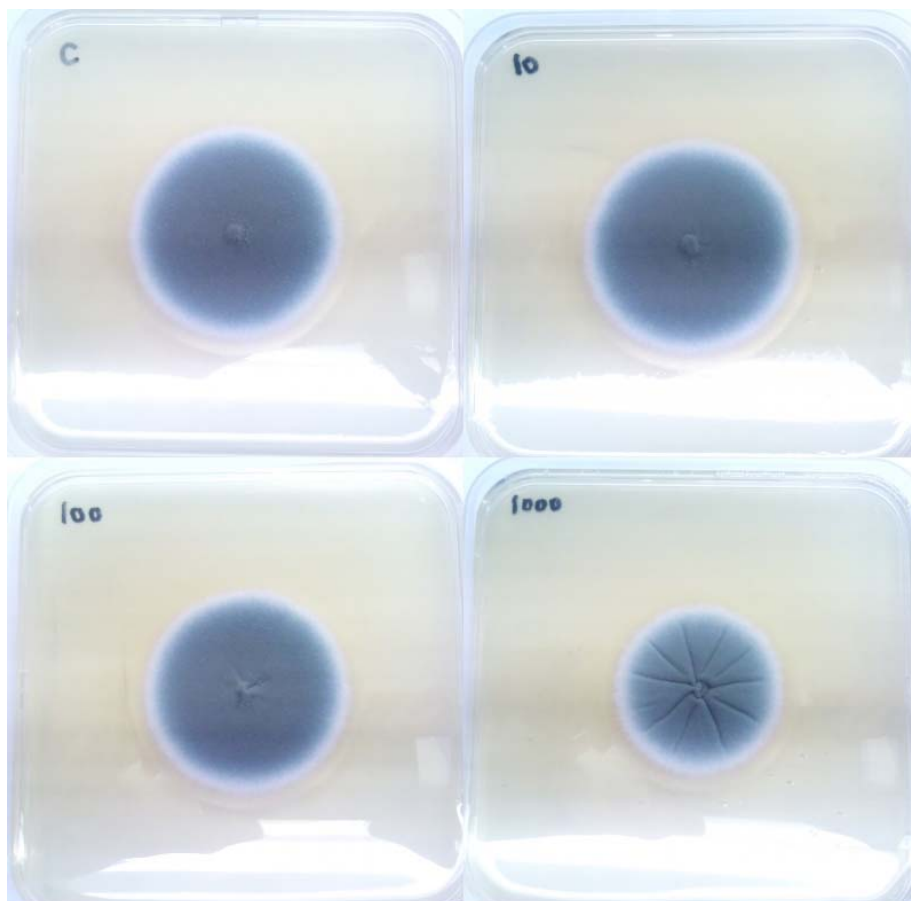
Figure 1. Macroscopic morphology of *A. fumigatus* colonies grown on agar plates containing 0 (control), 10, 100 and 1000 ng/ml T-2 at 37 °C for 3 days. The marks "C", "10", "100" and "1000" in the upper left corner of each plate indicate the T-2 concentrations, i.e. 0, 10, 100, 1000 ng/ml in the corresponding plate. Note the deep radial wrinkles and the depigmentation of the colony on the plate with 1000 ng/ml T-2, and the slight wrinkles at the centre of the colony on the plate with 100 ng/ml T-2.

3.2. Differential expression of proteins in T-2-exposed *A. fumigatus* conidia

Peptides from trypsin digested proteins were labeled with isobaric mass tag labels and analyzed by 2-D LC-MS/MS. Collision-induced dissociation resulted in the release of these isobaric tags, which allowed relative quantification of the peptides. A broad comparison between T-2-exposed and unexposed *A. fumigatus* conidia yielded the identification of 15

proteins significantly upregulated by T-2 (in two or more samples at the same or different concentrations) and 7 dose-dependently upregulated, resulting in 21 upregulated proteins in total (Table S1), while 19 proteins were found to be significantly downregulated by T-2 (in two or more samples at the same or different concentrations) and 16 dose-dependently downregulated, resulting in 33 downregulated proteins in total (Table S2). Yeast homologues of 8 upregulated proteins and 9 downregulated proteins were successfully mapped in metabolic pathways, and they were found to be mostly involved in the citric acid (TCA) cycle, DNA replication, the metabolisms of carbohydrates, nucleotides, proteins, lipids, lipoproteins, amino acids and derivatives (Table S3). As categorized according to FunCat, among all the 21 upregulated proteins, 11 were involved in metabolism, 7 were proteins with binding function or cofactor requirement (structural or catalytic), 4 were involved in cell surface, cellular communication/signal transduction mechanism, and so on. When the upregulated proteins were mapped in the metabolic pathways in Reactome database, a shift towards catabolism of lipids and amino acids emerged, and an increase in the anaerobic glycolysis could be assumed (agmatinase, aminotransferase, triosephosphate isomerase, 15-hydroxyprostaglandin dehydrogenase, cytochrome P450 monooxygenase). The upregulated proteins further include stress regulators such as antigenic mitochondrial protein HSP 60, GPI anchored cell wall protein (Dan 4), hydrophobin protein, MFS multidrug transporter, and so on. Among all the 33 downregulated proteins, 14 were involved in metabolism, 10 were proteins with binding function or cofactor requirement (structural or catalytic), 7 were associated with protein synthesis, protein fate and protein turnover, 6 functioned in cell rescue, defense and virulence, 5 were involved in energy, 5 were related to transcription, and so on. Pathway mapping of the downregulated metabolic proteins suggests a reduction in oxidative metabolism and a shift in the pentose phosphate cycle, potentially underlying reduced nucleotide synthesis and transcription (dihydrolipoamide succinyltransferase, fumarate hydratase, glucose-6-phosphate 1-dehydrogenase, mitochondrial chaperone frataxin, adenylosuccinate lyase, proteasome component Pre 4, pre-mRNA splicing factor Prp 8). The downregulated proteins further comprise antioxidant protein LsfA, CipC-like antibiotic response protein, conidial pigment biosynthesis oxidase Arb 2, BZIP transcription factor (LziP), C6 finger domain protein, polyadenylate-binding protein, and so on.

3.3. Minimal inhibitory concentration of antimycotics against *A. fumigatus* grown with T-2

MICs of amphotericin B, itraconazole, voriconazole and terbinafine were respectively 2, 0.5, 0.125, and 4 µg/ml for both control conidia and T-2-exposed conidia, whether in normal RPMI-1640 or in RPMI-1640 supplemented with 1000 ng/ml T-2. No difference was noticed in MICs of these antimycotics between control conidia and T-2-exposed conidia.

3.4. Susceptibility of *A. fumigatus* grown in the presence of T-2 to oxidative stress

Control conidia and T-2-exposed conidia were inoculated on blank medium and media containing oxidative agents, and the colonial diameters at 48 h are presented in Table 2. There was no significant difference between the control conidia and T-2-exposed conidia on blank medium or on medium with diamide (≤ 8 mM), but the colonies formed by T-2-exposed conidia were significantly smaller than those formed by control conidia when exposed to H_2O_2 (4 mM) or menadione (120 and 140 µM). Control conidia germinated in all the 6 replicates in 4 mM H_2O_2 or 140 µM menadione, while T-2-exposed conidia germinated respectively in only 2 and 1 replicate in the same conditions.

Table 2. Diameter of *A. fumigatus* colonies formed by control conidia and T-2-exposed conidia after 48 h growth on oxidant-containing agar plates at 37 °C.

Oxidant and concentration		Diameter of colony ± SD (cm) at 48 h (number of growth out of 6 replicates)	
		Control conidia	T-2-exposed conidia
Blank		3.91 ± 0.07 (6/6)	3.93 ± 0.08 (6/6)
H_2O_2	1 mM	3.78 ± 0.04 (6/6)	3.73 ± 0.04 (6/6)
	2 mM	3.63 ± 0.03 (6/6)	3.58 ± 0.08 (6/6)
	4 mM	2.23 ± 0.44 (6/6)	* 0.15 ± 0.37 (2/6)
	8 mM	0 ± 0 (0/6)	0 ± 0 (0/6)
Menadione	80 µM	2.27 ± 0.14 (6/6)	2.18 ± 0.11 (6/6)
	120 µM	1.58 ± 0.05 (6/6)	* 1.29 ± 0.12 (6/6)
	140 µM	0.76 ± 0.41 (6/6)	* 0.05 ± 0.12 (1/6)
	160 µM	0.05 ± 0.12 (1/6)	0 ± 0 (0/6)
Diamide	2 mM	2.94 ± 0.07 (6/6)	2.95 ± 0.08 (6/6)
	4 mM	2.11 ± 0.05 (6/6)	2.10 ± 0 (6/6)
	8 mM	0 ± 0 (0/6)	0.06 ± 0.14 (1/6)

Control conidia: *A. fumigatus* conidia harvested from 3 day old culture on Sabouraud dextrose agar without T-2; T-2-exposed conidia: *A. fumigatus* conidia harvested from 3 day old culture on Sabouraud dextrose agar with 1000 ng/ml T-2; The asterisk (*) indicates a significant difference from the control conidia.

3.5. Effect of T-2 exposure on the experimental infection of chickens with *A. fumigatus*

The average aspergillosis score per bird of each group is presented in Figure 2. Birds exposed to dietary T-2 and inoculated with T-2-exposed conidia (Group 6) had a significantly higher aspergillosis score compared to all the other groups. Besides, chickens fed blank feed and inoculated with T-2-exposed *A. fumigatus* conidia (Group 3) and chickens fed a diet supplemented with T-2 and inoculated with control conidia (Group 5) both showed a mild increase in the development of aspergillosis in comparison with chickens fed the blank feed and inoculated with control conidia (Group 2).
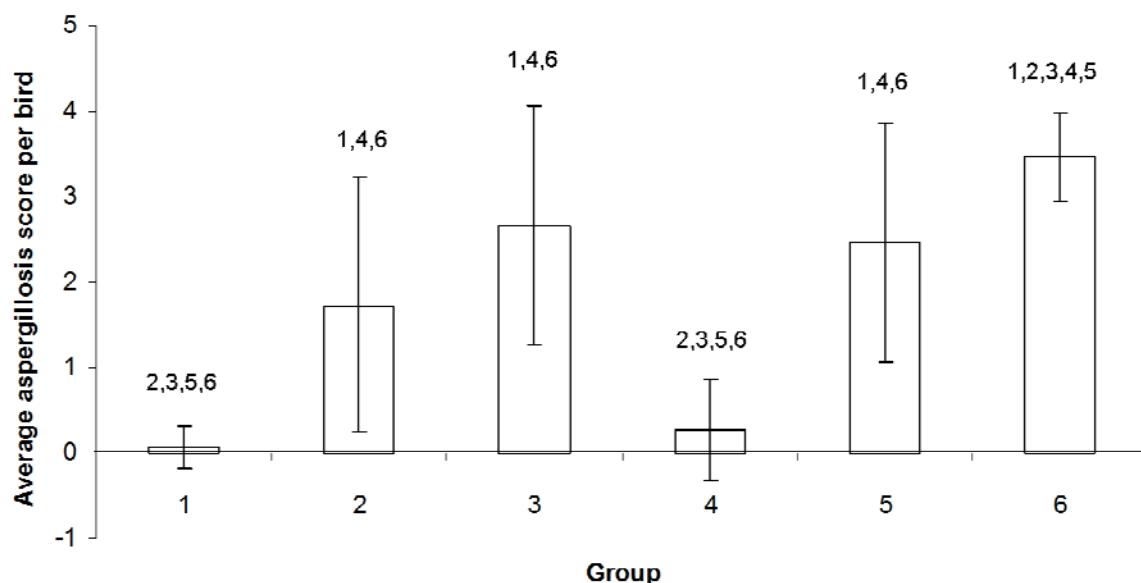
Figure 2. Average aspergillosis score per bird (± SD) in differently treated groups of chickens. Group 1: given blank feed and sham-inoculated; Group 2: given blank feed and inoculated with *A. fumigatus* conidia grown in absence of T-2; Group 3: given blank feed and inoculated with *A. fumigatus* conidia grown in presence of 1000 ng/ml T-2; Group 4: given feed containing 927 μg/kg T-2 and sham-inoculated; Group 5: given feed containing 927 μg/kg T-2 and inoculated with *A. fumigatus* conidia grown in absence of T-2; Group 6: given feed containing 927 μg/kg T-2 and inoculated with *A. fumigatus* conidia grown in presence of 1000 ng/ml T-2. Columns marked with 1, 2, 3, 4, 5 and/or 6 indicate a significant difference from the corresponding group.

4. Discussion

Levels of T-2 can reach 1200 μg/kg in grain dust (Nordby et al., 2004), and thousands of μg/kg in agricultural products (EFSA, 2011). In this study, the viability of *A. fumigatus* was not affected by the presence of T-2 (up to 1000 ng/ml) in the agar media. This result demonstrates that *A. fumigatus* can grow on substrates contaminated with T-2 even at high concentrations, and thus further justifies the necessity to evaluate the effects of T-2 on the fitness of *A. fumigatus*. The smaller colonies formed on media containing 100 and 1000 ng/ml suggest that T-2 inhibits the growth of *A. fumigatus* at these concentrations. This result is

compatible with the previously reported inhibitory effect of T-2 and other trichothecenes on the fungi *Penicillium digitatum*, *Mucor ramannianus*, *Kluyveromyces marxianus*, *Saccharomyces bayanus*, *S. carlsbergensis*, *S. pastorianus*, *Rhodotorula rubra* and *R. glutinus* (Madhyastha et al., 1994; Vesonder et al., 1981; Burmeister and Hesseltine, 1970). This inhibited growth of *A. fumigatus* probably resulted from the widely reported inhibitory effect of T-2 on protein and nucleic acid synthesis (EFSA, 2011). Trichothecene mycotoxins inhibit protein synthesis by binding to the ribosomal peptidyltransferase site in animal cells, leading to a ribotoxic stress response and as a result modulate a variety of physiological processes (Shifrin and Anderson, 1999). Proteome analysis and pathway mapping in this study revealed downregulation of dihydrolipoamide succinyltransferase and fumarate hydratase in T-2-exposed conidia, which likely hindered the citric acid (TCA) cycle and the oxidative metabolism. The downregulation of two transcription factors (i.e. BZIP transcription factor (LziP) and C6 finger domain protein), polyadenylate-binding protein, proteasome component Pre 4, pre-mRNA splicing factor (Prp 8) and RNA exonuclease Rex 2 might have interfered intensively with DNA replication, transcription and post-transcriptional processing in the T-2-exposed conidia. These changes probably contributed to the observed growth inhibition of *A. fumigatus* in the presence of T-2. As a toxic secondary metabolite released by the fungal genus *Fusarium*, T-2 might serve as an inhibitor against other fungal competitors. The radial wrinkles of the *A. fumigatus* colonies grown on agar medium containing T-2 appeared similar to those observed in *Aspergillus costaricaensis*, *A. piperis* and *A. sclerotioniger* colonies in the absence of T-2 (Samson et al., 2004), but the mechanism for how these wrinkles are formed has never been elucidated. The depigmentation of the *A. fumigatus* colonies exposed to 1000 ng/ml T-2 might play a role in the increased susceptibility to oxidative stress observed in this study, as pigment is thought to protect *A. fumigatus* conidia against ROS of the host (Sugareva et al., 2006). It is not sure whether the dose-dependent downregulation of conidial pigment biosynthesis oxidase Arb 2, revealed by the proteome analysis, contributed to the lightening of the blue-green colour of the colony, because deletion of the *abr2* gene in *A. fumigatus* reportedly lead to brown colony morphology (Sugareva et al., 2006).

The response of *A. fumigatus* to the stress posed by T-2 involved the upregulated expression of some anti-stress proteins and changes in metabolic pathways. The expression of antigenic mitochondrial protein HSP 60 in *A. fumigatus* was found to be increased upon T-2 exposure, and increased expression of heat shock proteins is known as a protective mechanism against stresses (Li and Srivastava, 2004). The upregulated GPI anchored cell

wall protein (Dan 4) and hydrophobin protein are cell wall structural proteins involved in maintenance of fungal cell wall integrity and resistance to environmental stress (Gautam et al., 2008). MFS multidrug transporter, the expression of which was also upregulated in T-2-exposed conidia, acts as an efflux pump removing toxic substances. Histones H2A and H3 were upregulated in T-2-exposed conidia, and histones are believed to play a central role in transcription regulation, DNA repair, DNA replication and chromosomal stability. Fifteen-hydroxyprostaglandin dehydrogenase (NAD(+)) was mapped in the metabolisms of peroxisomal lipid, alpha-linolenic acid, bile acids and bile salts, so the upregulated expression of this protein suggests the activation of metabolisms of lipids and lipoproteins. The upregulation of agmatinase and aminotransferase, and the downregulation of adenosylhomocysteinase, dihydrolipoamide succinyltransferase, and ornithine carbamoyltransferase also suggest pathway shifts in metabolisms of amino acids and derivatives in T-2-exposed *A. fumigatus* conidia. Taken together, the conidial proteome of the T-2-exposed *A. fumigatus* seems to have shifted away from the conventional oxidative metabolism in the mitochondria towards the glycolysis and catabolism of lipids. Both protein synthesis and degradation are downregulated, and the conidia have upregulated proteins that can convert amino acids.

In the antimycotic MIC assay, the control conidia and the T-2-exposed conidia did not exhibit any difference in MICs of amphotericin B, itraconazole, voriconazole and terbinafine, and the MICs were in the ranges reported in earlier studies (Beernaert et al., 2009; Moore et al., 2001; Dannaoui et al., 2004). Although expression of CipC-like antibiotic response protein was decreased in T-2-exposed conidia, this did not affect the MICs of the four antimycotics in the current study, possibly because this protein is not associated with the conidial response to these antimycotics. Possibly, the upregulated MFS multidrug transporter in T-2-exposed conidia contributes to their sustained resistance, and the upregulated expression of cytochrome P450 monooxygenase in T-2-exposed conidia may also promote the metabolism of voriconazole (Johnson and Kauffman, 2003).

When challenged with H_2O_2 or menadione, the T-2-exposed conidia showed lower viability and growth rate than the control conidia, indicating that growth in the presence of T-2 reduced the tolerance of *A. fumigatus* conidia to oxidative stress. This result is compatible with the decreased expression of the antioxidant protein LsfA and mitochondrial peroxiredoxin Prx1 in the proteome analysis. Tolerance to oxidative stress is important to the virulence of this fungus, because the host exerts fungicidal effects on the invading *A. fumigatus* conidia by ROS, such as H_2O_2, hydroxyl radicals, and superoxide anions (Juul-

Madsen et al., 2008; Xu et al., 2011). H_2O_2, menadione, and diamide often have been used to study the multiple modes of oxidative stress encountered by fungal pathogens (Qiao et al., 2010; Pócsi et al., 2005). Interestingly, the current study showed that growth with T-2 increased the susceptibility of *A. fumigatus* conidia to H_2O_2 and menadione, but not to diamide, which is probably due to the different mechanisms of these three oxidative stress-inducing agents. H_2O_2 increases levels of peroxide and hydroxyl radicals, and leads to the oxidation of sulfur containing amino acids and damage of the double-stranded super-coiled structure of DNA (Toledano et al., 2003; Branco et al., 2004). Menadione generates superoxide anions in a redox cycle, which destroys 4Fe-4S proteins, and through the consumption of NADPH, menadione also causes the conversion of molecular oxygen to superoxide, which is dismutased to H_2O_2 (Toledano et al., 2003; Sun et al., 1997). As a thiol-oxidizing agent, diamide affects redox balance of glutathione/glutathione disulfide by fast oxidation of glutathione (Toledano et al., 2003).

The *in vivo* experiment showed that pre-exposure of *A. fumigatus* to T-2 mildly and non significantly exacerbated aspergillosis in chickens fed the blank feed, but significantly exacerbated aspergillosis in chickens exposed to dietary T-2. This exacerbation suggests that the T-2 induced stress response in *A. fumigatus* results in enhanced *A. fumigatus* virulence, which is most noticeable in the T-2-exposed chickens. Immunosuppression caused by T-2 probably accounts for the observed increase of chicken susceptibility, since T-2 has been shown to suppress phagocytosis of *A. fumigatus* conidia by chicken macrophages (Li et al., 2013b) and increase *Salmonella* Typhimurium infection in chickens in previous studies (Ziprin and Elissalde, 1990; Kubena et al., 2001).

In conclusion, *A. fumigatus* is capable of surviving and growing in substrates containing levels of T-2 up to 1000 ng/ml. Growth in presence of T-2 results in a stress response, with reduced fitness of *A. fumigatus in vitro*, as evidenced by growth inhibition, altered colonial morphology and conidial proteome, and decreased oxidative stress tolerance, but increased virulence *in vivo*, as evidenced by exacerbation of aspergillosis in chickens.

Acknowledgements

Table S1. Conidial proteins of *A. fumigatus* upregulated upon T-2 exposure

Significantly upregulated protein (by Rover)	Dose-dependenct upregulation (by Qlucore) or not	Sample and concentration of T-2 treatment (ng/ml)	Category [F]	Function [U]	UniProtKB/Swiss-Prot ID of yeast homologue
15-hydroxyprostaglandin dehydrogenase (NAD(+))		100 (Sample 3) 1000 (Sample 3)	1, 18	Prostaglandin inactivation. Contributes to the regulation of events that are under the control of prostaglandin levels. Catalyzes the NAD-dependent dehydrogenation of lipoxin A4 to form 15-oxo-lipoxin A4.	Q02207
Agmatinase		100 (Sample 1) 1000 (Samples 1, 2)	18	Catalytic activity: Agmatine + H_2O = putrescine + urea.	P00812
Antigenic mitochondrial protein HSP 60		10 (Sample 2) 1000 (Sample 2)	32	Participates in assembly and/or disassembly of proteins imported into the mitochondrion. HSP 60 are ATPases and have affinity for unfolded proteins By similarity. Involved in osmoadaptation.	P19882
C6 finger domain protein		10 (Samples 1, 2)	11, 14	A transcription factor required for protein utilization and degradation. Regulates transcription of major secreted proteases including a serine alkaline protease (alp 1) and a metalloprotease (Npl).	P40467
Cytochrome P450 monooxygenase Sir B-like		10 (Sample 3) 100 (Sample 3) 1000 (Sample 3)	1	Cytochrome P450 superfamily of monooxygenases, involved in metabolism of voriconazole (Johnson and Kauffman, 2003).	P10614
DMRL synthase family protein		10 (Sample 2) 100 (Sample 2)	1	Catalyzes the formation of 6,7-dimethyl-8-ribityllumazine by condensation of 5-amino-6-(D-ribitylamino)uracil with 3,4-dihydroxy-2-butanone 4-phosphate. This is the penultimate step in the biosynthesis of riboflavin. Catalytic activity: 1-deoxy-L-glycero-tetrulose 4-phosphate + 5-amino-6-(D-ribitylamino)uracil = 6,7-dimethyl-8-(D-ribityl)lumazine + 2 H_2O + phosphate.	P50861
Glutamate/Leucine/Phenylalanine/Valine dehydrogenase		1000 (Samples 2, 3)	1, 2, 16, 42	Catalytic activity: L-glutamate + H_2O + $NADP^+$ = 2-oxoglutarate + NH_3 + NADPH. L-leucine + H_2O + NAD^+ = 4-methyl-2-oxopentanoate + NH_3 + NADH. L-phenylalanine + H_2O + NAD^+ = phenylpyruvate + NH_3 + NADH. L-valine + H_2O + NAD^+ = 3-methyl-2-oxobutanoate + NH_3 + NADH.	P07262
GPI anchored cell wall protein (Dan 4)		100 (Sample 3) 1000 (Sample 3)	30	Component of the cell wall. Induced during anaerobic growth and completely repressed during aerobic growth.	P53185

Histone H2A		100 (Samples 1, 2)	10, 11, 16, 42	Core component of nucleosome which plays a central role in DNA double strand break (DSB) repair. Histones thereby play a central role in transcription regulation, DNA repair, DNA replication and chromosomal stability. DNA accessibility is regulated via a complex set of post-translational modifications of histones, also called histone code, and nucleosome remodeling.	P04912
Histone H3		10 (Sample 2) 100 (Sample 2) 1000 (Sample 2)	10, 11, 16, 42	Core component of nucleosome. Histones play a central role in transcription regulation, DNA repair, DNA replication and chromosomal stability. DNA accessibility is regulated via a complex set of post-translational modifications of histones, also called histone code, and nucleosome remodeling.	P61830
Hydrophobin protein		1000 (Samples 1, 3) 100 (Sample 3)	30	Cell wall protein regularly arranged in interwoven fascicules of clustered proteinaceous microfibrils, or rodlets, to form the outer spore coat protein. Involved in resistance to environmental stress and may be associated with conidial hydrophobicity.	not found
MFS multidrug transporter		10 (Sample 2) 100 (Sample 2) 1000 (Sample 2)	20, 30	Energy-dependent efflux pump responsible for decreased drug accumulation in multi-drug-resistant cells. Probably uses a transmembrane proton gradient as the energy source. Causes the efflux of a variety of toxic substances, including such structurally diverse compounds as ethidium bromide, rhodamine and acridine dyes, tetraphenylphosphonium, puromycin, chloramphenicol, doxorubicin, and fluoroquinolone antibiotics.	not found
Probable aspartic-type endopeptidase opsB		10 (Sample 3) 100 (Sample 3)	12, 30, 32	Probable GPI-anchored aspartic-type endopeptidase which contributes to virulence	Q12303
RNA binding protein		100 (Sample 1) 10 (Sample 2 [B])	16	Not specified in *Aspergillus* spp.	not found
Triosephosphate isomerase	Qlucore	10 (Sample 2) 1000 (Sample 2)	1	Catalytic activity: D-glyceraldehyde 3-phosphate = glycerone phosphate.	P00942
Aminotransferase	Qlucore	1000 (Sample 2)	1, 16	not reported	P47039
ATP sulphurylase	Qlucore		1	Catalyzes the first intracellular reaction of sulfate assimilation, forming adenosine-5'-phosphosulfate (APS) from inorganic sulfate and ATP. Plays an important role in sulfate activation as a component of the biosynthesis pathway of sulfur-containing amino acids. Catalytic activity: ATP + sulfate = diphosphate + adenylyl sulfate.	P08536
Isocitrate dehydrogenase Idp1	Qlucore		1, 2, 4, 16	Catalytic activity: Isocitrate + $NADP^+$ = 2-oxoglutarate + CO_2 + NADPH.	P21954

Oxidoreductase, zinc-binding dehydrogenase family	Qlucore		1	not reported	Q03102
Sorbitol/xylulose reductase Sou 1-like	Qlucore		1	Xylitol dehydrogenase which catalyzes the conversion of xylitol to D-xylulose. Xylose is a major component of hemicelluloses such as xylan. Catalytic activity: Xylitol + NAD$^+$ = D-xylulose + NADH.	P32573
Transaldolase	Qlucore		1, 2, 16	Important for the balance of metabolites in the pentose-phosphate pathway. Catalytic activity: Sedoheptulose 7-phosphate + D-glyceraldehyde 3-phosphate = D-erythrose 4-phosphate + D-fructose 6-phosphate.	P15019

[B] The protein was identified by BLAST in NCBI database.

[U] Protein functions were found in UniProtKB/Swiss-Prot database.

[F] Proteins were functionally categorized according to FunCat. 1, Metabolism; 2, Energy; 4. Storage protein; 10, Cell cycle and DNA processing; 11, Transcription; 12, Protein synthesis, protein fate and protein turnover; 16, Protein with binding function or cofactor requirement (structural or catalytic); 18, Regulation of metabolism and protein function; 20, Cellular transport, transport facilities and transport routes; 30, Cell surface, cellular communication/signal transduction mechanism; 32, Cell rescue, defense and virulence; 34, Interaction with the environment; 42, Biogenesis of cellular components; 99, Unclassified proteins.

Table S2. Conidial proteins of *A. fumigatus* downregulated upon T-2 exposure

Significantly downregulated protein (by Rover)	Dose-dependent downregulation (by Qlucore) or not	Sample and concentration of T-2 treatment (ng/ml)	Category [F]	Function [U]	UniProtKB/Swiss-Prot ID of yeast homologue
Adenosylhomocysteinase		10 (Sample 3)	1, 16	Adenosylhomocysteine is a competitive inhibitor of S-adenosyl-L-methionine-dependent methyl transferase reactions; may play a key role in the control of methylations via regulation of the intracellular concentration of adenosylhomocysteine.	P39954
		100 (Samples 1, 3)			
		1000 (Sample 3)			
Antioxidant protein LsfA	Qlucore	1000 (Samples 1, 3)	32	not reported	P34227
BZIP transcription factor (LziP)		100 (Sample 3)	11, 16	Transcription factor that mediates sequence specific DNA binding properties and the leucine zipper that is required to hold together (dimerize) two DNA binding regions (Ellenberger, 1994; Hurst, 1994).	Q02100
		1000 (Sample 3)			
C6 finger domain protein		100 (Samples 2, 3 [B])	11, 14	Transcription factor required for protein utilization and degradation. Regulates transcription of major secreted proteases including a serine alkaline protease (alp 1) and a metalloprotease (Npl).	Q12180
Cell surface protein	Qlucore	1000 (Samples 1, 2)	30	not specified	not found
CipC-like antibiotic response protein		10 (Sample 1)	32	not reported	not found
		100 (Sample 3)			
		1000 (Samples 1, 2, 3)			
Cytochrome C oxidase subunit 5A		10 (Sample 2)	1	Catalytic activity: 4 ferrocytochrome C + O_2 + 4 H^+ = 4 ferricytochrome C + $2H_2O$.	P00427
		100 (Samples 2, 3)			
		1000 (Samples 2, 3)			
Dihydrolipoamide succinyltransferase		100 (Sample 1)	1, 2	Dihydrolipoamide succinyltransferase component of 2-oxoglutarate dehydrogenase complex . The 2-oxoglutarate dehydrogenase complex catalyzes the overall conversion of 2-oxoglutarate to succinyl-CoA and CO_2. Catalytic activity: Succinyl-CoA + enzyme N(6)-(dihydrolipoyl)lysine = CoA + enzyme N(6)-(S-succinyldihydrolipoyl)lysine.	P19262
		1000 (Sample 1)			
Fumarate hydratase		10 (Sample 3)	1, 2, 42	Catalytic activity: (S)-malate = fumarate + H_2O.	P08417
		100 (Sample 3)			

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		1000 (Sample 3)			
Lipid transfer protein		100 (Sample 2)	16, 20	not reported	not found
		1000 (Sample 2)			
NAD dependent epimerase/dehydratase		10 (Sample 1 [B])	1, 2, 16	Catalyzes the epimerization of the S- and R-forms of NAD(P)HX, a damaged form of NAD(P)H that is a result of enzymatic or heat-dependent hydration. This is a prerequisite for the S-specific NAD(P)H-hydrate dehydratase to allow the repair of both epimers of NAD(P)HX.	P32527
		1000 (Sample 1 [B])			
Ornithine carbamoyltransferase		100 (Sample 3)	1	Catalytic activity: Carbamoyl phosphate + L-ornithine = phosphate + L-citrulline.	P05150
		1000 (Sample 3)			
Peptidase inhibitor I 9, partial		1000 (Samples 2 [B], 3 [B])	12, 18	not reported	P0CT04
		100 (Sample 3 [B])			
Polyadenylate-binding protein, cytoplasmic and nuclear		100 (Sample 1)	11, 16	Binds the poly(A) tail of mRNA. Appears to be an important mediator of the multiple roles of the poly(A) tail in mRNA biogenesis, stability and translation.	P04147
		1000 (Sample 1)			
Pre-mRNA splicing factor (Prp 8)		10 (Sample 1)	10, 11, 12	Involved in pre-mRNA splicing and cell cycle progression. Required for the spliceosome assembly and initiation of the DNA replication.	P33334
		1000 (Sample 1)			
Proteasome component Pre 4		100 (Sample 3)	2, 14, 16, 32	Degrades poly-ubiquitinated proteins in the cytoplasm and in the nucleus. Essential for the regulated turnover of proteins and for the removal of misfolded proteins. Able to cleave peptides with Arg, Phe, Tyr, Leu, and Glu adjacent to the leaving group at neutral or slightly basic pH. Has an ATP-dependent proteolytic activity. Necessary for the peptidyl-glutamyl-peptide-hydrolyzing activity.	P30657
		1000 (Samples 2, 3)			
Protein pal 1		10 (Sample 1 [B])	30	Involved in the early step of endocytosis	Q05518
		1000 (Sample 1 [B])			
RNA exonuclease Rex 2		10 (Sample 2)	11	Possesses 5'->3' exoribonuclease activity. Required for the processing of nuclear mRNA and rRNA precursors. May promote termination of transcription by RNA polymerase II By similarity. Catalytic activity Exonucleolytic cleavage in the 5'- to 3'-direction to yield nucleoside 5'-phosphates.	P54964
		100 (Sample 2)			
		1000 (Sample 2)			
Signal transduction protein Syg 1		10 (Sample 1)	30	Functions in G-protein coupled signal transduction	P40528
		100 (Sample 1)			

Adenylosuccinate lyase	Qlucore [B]	1000 (Sample 1 [B])	1	Catalytic activity: N(6)-(1,2-dicarboxyethyl)AMP = fumarate + AMP. (S)-2-(5-amino-1-(5-phospho-D-ribosyl)imidazole-4-carboxamido)succinate = fumarate + 5-amino-1-(5-phospho-D-ribosyl)imidazole-4-carboxamide.	Q05911
C2 domain protein	Qlucore [B]	100 (Sample 3)	16	key membrane-localization modules, in multiple ciliary proteins, including those from the NPHP1-4-8 and the MKS complexes (Zhang and Aravind, 2012)	not found
HHE domain protein	Qlucore	1000 (Sample 1)	99	not reported	not found
FK506-binding protein 1 A	Qlucore		12, 16	PPIases accelerate the folding of proteins. It catalyzes the cis-trans isomerization of proline imidic peptide bonds in oligopeptides. Catalytic activity: Peptidylproline (omega=180) = peptidylproline (omega=0).	P20081
Mitochondrial chaperone Frataxin	Qlucore		1, 12, 32	Promotes the biosynthesis of heme as well as the assembly and repair of iron-sulfur clusters by delivering Fe^{2+} to proteins involved in these pathways. Plays a role in the protection against iron-catalyzed oxidative stress through its ability to catalyze the oxidation of Fe^{2+} to Fe^{3+}. Can store large amounts of the metal in the form of a ferrihydrite mineral by oligomerization. May be involved in regulation of the mitochondrial electron transport chain. Catalytic activity: $4Fe^{2+} + 4H^+ + O_2 = 4Fe^{3+} + 2H_2O$.	Q07540
Aldehyde reductase (AKR1)	Qlucore		1, 2, 16	Catalyzes the asymmetric reduction of aliphatic and aromatic aldehydes and ketones to an R-enantiomer. Reduces ethyl 4-chloro-3-oxobutanoate to ethyl (R)-4-chloro-3-hydroxybutanoate. Catalytic activity: An alcohol + $NADP^+$ = an aldehyde + NADPH.	P14065
Mannitol-1-phosphate 5-dehydrogenase	Qlucore		1	Catalyzes the NAD(H)-dependent interconversion of D-fructose 6-phosphate and D-mannitol 1-phosphate in the mannitol metabolic pathway. Has a strong preference for NADH over NADPH. Catalytic activity: D-mannitol 1-phosphate + NAD^+ = D-fructose 6-phosphate + NADH.	P0CX08
Metallo-dependent phosphatase	Qlucore [B]		99	not reported	P40002
Mitochondrial peroxiredoxin Prx1	Qlucore		1, 32	Has a thioredoxin peroxidase activity with a role in reduction of hydroperoxides. Catalytic activity: 2 R'-SH + ROOH = R'-S-S-R' + H_2O + ROH.	P34227
Glucose-6-phosphate 1-dehydrogenase	Qlucore		1	Catalytic activity: D-glucose 6-phosphate + $NADP^+$ = 6-phospho-D-glucono-1,5-lactone + NADPH	P11412
Eukaryotic translation elongation factor 1 subunit Eef 1-β	Qlucore		12, 16, 18	Catalytic subunit of the guanine nucleotide exchange factor (GEF) (eEF1B subcomplex) of the eukaryotic elongation factor 1 complex (eEF1). Stimulates the exchange of GDP for GTP on elongation factor 1A (eEF1A), probably by displacing GDP from the nucleotide binding pocket in eEF1A. The 30-fold higher concentration of GTP compared to GDP in cells favors the formation of eEF1A-GTP, which rapidly forms a ternary complex with aminoacyl-tRNA that in turn displaces eEF1B from the complex.	P32471

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Dienelactone hydrolase family protein	Qlucore		1	Catalytic activity: 4-carboxymethylenebut-2-en-4-olide + H_2O = 4-oxohex-2-enedioate	P39721
Conidial pigment biosynthesis oxidase Arb 2	Qlucore		1	A polyketide synthase which condenses acetate units to form a heptaketide naphthopyrene YWA 1, via a polyketomethylene intermediate step. YWA 1 is a yellow pigment found in mature asexual spores (conidia).	P38993
Asp hemolysin-like protein	Qlucore		32	Belongs to aegerolysin family. Expressed during fruiting initiation in primordia and immature fruiting bodies.	not found

[B] The protein was identified by BLAST in NCBI database.

[U] Protein functions were found in UniProtKB/Swiss-Prot database.

[F] Proteins were functionally categorized according to FunCat. 1, Metabolism; 2, Energy; 4. Storage protein; 10, Cell cycle and DNA processing; 11, Transcription; 12, Protein synthesis, protein fate and protein turnover; 16, Protein with binding function or cofactor requirement (structural or catalytic); 18, Regulation of metabolism and protein function; 20, Cellular transport, transport facilities and transport routes; 30, Cell surface, cellular communication/signal transduction mechanism; 32, Cell rescue, defense and virulence; 34, Interaction with the environment; 42, Biogenesis of cellular components; 99, Unclassified proteins.

Table S3. Pathways involving proteins differentially expressed in *A. fumigatus* upon T-2 exposure

Pathway	Sub-pathway	Reaction	UniProtKB/Swiss-Prot ID of yeast homologue of involved protein	
			upregulated	downregulated
The citric acid (TCA) cycle and respiratory electron transport	Citric acid cycle (TCA cycle)	alpha-ketoglutarate + CoASH + NAD$^+$ => succinyl-CoA + CO$_2$ + NADH + H$^+$		P19262
The citric acid (TCA) cycle and respiratory electron transport	Citric acid cycle (TCA cycle)	(S)-Malate <=> Fumarate + H$_2$O		P08417
The citric acid (TCA) cycle and respiratory electron transport	Citric acid cycle (TCA cycle)	Fumarate + H$_2$O <=> (S)-Malate		P08417
Metabolism of carbohydrates	Pentose phosphate pathway (hexose monophosphate shunt)	alpha-D-glucose 6-phosphate + NADP+ => D-glucono-1,5-lactone 6-phosphate + NADPH + H$^+$		P11412
Metabolism of carbohydrates	Pentose phosphate pathway (hexose monophosphate shunt)	D-fructose 6-phosphate + D-erythrose 4-phosphate <=> sedoheptulose 7-phosphate + D-glyceraldehyde 3-phosphate	P15019	
Metabolism of carbohydrates	Pentose phosphate pathway (hexose monophosphate shunt)	sedoheptulose 7-phosphate + D-glyceraldehyde 3-phosphate <=> D-erythrose 4-phosphate + D-fructose 6-phosphate	P15019	
Metabolism of carbohydrates	Glucose metabolism	D-glyceraldehyde 3-phosphate <=> dihydroxyacetone phosphate	P00942	
Metabolism of carbohydrates	Glucose metabolism	dihydroxyacetone phosphate <=> D-glyceraldehyde 3-phosphate	P00942	
Metabolism of lipids and lipoproteins	Peroxisomal lipid metabolism	trans-2,3-dehydropristanoyl-CoA + H$_2$O => 3-hydroxypristanoyl-CoA	Q02207	
Metabolism of lipids and lipoproteins	Peroxisomal lipid metabolism	3-hydroxypristanoyl-CoA + NAD+ => 3-ketoxypristanoyl-CoA + NADH + H$^+$	Q02207	
Metabolism of lipids and lipoproteins	Peroxisomal lipid metabolism	trans-2,3-dehydrohexacosanoyl-CoA + H2O => 3-hydroxyhexacosanoyl-CoA	Q02207	
Metabolism of lipids and lipoproteins	Peroxisomal lipid metabolism	3-hydroxyhexacosanoyl-CoA + NAD$^+$ => 3-ketohexacosanoyl-CoA + NADH + H$^+$	Q02207	
Metabolism of lipids and lipoproteins	alpha-linolenic acid (ALA) metabolism	Hydration of delta2-tetracosaheptaenoyl-CoA to 3-hydroxy tetracosahexaenoyl-CoA	Q02207	
Metabolism of lipids and lipoproteins	alpha-linolenic acid (ALA) metabolism	Dehydrogenation of 3-hydroxy tetracosahexaenoyl-CoA	Q02207	

Metabolism of lipids and lipoproteins	Synthesis of bile acids and bile salts	25(S) 3alpha,7alpha-dihydroxy-5beta-cholest-24-enoyl-CoA is hydrated to (24R, 25R) 3alpha,7alpha,24-trihydroxy-5beta-cholestanoyl-CoA	Q02207	
Metabolism of lipids and lipoproteins	Synthesis of bile acids and bile salts	(24R, 25R) 3alpha,7alpha,12alpha,24-tetrahydroxy-5beta-cholestanoyl-CoA is oxidized to 3alpha,7alpha,12alpha-trihydroxy-5beta-cholest-24-one-CoA	Q02207	
Metabolism of lipids and lipoproteins	Synthesis of bile acids and bile salts	(24R, 25R) 3alpha,7alpha,24-trihydroxy-5beta-cholestanoyl-CoA is oxidized to 3alpha,7alpha-dihydroxy-5beta-cholest-24-one-CoA	Q02207	
Metabolism of lipids and lipoproteins	Synthesis of bile acids and bile salts	3alpha,7alpha,12alpha-trihydroxy-5beta-cholest-24-enoyl-CoA (THCA-CoA) is hydrated to (24R, 25R) 3alpha,7alpha,12alpha,24-tetrahydroxy-5beta-cholestanoyl-CoA	Q02207	
Metabolism of lipids and lipoproteins	Cholesterol biosynthesis	Lanosterol is oxidatively demethylated to 4,4-dimethylcholesta-8(9),14,24-trien-3beta-ol	P10614	
Metabolism of proteins	Mitochondrial Protein Import	Precursor Proteins Enter TIM23 PAM Complex	P19882	
Metabolism of proteins	Mitochondrial Protein Import	TOM40:TOM70 Complex Imports Proteins Across Mitochondrial Outer Membrane	P19882	
Metabolism of proteins	Mitochondrial Protein Import	MPP Cleaves Presequence of Matrix Precursors	P19882	Q07540
Metabolism of proteins	Mitochondrial Protein Import	TIM23 PAM Complex Imports Proteins to Mitochondrial Matrix	P19882	Q07540
Mitochondrial Iron-Sulfur Cluster Biogenesis		FXN:NFS1:ISD11:ISCU Synthesizes Iron-Sulfur Cluster		Q07540
Mitochondrial Iron-Sulfur Cluster Biogenesis		Frataxin Binds Iron		Q07540
Adaptive Immune System	Antigen processing-Cross presentation	Proteasomal cleavage of substrate		P30657
Adaptive Immune System	Antigen processing-Cross presentation	Proteasomal clevage of exogenous antigen		P30657
Adaptive Immune System	Antigen processing: Ubiquitination & Proteasome degradation	Proteasomal cleavage of substrate		P30657
DNA Repair	ATM mediated response to DNA double-strand break	Phosphorylation of histone H2AX at Serine-139 by ATM at the site of DSB	P04912	
Metabolism of nucleotides	Purine ribonucleoside monophosphate biosynthesis	adenylosuccinate => adenosine 5'-monophosphate + fumarate		Q05911
Metabolism of nucleotides	Purine ribonucleoside monophosphate biosynthesis	SAICAR => AICAR + Fumarate		Q05911

Cell Cycle, Mitotic / DNA replication	Switching of origins to a post-replicative state	Ubiquitinated Orc1 is degraded by the proteasome		P30657
Cell Cycle, Mitotic / DNA replication	Switching of origins to a post-replicative state	Ubiquitinated Cdc6 is degraded by the proteasome		P30657
Cell Cycle, Mitotic / DNA replication	Removal of licensing factors from origins	Ubiquitinated Orc1 is degraded by the proteasome		P30657
Cell Cycle, Mitotic / DNA replication	Removal of licensing factors from origins	Ubiquitinated Cdc6 is degraded by the proteasome		P30657
Regulation of Apoptosis	Regulation of activated PAK-2p34 by proteasome mediated degradation	Proteasome mediated degradation of PAK-2p34		P30657
Gene Expression	Regulation of mRNA Stability by Proteins that Bind AU-rich Elements	Destruction of AUF1 and mRNA		P30657
Gene Expression	mRNA Splicing	ATAC spliceosome mediated 3' splice site cleavage, exon ligation		P33334
Gene Expression	mRNA Splicing	ATAC spliceosome mediated Lariat formation,5' splice site cleavage		P33334
Gene Expression	mRNA Splicing	Formation of AT-AC B Complex		P33334
Gene Expression	mRNA Splicing	Formation of AT-AC C complex		P33334
Metabolism of amino acids and derivatives	Lysine catabolism	alpha-ketoadipate + CoASH + NAD$^+$ => glutaryl-CoA + CO$_2$ + NADH + H$^+$		P19262
Metabolism of amino acids and derivatives	Sulfur amino acid metabolism	S-adenoylhomocysteine is hydrolyzed		P39954
Metabolism of amino acids and derivatives	Urea cycle	carbamoyl phosphate + ornithine => citrulline + orthophosphate		P05150
Metabolism of amino acids and derivatives	Urea cycle	arginine + H$_2$O => ornithine + urea [ARG1]	P00812	
Metabolism of amino acids and derivatives	Urea cycle	arginine + H$_2$O => ornithine + urea [ARG2]	P00812	
Metabolism of amino acids and derivatives	Amino acid synthesis and interconversion (transamination)	glutamine + pyruvate => 2-oxoglutaramate + alanine	P47039	
Metabolism of amino acids and derivatives	Phenylalanine and tyrosine catabolism	phenylalanine + pyruvate => 3-(indol-3-yl)pyruvate + alanine	P47039	

Metabolism of amino acids and derivatives	Tryptophan catabolism	kynurenine + pyruvate => 4-(2-aminophenyl)-2,4-dioxobutanoic acid + alanine [CCBL1]	P47039	
Metabolism of amino acids and derivatives	Tryptophan catabolism	kynurenine + pyruvate => 4-(2-aminophenyl)-2,4-dioxobutanoic acid + alanine [CCBL2]	P47039	
Abnormal metabolism in phenylketonuria		kynurenine + pyruvate => 4-(2-aminophenyl)-2,4-dioxobutanoic acid + alanine [CCBL1]	P47039	
Abnormal metabolism in phenylketonuria		phenylalanine + oxaloacetate => phenylpyruvate + aspartate [CCBL1]	P47039	

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General Discussion

1. Occurrence and control of mycotoxins in bird feeds

Mycotoxins occur typically in agricultural products and their derived food- and feed-stuffs, probably because agricultural products provide nutritional substrates for fungal growth. Due to the adverse effects of dietary mycotoxins on humans and other animals, surveys on mycotoxin occurrence have been focused on foods and feeds, but only limited studies have been carried out on bird feeds. Scudamore et al. (1997) analyzed 15 samples of pet bird feeds for aflatoxins, fumonisins and ochratoxin A, and detected ochratoxin A in 4 feed samples. Maia and Pereira Bastos de Siqueira (2002) detected aflatoxins in 8 out of 30 pet bird feed samples. Martins et al. (2003) analyzed aflatoxins, ochratoxin A, fumonisin B1 and deoxynivalenol (DON) in 20 domestic bird feeds but no mycotoxins were detected. In this thesis, mycotoxins in 10 commercial parrot feeds (5 seed mixed feeds and 5 pelleted feeds) were analyzed by LC-MS/MS. Four pelleted feeds and all the 5 seed mixed feeds were found to be contaminated with mycotoxins, and there were more types of mycotoxins in pelleted feeds than in seed mixed feeds on average. Low levels of mycotoxins seem to be prevalent in bird feeds. Due to the drying process allowing to reduce moisture content, extruded feeds are supposed to be less susceptible to mold growth and mycotoxin contamination (Binder et al., 2007; Bullerman and Bianchini, 2007), but mycotoxin levels in bird feeds are probably more related to the quality of the raw materials than to the production process. Although none of the detected mycotoxins in our parrot feeds exceeded the maximum levels regulated or recommended for poultry and other animals by the European Commission (EC, 2003; EC, 2006; EC, 2013), the pathological changes that occasionally occurred in cockatiels imply that exotic pet birds might be more vulnerable to mycotoxins than domesticated animals, so stricter regulations for exotic pet birds might be necessary. Prevention of fungal growth and invasion is primarily important in preventing mycotoxin contamination in agricultural commodities (FAO, year unknown). The inhibition of fungal growth can be achieved by physical, chemical and biological treatments. Physical treatments include storage at low temperature and humidity (FAO, 1979) and application of gamma-irradiation (WHO, 1988). Chemical treatments involve the application of synthetic antifungal agents such as sodium acetate (Buchanan and Ayres, 1979), malonic acid (Megalla and Hafez, 1982), benzoic acid and derivatives (Chipley and Uraih, 1980; Uraih and Offonre, 1981), or natural phytochemicals such as allicin (Appleton and Tansey, 1977), cinnamon and clove oils (Bullerman et al., 1977). Commercial fungicides containing tebuconazole, prothioconazole or prochloraz also showed good efficacy in inhibiting fungal growth and reducing production of fumonisins and aflatoxins by *Fusarium* and *Aspergillus* (Formenti et al., 2012). Biological

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control methods involve some bacterial and fungal antagonists of the mycotoxigenic fungi (Palumbo et al., 2008). The bacterium *Bacillus subtilis* has been shown to be effective in control of mycotoxin production by *Fusarium* and *Aspergillus* (Formenti et al., 2012). Another effective biocontrol example is the use of nontoxigenic strains of *A. flavus* and *A. parasiticus* in fields of cotton, peanut, maize and pistachio to competitively exclude the aflatoxin-producing strains (Yin et al., 2008). Foods and feeds contaminated with mycotoxins should be disposed, or alternatively decontaminated or detoxified. Heating (particularly under pressure) and gamma-irradiation can reduce aflatoxins to some extents (Chipley and Uraih, 1980; Coomes et al., 1966), and chemicals such as formaldehyde, calcium hydroxide, hydrogen peroxide, sodium bisulfite, sodium hydroxide and aqueous ammonia can also reduce aflatoxins in peanut and corn in lab settings (Codifier et al., 1976; Moerch et al., 1980; Spreenivasamurthy et al., 1967), but the authorization of using chemical additives in feed is strictly regulated by EC (2003, 2009). Commercial mycotoxin binders have been developed to detoxify mycotoxin-contaminated feeds, and are widely used. Bentonite clays are inorganic mycotoxin binders and exhibit a high affinity towards aflatoxins (Diaz-Llano and Smith, 2006; Phillips et al., 1988). A commercial glucomannan mycotoxin binder claims to bind T-2 toxin (T-2) and DON (Goossens et al., 2012). However, the efficacy of mycotoxin binders seems to be inconsistent in different tests, and special attention has to be paid to their interactions with other veterinary medicinal products (Devreese et al., 2013; Goossens et al., 2012). Besides the binding strategy, biotransformation of mycotoxins is also effective in mycotoxin detoxification. For example, fermentation with lactic acid bacteria reduces the toxicity of mycotoxins, and *Eubacteria* spp. can detoxify trichothecenes by cleaving the 12,13-epoxy group with de-epoxidases (Kolosova and Stroka, 2011). A fumonisin esterase produced by a strain of yeast *Komagataella pastoris* can partially degrade fumonisins by cleavage of the diester bonds (EFSA, 2014).

2. Effects of mycotoxins on microorganisms

Among different secondary metabolites of fungi, mycotoxins are grouped together only because of their toxicities on humans and animals (Bennett and Klich, 2003). In the exploration on the nature and biological functions of mycotoxins, it has been found that mycotoxins and other secondary metabolites confer a competitive advantage for the producers over other competitor organisms (Magan and Aldred, 2007). Therefore, the primary targets of mycotoxins seem to be microorganisms instead of animals, although to a broader extent, humans and animals can also be considered the fungi's competitors for food (Janzen, 1977).

Indeed, many mycotoxins such as trichothecenes, zearalenone, patulin, aflatoxin B1, ochratoxin A, citrinin, penicillic acid, cyclopiazonic acid and penitrem A have shown adverse effects on different fungi and/or bacteria (Elmholt, 2008). Other studies also suggested that trichothecenes, e.g. T-2, HT-2 toxin, diacetoxyscirpenol (DAS) and DON have stronger antimicrobial activity on fungi than on bacteria (Burmeister and Hesseltine, 1970; Madhyastha et al., 1994; Vesonder et al., 1981). Interestingly, T-2 and diacetoxyscirpenol have stronger antimicrobial activity than DON on fungi (Vesonder et al., 1981), which is compatible to the higher toxicity of type-A trichothecenes than type-B trichothecenes on humans and animals (Yazar and Omurtag, 2008), and might be associated with the common characteristics of eukaryotic cells. Potential effects of T-2, produced by *Fusarium* spp., on the ubiquitous fungal pathogen *Aspergillus fumigatus* were assessed for the first time in this thesis. Both fungal genera live saprophytically in organic debris in nature and share soil as the common ecological niche (Latgé, 1999; Smith et al., 2007). The co-occurrence of *Fusarium* and *Aspergillus* as well as their mycotoxins (e.g. trichothecenes, fumonisins, and aflatoxins) in the previous soil, food and feed samples further evidenced the overlapping ecological niche of these two fungal genera (Ali et al., 1998; Clarke and Christensen, 1981; Hagler et al., 1984; Reddy and Salleh, 2011; Wang et al., 1995). In this thesis, the growth of *A. fumigatus* was inhibited by T-2 exposure, and as responses to the stress of T-2, *A. fumigatus* modified its proteome expression, resulting in changes of colonial morphology, lower resistance against oxidative stress, and higher virulence in poultry. Although T-2 exposure reduced the tolerance of *A. fumigatus* conidia against oxidative stress, the upregulated expression of some virulence and resistance associated proteins such as heat shock proteins, hydrophobin protein, MFS multidrug transporter and cytochrome P450 monooxygenase might have compensated the tolerance of *A. fumigatus* against antifungal agents (amphotericin B, itraconazole, voriconazole and terbinafine) and the virulence of *A. fumigatus* in infected chickens. Previous studies also demonstrated that the virulence of insect-pathogenic fungus *Metarhizium anisopliae* was influenced by medium nutrients (Maldonado-Blanco et al., 2014; Shah et al., 2005), and an unidentified antifungal metabolite of *Fusarium chlamydosporum* reduced rust disease in groundnut by inhibiting the spore germination of the fungal pathogen *Puccinia arachidis* (Mathivanan and Murugesan, 1999). Studies on mycotoxins have largely focused on their toxicities to humans and animals. However, considering their toxicities on animals and microbes, mycotoxins might have promising potential to be used as pesticides, fungicides, antibiotics and preservatives in areas such as agriculture and pharmacy. For example, since many fungi are more sensitive to trichothecenes than bacteria, use of trichothecenes or their

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detoxified derivatives might be a potential option to prevent fungal contamination during bacterial fermentation processes. A possible disadvantage of a detoxified mycotoxin may be the loss of its antifungal activity, so this is a challenge to solve in the potential application of mycotoxins.

3. The impact of dietary T-2 on birds' health and susceptibility to *Aspergillus* infection

Intake of feed containing T-2 has exhibited different adverse effects on chickens. Reduced feed consumption and weight gain have been widely reported in poultry exposed to 0.1 to 20 mg T-2/kg feed for 2 to 7 weeks (Diaz et al., 2005; Osselaere et al., 2013; Rezar et al., 2007; Wyatt et al., 1975), and necrosis of mucous membranes of the oral cavity, tongue and intestines was also frequently observed (Rafai et al., 2000; Sklan et al., 2001; Weber et al., 2010). In laying hens, exposure to 0.2 to 20 mg T-2/kg feed for 2 to 4 weeks may reduce egg production (Diaz et al., 1994; Ványi et al., 1994; Wyatt et al., 1975). Osselaere et al. (2013) studied the responses of enzymes and proteins associated with the metabolism of T-2 in chickens. Genes encoding drug-metabolizing enzymes CYP1A4, CYP1A5 and CYP3A37, and multidrug resistance-associated protein 2 (MRP2) were downregulated in the liver, and the activity of the drug-metabolizing enzyme CYP3A was increased in the ileum and the liver after 3 weeks exposure to 752 μg T-2/kg feed (Osselaere et al., 2013). Like other trichothecenes, T-2 can affect the course of infectious diseases. Dietary T-2 caused immunosuppression against Newcastle disease virus in broiler chickens (Kamalavenkatesh et al., 2005; Weber et al, 2006), and also presented a profound negative effect on the ability of the chickens to resist *Salmonella* Typhimurium infection, as indicated by the increased mortality of chickens (Ziprin and Elissalde, 1990). This thesis revealed that dietary T-2 increased aspergillosis development in chickens infected with *A. fumigatus* conidia, and the increase was most noticeable in chickens infected with conidia pre-exposed to T-2. The impaired antifungal activities of macrophages seen in this thesis are believed to contribute to the increase of aspergillosis in T-2 exposed chickens, as avian macrophages form the first immune defense against *Aspergillus* infection (Arné et al., 2011; Beernaert et al., 2010). This thesis demonstrated that exposure to T-2 also stimulated the expression of some pro-inflammatory cytokines and chemokines in both *Aspergillus* infected and uninfected chicken macrophages. These results therefore support the view that T-2 can both stimulate and suppress immunity (Sokolovic et al., 2008). Macrophages sense T-2 and other trichothecenes as danger signals, which stimulate the immune response including the activation of genes important for the inflammatory response (Kankkunen et al., 2009; Sokolovic et al., 2008).

Immunosuppression caused by T-2 is thought to be due to the damage of bone marrow, lymph nodes, spleen, thymus, intestinal mucose, leucopenia and the inhibition of the functions of immune cells (EFSA, 2011; Sokolovic et al., 2008). For example, exposure to T-2 induced apoptosis in thymus (Venkatesh et al., 2005) and increased DNA fragmentation in spleen leukocytes in chickens (Frankic et al., 2006; Rezar et al., 2007). Decrease in the size of bursa of Fabricius and accelerated thymus involution were observed in turkeys dieted with T-2 (Richard et al., 1978). Lymphocyte depletion was observed histologically in spleen and bursa of Fabricius in Pekin ducks exposed to T-2 (Rafai et al., 2000). However, the specific mechanisms by which T-2 influences the avian innate and adaptive immunity against *Aspergillus* need to be studied further.

4. Effects of mycotoxins on the hosts VS effects of mycotoxins on the pathogens

In contrast with most studies previously conducted, this thesis describes the effects of mycotoxins both from a host and pathogen perspective. Pathogen virulence may be altered by exposure to mycotoxins either before infection (in the environment) or during infection of an intoxicated host. Reports of direct antimicrobial activities of mycotoxins on pathogens are scarce. Neither DON, T-2 or DAS exerted any obvious antimicrobial effect on the pathogenic bacterium *Bordetella bronchiseptica* (Vesonder et al., 1981). ZEA proved toxic to the opportunistic respiratory fungal pathogen *Alternaria alternata* (Utermark and Karlovsky, 2007, Wiest et al., 1987). However, *in vivo* relevance of this finding was not demonstrated. Here, we provide evidence that environmental exposure of *A. fumigatus* to T-2 levels up to 1 μg/ml reduces growth, dramatically alters the fungal proteome without affecting fungal viability, and increases fungal virulence for the avian host.

Although exposure of pathogens to these relatively high mycotoxin concentrations in a host is not unlikely, the direct toxic effects of these levels on host health, such as reduced appetite and weight gain, and lesions in the digestive tract in pigs and poultry (EFSA, 2004; EFSA, 2011a; EFSA, 2011b; Fairchild et al., 2005), will probably outweigh the more subtle impact they may exert on host-pathogen interactions. However, T-2 exerted a marked negative impact on avian macrophage functioning and thus on bird – *A. fumigatus* interaction at concentrations far below those known to directly affect fungal fitness or overall avian health. Indeed, very low level exposure of avian macrophages to T-2 reduced their ability to limit *A. fumigatus* growth. Similar effects of low levels of T-2 on a.o. macrophage functioning have been demonstrated in pigs (Verbrugghe et al., 2012). These results thus add to the

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evidence that T-2 already severely affects animal health at concentrations significantly below those allowed in animal feed, by interfering with host-pathogen interactions

5. Suggestions for further studies

Based on the results of this thesis and on literature, the following topics are recommended for future studies:

1. Effects of other mycotoxins and fungal secondary metabolites on the growth and virulence of *Aspergillus*. This thesis initiated this topic with the effect of *Fusarium* T-2 toxin on *A. fumigatus*. This thesis has shown the influence of T-2 on the fitness and virulence of *A. fumigatus*, but the effects of other frequently detected mycotoxins (e.g. ZEA, DON, and fumonisins) and microbial secondary metabolites are not known, so more studies on this topic are needed.

2. The innate and adaptive immunity of avian species in response to fungal infections. The immune responses of avian species against fungal infections are not as clear as those of humans and mammals. For example, a better understanding of the exact roles of avian heterophils and dendritic cells in fungal infection may help to develop better control measures for avian mycosis.

3. The mechanisms by which T-2 and other mycotoxins affect the avian immune functions. Many mycotoxins are known to be immunotoxic, but their immunotoxicity has mainly been studied in mammals, while birds have largely been neglected.

4. Potential commercial value of mycotoxins. Antibiotics, the bactericidal secondary metabolites of fungi, have been widely used in medication. A question can be asked whether environment friendly products can be developed from mycotoxins to control pests, bacteria and fungi in agriculture. For example, trichothecenes might get huge commercial value if they could be modified in a way that they become non-toxic for humans and animals while keeping their antifungal activity.

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Summary/Samenvatting

Mycotoxins are toxic secondary metabolites of fungi. They can be produced by fungi in a variety of environments, affecting the growth and virulence of other co-inhabiting microorganisms. Agricultural products and their derived food- and feed- stuffs can be easily invaded by fungi during production and storage, resulting in mycotoxin contamination. Mycotoxins can be oestrogenic, carcinogenic, neurotoxic, and immunotoxic. Ingestion of mycotoxins from contaminated agricultural products can impair human and animal health. Aspergillosis is an opportunistic fungal disease mainly caused by *Aspergillus fumigatus*. Avian species are highly susceptible to aspergillosis, which strikes depending on factors including the amount and virulence of inhaled *Aspergillus* conidia and the immune status of the birds. The hypothesis of this thesis was that mycotoxins in avian feed alter the bird's susceptibility to aspergillosis. This thesis thus aimed to assess the effects of mycotoxins on avian health and avian aspergillosis.

The first study investigated the occurrence of mycotoxins in commercial bird feeds, and their pathological effects in pet birds. The occurrence of mycotoxins in commercial parrot feeds (5 seed mixes and 5 pelleted feeds) was analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS). The following 12 mycotoxins were detected: zearalenone, deoxynivalenol, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, fusarenon-X, aflatoxin B1, sterigmatocystin, alternariol, alternariol methylether, fumonisin B1, fumonisin B3, and ochratoxin A. Zearalenone was found to be the most prevalent. Pathological effects after 21 days feeding mycotoxin-contaminated diets were examined in an *in vivo* trial with 3 groups of 5 cockatiels: Group 1 (control) was fed a non-contaminated extruded feed; Group 2 was fed a extruded feed containing zearalenone, deoxynivalenol, 15-acetyldeoxynivalenol, and fumonisins; and Group 3 was fed a extruded feed containing fumonisins. Average body weight gain and relative organ weight were not significantly different between the treatment groups and the control group. Apoptosis of renal tubular cells, diarrhoea, reduced appetite, enlargement of liver, kidney and proventriculus were occasionally observed in the birds from Groups 2 and 3. In summary, contamination with mycotoxins is common in parrot feeds. The mycotoxin levels did not reach toxic levels, but might pose a threat to some cockatiels.

The respiratory macrophages of birds provide an important first line of defense against *Aspergillus* infection. Therefore, the second study evaluated the impact of T-2 toxin (T-2), a potent immunosuppressive mycotoxin produced by *Fusarium* spp., on the antifungal response of chicken macrophages against *A. fumigatus*. The chicken macrophage cell line HD-11 was first exposed to 0.5 to 10 ng/ml T-2 for 24 h, and then the viability, antifungal activity, and cytokine expression of the macrophages in response to *A. fumigatus* conidial infection were

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determined. Macrophage viability decreased when cells were exposed to T-2 at concentrations higher than 1 ng/ml. One hour after conidial infection, phagocytosed conidia were observed in 30% of the non-T-2-exposed macrophages, but in only 5% of the macrophages exposed to 5 ng/ml T-2. Seven hours after infection, 24% of the conidia associated with non-T-2-exposed macrophages germinated, in contrast to 75% of those with macrophages exposed to 5 ng/ml T-2. *A. fumigatus* infection induced upregulation of interleukin (IL)-1β, CXCLi1, CXCLi2 and IL-12β, and downregulation of transforming growth factor-β4 (TGF-β4) in chicken macrophages. Pre-exposure of *A. fumigatus*-infected macrophages to T-2 at 1 to 5 ng/ml further upregulated the expression of IL-1β, IL-6, CCLi2, CXCLi1, CXCLi2, IL-18 (at 1 and 2 ng/ml) and IL-12β, and further downregulated that of TGF-β4 (at 5 ng/ml). In summary, T-2 impaired the antifungal activities of chicken macrophages against *A. fumigatus* conidia, but might stimulate immune response by upregulating the expression of pro-inflammatory cytokines, chemokines and T-helper 1 cytokines.

The third study assessed the influence of environmental T-2 on the fitness and virulence of *A. fumigatus*, and examined the effect of dietary T-2 on the development of aspergillosis in experimentally infected chickens. *A. fumigatus* was first cultured on agar media containing 0 to 1000 ng/ml of T-2. Viability, morphology, growth rate, proteome expression, and susceptibility to antimycotics and oxidative stress were deteremined. Results showed that exposure to 1000 ng/ml T-2 did not reduce the viability of *A. fumigatus*, but inhibited its growth, with wrinkling and depigmentation of the colonies. Proteomic analysis revealed 21 upregulated proteins and 33 downregulated proteins, including those involved in stress response, pathogenesis, metabolism, and transcription. The proteome seems to have shifted to enhance the glycolysis, catabolism of lipids, and amino acid conversion. Assays on fungal susceptibility to antimycotics and oxidative stress showed that T-2 exposure did not affect the minimal inhibitory concentrations of amphotericin B, itraconazole, voriconazole and terbinafine against *A. fumigatus*, but increased the susceptibility of *A. fumigatus* to H_2O_2 and menadione. Experimental inoculation of chickens with *A. fumigatus* showed that pre-exposure of *A. fumigatus* to T-2 exacerbates aspergillosis, especially when chickens were fed a T-2-containing diet. In summary, *A. fumigatus* is capable of surviving and growing in substrates containing levels of T-2 up to 1000 ng/ml. Growth in the presence of T-2 induces a stress response in *A. fumigatus*, which is associated with exacerbation of aspergillosis *in vivo*.

In conclusion, the impact of T-2 on avian aspergillosis is not unambiguous since the toxin exerts negative effects both on innate immune mechanisms of the host and on fungal fitness. However, the fungal stress response induced by exposure to T-2 and the suppression

of antifungal host macrophage activity appear to outweigh the pro-inflammatory host response, resulting in exacerbation of avian aspergillosis if the fungus and/or the birds have been exposed to T-2.

Samenvatting

Mycotoxines zijn secundaire metabolieten van fungi die geproduceerd kunnen worden in tal van situaties en omgevingen. Grondstoffen voor voeder- en voedingsproducten zijn vaak gecontamineerd met mycotoxines en komen op deze manier in het voedsel en voeder van mens en dier terecht. Mycotoxines kunnen onder andere carcinogene, neurotoxische en immunotoxische eigenschappen bezitten. Opname ervan kan dan ook zowel bij mens als dier nadelige effecten hebben. Anderzijds kunnen ook micro-organismen, zoals andere schimmels, beïnvloed worden door mycotoxines. *Aspergillus fumigatus* is een vaak voorkomende omgevingscontaminant. Deze schimmel kan een opportunistische mycose veroorzaken die aangeduid wordt als aspergillose en vooral voorkomt bij vogels.

De hypothese van deze thesis is dat mycotoxines in het voeder van vogels een invloed hebben op de gevoeligheid van deze dieren voor aspergillose. Daarom werd nagegaan wat de effecten zijn van mycotoxines op de gezondheidsstatus van vogels en op een infectie met *A. fumigatus*.

In een eerste studie werd nagegaan of commerciële vogelvoeders gecontamineerd zijn met mycotoxines en wat de pathologische gevolgen hiervan zijn bij gezelschapsvogels. Tien verschillende commerciële voeders voor papegaaien (5 zadenmengsels en 5 gepelleteerde voeders) werden via LC-MS/MS onderzocht op de aanwezigheid van mycotoxines. In totaal werden 12 verschillende mycotoxines gedetecteerd, namelijk zearalenone, deoxynivalenol, 3-acetyldeoxynivalenol, 15-acetyldeoxynivalenol, fusarenone X, aflatoxine B1, sterigmatocystine, alternariol, alternariol methylether, fumonisine B1, fumonisine B3 en ochtratoxine A. Zearalenone bleek de meest voorkomende contaminant te zijn. Om na te gaan wat de pathologische gevolgen zijn van de opname van met mycotoxine gecontamineerd voeder, werden 3 geëxtrudeerd voeders van de 5 hierboven vernoemde gepelleteerde voeders getest in telkens 5 valkparkieten. Groep 1 (controle groep) kreeg mycotoxine vrij voeder, groep 2 kreeg voeder gecontamineerd met zearalenone, deoxynivalenol, 15-acetyldeoxynivalenol en fumonisines, en het voeder van groep 3 was gecontamineerd met fumonisines. Na 21 dagen voederen bleek dat de gemiddelde gewichtstoename en de relatieve orgaangewichten in groep 2 en 3 niet significant verschilden van de controle groep. Toch werden er in groep 2 en 3 bij enkele dieren abnormaliteiten waargenomen zoals apoptose van tubulaire niercellen, diarree, verminderde eetlust en een vergroting van de lever, nier en proventriculus. Samengevat kunnen we concluderen dat contaminatie van papegaaienvoer met mycotoxines een vaak voorkomend probleem is. Deze contaminatie zou bij langdurig voederen gezondheidsproblemen bij gezelschapsvogels kunnen veroorzaken.

De alveolaire macrofagen van vogels vormen een belangrijke eerstelijnsdefensie tegen *A. fumigatus* infecties. In een tweede studie werd daarom nagegaan wat de invloed is van T-2 toxine (T-2), een sterk immunosuppressief mycotoxine, op de antifungale respons van kippenmacrofagen tegen *A. fumigatus*. Hierbij werd gebruik gemaakt van een HD-11 kippenmacrofagen cellijn. Deze cellen werden eerst gedurende 24 uur blootgesteld aan T-2 (0.5-10 ng/ml) en daarna geïnoculeerd met *A. fumigatus* conidia. De antifungale activiteit en de cytokine expressie werden vervolgens bepaald. Reeds heel lage concentraties aan T-2 (> 1 ng/ml) waren toxisch voor de HD-11 cellen. Daarenboven had T-2 een invloed op de fagocytose en ontkieming van *A. fumigatus* conidia. Eén uur na de infectie werden in 30% van de onbehandelde macrofagen gefagocyteerde conidia geobserveerd, tegenover slechts in 5% van de met T-2 (5 ng/ml) behandelde macrofagen. T-2 heeft dus een negatieve invloed op het vermogen van macrofagen om conidia te fagocyteren. Zeven uur na infectie bleek 24% van de conidia zich te ontkiemen tot hyfen in de onbehandelde macrofagen, terwijl dit steeg tot 75% bij de met T-2 behandelde macrofagen. T-2 zorgt er dus voor dat meer conidia ontkiemen tot hyfen, wat erop kan wijzen dat *A. fumigatus* gemakkelijker kan aanslaan bij vogels die blootgesteld zijn aan T-2. Blootstelling van HD-11 cellen aan *A. fumigatus* conidia veroorzaakte een opregulatie van IL-1β, CXCLi1, CXCLi2 en IL-12β, en een downregulatie van de TGF- β4. Een voorafgaande blootstelling van de macrofagen aan T-2 (1-5 ng/ml) resulteerde na inoculatie met conidia in een verdere stijging van de expressie van IL-1β, IL-6, CCLi2, CXCLi1, CXCLi2, IL-18 (bij T-2 concentraties van 1 en 2 ng/ml) en IL-12β. Bij een behandeling met 5 ng/ml T-2 werd een verdere daling in de expressie van TGF-β4 waargenomen. Samengevat kunnen we besluiten dat T-2 een negatieve invloed heeft op de antifungale eigenschappen van kippenmacrofagen tegen *A. fumigatus* conidia, maar ook de expressie van pro-inflammatoire cytokines, chemokines en T-helper 1 cytokines stimuleert.

In een derde studie werd de invloed nagegaan van T-2 als omgevingscontaminant op de "fitness" en virulentie van *A. fumigatus*. Daarenboven werd in deze studie onderzocht wat het effect is van het voorkomen van T-2 in kippenvoeder op de ontwikkeling van aspergillose bij experimenteel geïnfecteerde kippen. Om het effect van T-2 op de viabiliteit, morfologie, groei, eiwitexpressie en gevoeligheid tegen antimycotica en oxidatieve stress na te gaan, werd *A. fumigatus* gecultiveerd op agar media, al dan niet gesupplementeerd met T-2 (0-1000 ng/ml). De resultaten tonen aan dat een blootstelling van *A. fumigatus* aan T-2, tot een concentratie van 1000 ng/ml, geen effect heeft op de leefbaarheid van de schimmel, maar wel de groei ervan inhibeert. Dit fenomeen ging gepaard met rimpeling en depigmentatie van de kolonies. Via een proteoomanalyse werd aangetoond dat T-2 een opregulatie en

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downregulatie van respectievelijk 21 en 33 eiwitten veroorzaakt, waaronder eiwitten betrokken in de stress respons, pathogenese en het transcriptie metabolisme. Er werd een verschuiving van het proteoom waargenomen richting een verhoogde glycolyse, katabolisme van lipiden en aminozuur metabolisme. Blootstelling van *A. fumigatus* aan T-2 bleek geen invloed te hebben op de minimale inhibitorische concentraties van amfotericine B, itraconazole, voriconazole en terbinafine tegen de schimmel, maar het induceerde wel een verhoogde gevoeligheid voor H_2O_2 en menadione. Kippen die experimenteel besmet werden met *A. fumigatus* die opgegroeid werd in aanwezigheid van T-2 ontwikkelden ergere vormen van aspergillose dan wanneer de inoculatie gebeurde met *A. fumigatus* die niet behandeld werd met T-2, vooral wanneer de kippen ook T-2 gecontamineerd voeder kregen. Samengevat kan gesteld worden dat *A. fumigatus* in staat is om te overleven en groeien in substraten die gecontamineerd zijn met T-2, zelfs wanneer dit mycotoxine in heel hoge concentraties aanwezig is (1000 ng/ml). De groei van *A. fumigatus* in de aanwezigheid van T-2 induceert echter een stressrespons in de schimmel, wat gepaard gaat met een ernstiger verloop van de infectie bij kippen.

De resultaten van deze thesis tonen aan dat commerciële vogelvoeders vaak gecontamineerd zijn met lage hoeveelheden van verschillende mycotoxines. Dit kan negatieve gevolgen hebben op de gezondheid van gevoelige gezelschapsvogels. De impact van T-2 op aspergillose bij vogels is echter dubbelzinnig aangezien het toxine zowel negatieve effecten uitoefent op de aangeboren afweermechanismen van de gastheer, als op de algemene toestand van de schimmel. Toch blijkt de T-2-geïnduceerde stressrespons in de schimmel en de suppressie van de antifungale activiteit van gastheermacrofagen een overwicht te hebben. Finaal resulteert dit in een ernstiger verloop van de *Aspergillus* infectie bij vogels als de schimmel en/of de vogels blootgesteld zijn aan T-2.

Curriculum Vitae

Shaoji Li was born on February 7, 1982, in Xinhui County (Currently Xinhui District of Jiangmen City), on the beautiful subtropical coast of Guangdong Province, China. He studied in Hongwei Primary School and then in Xinhui No. 1 Middle School for his primary and secondary educations. From 2001, he studied Biological Engineering at Northeastern University, in Shenyang, the capital city of Liaoning Province, and obtained a bachelor's degree in 2005. He continued his study on the same major in the graduate school of Inha University, Incheon, South Korea, where he was supervised by Prof. Jae-Seong So and specifically studied probiotic *Lactobacillus*. After graduation in 2007, he worked as a researcher in Gil Medical Center shortly and then in the Korea Centers for Disease Control and Prevention (CDC) until 2009. After that, he was employed as a product specialist by Tegent Technology in Guangzhou City, Guangdong Province, China until 2010.

In July 2010, he started his doctoral study at the Department of Pathology, Bacteriology and Avian Diseases, Faculty of Veterinary Medicine, Ghent University, Belgium. His doctoral research was funded by the Special Research Fund (BOF) of Ghent University, under the guidance of Prof. An Martel, Prof. Frank Pasmans and Prof. Freddy Haesebrouck. He performed 4 years of research on mycotoxicosis and aspergillosis in birds, and his research resulted in several scientific publications in international journals and several presentations at academic conferences.

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