Mycotoxins in swine production

Isabelle P. Oswald

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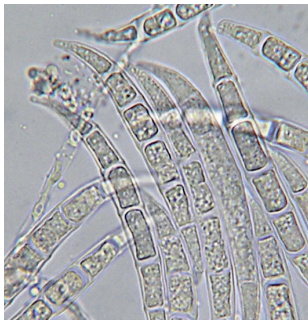

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MYCOTOXINS IN SWINE PRODUCTION

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FOREWORD

Mycotoxins are not a new topic and they are certainly not a recent worry. In fact, this year it is a decade since the *Council for Agricultural Science and Technology* (CAST) released its scientific task force report on mycotoxins and their impacts on plant, animal and human systems. Back then, economic impacts of mycotoxins in the United States alone were estimated to exceed 1.4 billion dollars, through losses in commodity quality and health of livestock. Actually, it is quite surprising to see how these very low molecular weight molecules have affected humans for thousands of years. In the 7th and 8th centuries BC god Robigus was honored in an annual festival aiming to protect cereal crops and trees from rust and mildew. Between 1692 and 1693, the Salem witch trials culminated in dozens of hangings and terrible suffering to hundreds of people accused of witchcraft. Medical explanations based on today's scientific knowledge suggest ingestion of rye bread infected with *Claviceps purpurea* fungus and derived ergot alkaloid-mycotoxins as the most plausible causative agent for the convulsive symptoms presented by the presumed witches.

As far as livestock is concerned, general interest in this field arose later on, in the 20th century, as aflatoxins were identified as the cause of death of thousands of turkey poults in England. Since then hundreds of other secondary metabolites of fungi have been identified and swine have been generally acknowledged as one of the most sensitive species to their effects amongst livestock animals. Also, Food and Agriculture Organization of the United Nations (FAO) estimates that by 2050 global agricultural production must grow 60 % above 2005-07 levels in order to meet the increasing demand of an escalating world population. By then, world meat production is expected to double. Livestock systems will have to become more efficient at a time of ever-increasing prices for commodities, labor and natural resources. Nutrition has a crucial role in the maximization of animal genetic potential, representing around 60 to 80 % of total production costs and poses a constant challenge: rations should satisfy the nutritional needs of animals, without posing a risk to their health. As increasing prices of raw materials make cheaper commodities more appealing to the producer, the well-documented increase in mycotoxin occurrence around the world, highlights the risks associated with this strategy.

These were some of the issues behind our choice to combine two topics - swine and mycotoxins - into one publication. This book is intended to be read by those involved in swine production, whether nutritionists or veterinarians, by students aiming to increase their understanding of the topic, or merely by curious minds.

We have gathered the knowledge of several experts and compiled information on a wide range of topics - from general concepts on production of mycotoxins by fungi, to their effects on swine performance, fertility and immunity. We have looked at worldwide existing legislation for mycotoxins, on how to make analysis of these toxic substances more accurate and we have included an overview on forefront mycotoxin management strategies.

I sincerely thank all who have made this book possible, especially Dr. med. vet. Maximilian Schuh, *Professor emeritus* at the department of Farm Animals and Herd Management, University of Veterinary Medicine, Vienna, Austria and Dr. Isabelle Oswald, research director at the Research Center in Food Toxicology (ToxAlim) in INRA, the leading European agricultural research institute located in France. Besides providing crucial information on specific topics related to their fields of expertise and years of research, they provided valuable inputs to the whole publication. I would also like to acknowledge my colleagues Roman Labuda, Project Leader at the R&D Department of Romer Labs

Holding GmbH, Austria and Georg Häubl R&D Manager at Romer Labs Holding GmbH, Austria, for sharing their expertise in the fields of mycology, microbiology and chemistry. A very big word of appreciation goes to my fellow workers Elisabeth Pichler (Romer Labs, Austria), Roger Berríos and my very good colleague and great team player Karin Nährer for managing this bold project amidst their very busy schedules. Thank you to other contributors to the book including Ursula Hofstetter, Eva Maria Binder and Yin-Jung Liu for their inputs on the whole manuscript and other colleagues for allowing us to use photographs gathered during their regular encounters with mycotoxicoses in the field. Last but not least, thank you Sarah Keeling of Context Products Ltd publishers for handling all my requests and pickiness in such a careful, professional and friendly manner.

It is BIOMIN's duty as a leader in Mycotoxin Risk Management strategies and a worldwide expert in animal nutrition to identify the enemies hindering efficient animal nutrition and to present you tools to counteract them.

We hope you enjoy reading this book and above all that you find it helpful in your daily activities.

Inês Rodrigues

CONTENTS

1. MYCOTOXINS – GENERAL CONCEPTS

KARIN NÄHRER

(Adapted from Chapter 2 of Guide to Mycotoxins: Näehrer, 2012)

1.1. What are mycotoxins?

Mycotoxins are naturally occurring secondary metabolites produced by certain moulds/fungi as a result of their organic processes. They are chemical compounds of low molecular weight and low immunogenic capacity (Mallmann and Dilkin, 2007). There are some secondary metabolites of fungal origin with medicinal or industrial applications, for example Penicillin. Unfortunately, most mycotoxins are known to hazardously contaminate crops and consequently animal feeds and animal products, causing significant economic losses associated with their impact on animal and human health, animal productivity and domestic and international trade.

In practical terms, the mycotoxins which cause bigger economic impacts in animal production are aflatoxins (Afla), trichothecenes (namely deoxynivalenol (DON) and T-2 toxin (T-2)), zearalenone (ZEN), ochratoxin A (OTA), fumonisins (FUM) and ergot alkaloids (Ergots). These will be the focus in the chapters of this book.

1.2. Mycotoxin producing fungi

The process of mycotoxin production by fungi is, in general, not well known. However, taking into account that 1) fungi, just like any living organism need nutrients to survive and 2) crops subjected to stress (namely droughts, poor fertilization, water excess etc.) usually present higher mycotoxin contamination, a possible explanation is that mycotoxins are produced by fungi so that they are able to prevail in adverse conditions. To put it simply, mycotoxins are produced so that fungi win a competitive advantage on other organisms (Rankin and Grau, 2002). Unfortunately, even if we cannot accurately explain the reasons for their existence, mycotoxins are produced in several stages of production of agricultural products, representing a serious problem of worldwide occurrence.

Despite the fact that proper conditions for growth of fungi can occur at all times during crop growth, harvest and storage, fungal species can be roughly divided into field moulds, which infect crops as parasites, and storage fungi which grow in feedstuffs stored under sub-optimal conditions.

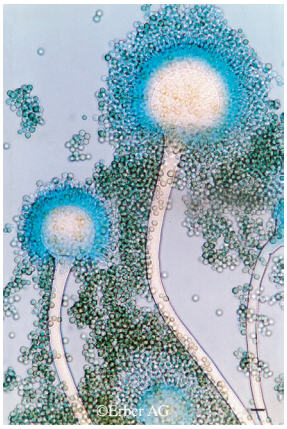
Field fungi are those which in general require higher moisture to grow and produce mycotoxins (> 0.9 water activity), infecting seeds and plants in the field, namely *Fusarium* sp. Storage fungi are those which require lower water activity, thus being more prominent after harvest and during storage, such as *Aspergillus* and *Penicillium* sp.

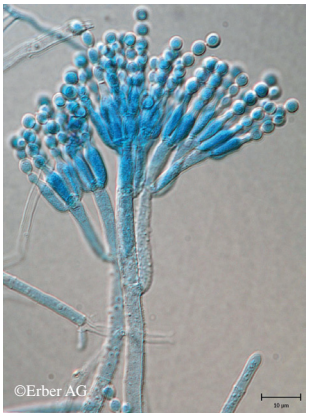
Infection by *Claviceps* sp. and *Neotyphodium* sp. occurs only in the field. *Claviceps* are plant pathogens that replace plant structures such as grain kernels with hardened fungal tissues called ergots or sclerotia (Tudzynski et al., 2001). These fungal bodies often contain a broad

range of toxic compounds called ergot alkaloids (more than 40 different ergot alkaloids are known) leading to a disease known as ergotism (one of the oldest recognized mycotoxicoses) (CAST, 2003; Flieger et al., 1997). The alkaloid pattern and individual alkaloid contents in sclerotia vary largely, due to differences in the maturity of the sclerotia and to other factors e.g.: fungal strain, host plant, geographical region and general weather conditions (EFSA, 2011). *Claviceps* genus, mainly *Claviceps purpurea*, parasitizes more than 600 plants, including some of the economically important cereal grains such as rye, wheat, barley, millet, and oats (Strickland et al., 2011). In addition, ergot contamination in sorghum due to *Claviceps africana* has been discovered in which the *Claviceps* spores germinate and grow into the unfertilized seed producing a sclerotium (Krska and Crews, 2008). Toxic alkaloids are also produced by fungal endophytes such as *Neotyphodium* sp. that colonize vegetative tissue as well as reproductive tissue in certain plant species such as perennial rye grass and tall fescue; thus only a concern for grazing animals (EFSA, 2005).

In Table 1 the most important fungal species are listed together with their respective produced mycotoxins.

Table 1 – The most important mycotoxins and respective producing fungi (Source: www.mycotoxins.info)

Major classes of mycotoxin-producing fungi	Fungi species	Mycotoxins
Aspergillus 	*A. flavus* *A. parasiticus* *A. nomius* *A. pseudotamarii*	Aflatoxin (B_1, B_2, G_1, G_2)
	A. ochraceus	Ochratoxin (Ochratoxin A)
	A. clavatus *A. terreus*	Patulin
	A. flavus *A. versicolor*	Cyclopiazonic acid
Claviceps 	*C. purpurea* *C. fusiformis* *C. paspali* *C. africana*	<u>Ergot alkaloids:</u> Clavines (Agroclavine) Lysergic acids Lysergic acid amids (Ergin) Ergopeptines (Ergotamine, Ergovaline)

Fusarium  	*F. verticillioides* *(syn. F. moniliforme)* *F. proliferatum*	Fumonisin (B_1, B_2, B_3) Fusaric Acid Moniliformin
	F. graminearum *F. avenaceum* *F. culmorum* *F. poae* *F. equiseti* *F. crookwellense* *F. acuminatum* *F. sambucinum* *F. sporotrichioides*	<u>Type-A Trichothecenes</u> T-2 toxin, HT-2 toxin, diacetoxyscirpenol <u>Type-B Trichothecenes</u> Nivalenol, deoxynivalenol, 3- and 15-acetyldeoxynivalenol, fusarenon-X Moniliformin
	F. graminearum *F. culmorum* *F. sporotrichioides*	Zearalenone
Penicillium  	*P. verrucosum* *P. viridicatum*	Ochratoxin (Ochratoxin A)
	P. citrinum *P. verrucosum*	Citrinin
	P. roqueforti	Roquefortine PR toxin
	P. cyclopium *P. camemberti*	Cyclopiazonic acid
	P. expansum *P. claviforme* *P. roquefortii*	Patulin
Neotyphodium ***(formerly Acremonium)***	*N. coenophialum*	<u>Tall fescue toxins:</u> Ergot alkaloids, lolines, peramine
	N. lolii	<u>Tall fescue toxins:</u> Lolitrems, peramine, ergot alkaloid (ergovaline)

1.3. Conditions for fungal growth and mycotoxin production

Although there are geographic and climatic differences in the production and occurrence of mycotoxins, exposure to these substances is worldwide (Kuiper-Goodman, 2004). Nevertheless, a preferred pattern in terms of temperature and water activity for fungal growth and mycotoxin production can be established to some extent (Table 2) (Marth, 1992; Sweeney and Dobson, 1998; FAO, 2003; Hussein and Brassel, 2001; CAST, 2003; Sanchis, 2004; Ribeiro et al., 2006).

Table 2 - Preferred temperatures and water activity values for fungal growth and mycotoxin production

Fungus species	Temperature range for fungal growth [°C]		
	Minimum	Optimum	Maximum
Aspergillus flavus	10-12	25-35	42-43
A. parasiticus	10-12	32-35	42-43
A. ochraceus	8	24-37	37
Penicillium verrucosum	0	20	31-35
Fusarium verticillioides	2-5	22.5-30	32-37
F. proliferatum	4	30	37
F. culmorum	0–10	20–25	31-35
F. poae	5–10	20–25	35
F. avenacum	5–10	20–25	35
F. tricinctum	5–10	20–25	35
F. graminearum	-	24-26	-
F. sporotrichioides	-2	21-27.5	35
Claviceps purpurea	9-10	18-22	-

Fungus species	Temperature range for mycotoxins formation [°C]		
	Minimum	Optimum	Maximum
Aspergillus flavus	12-15	30-33	37-40
A. parasiticus	12	33	40
A. ochraceus	12-15	25-31	37
Penicillium verrucosum	4	20-25	-
Fusarium verticillioides	10	15-30	37
F. proliferatum	10	15-30	37
F. culmorum	11	29-30	-
F. graminearum	11	29-30	-
Claviceps purpurea	-	18-20	-

Table 2 - Contd.

Fungus species	Water activity (a_w) for fungal growth		
	Minimum	Optimum	Maximum
Aspergillus flavus	0.80	0.95-0.99	-
A. parasiticus	0.83-0.84	0.95- 0.99	-
A. ochraceus	0.77-0.79	0.95-0.99	-
Penicillium verrucosum	0.80	0.95	-
Fusarium verticillioides	0.87-0.9	-	0.99
F. proliferatum	0.9	-	-
F. culmorum	0.90-0.91	0.98-0.995	-
F. poae	0.90-0.91	0.98-0.995	-
F. avenacum	0.90-0.91	0.98-0.995	-
F. tricinctum	0.90-0.91	0.98-0.995	-
F. graminearum	0.9	-	0.99
F. sporotrichioides	0.88	-	0.99

Fungus species	Water activity (a_w) for mycotoxins formation		
	Minimum	Optimum	Maximum
Aspergillus flavus	0.82	0.99-0.996	0.998
A. parasiticus	0.87	0.99	-
A. ochraceus	0.80-0.85	0.98	-
Penicillium verrucosum	0.83-0.86	0.90-0.95	-
Fusarium verticillioides	0.92-0.93	-	-
F. proliferatum	0.93	-	-
F. graminearum	0.9-0.91	0.98	-

For example, aflatoxins are produced by *A. flavus* over the temperature range of 12 – 48°C (optimum growth is at 37°C). It is not possible to specify an optimum temperature for the production of the toxins, although production between 25 – 30°C is reported to be significantly greater at higher rather than at lower temperatures. In general, temperature and drought stress are also likely to predispose the plant to increased infection (CAST, 2003).

In the case of *F. graminearum* growth, the optimum temperature has been estimated at 24 – 26°C and a minimum water activity of 0.90 (Sweeney and Dobson, 1998). Different information is available regarding the most favorable temperature for the production of trichothecenes and zearalenone by this fungus. In general, the production of these mycotoxins is ubiquitous, more prevalent in warm and moderate climates; however, trichothecenes and zearalenone may equally be produced at lower temperatures.

A. ochraceus grows at temperatures ranging from 8 – 38°C (optimum 24 – 37°C). Ochratoxin A is produced by *A. ochraceus* within the temperature range of 12 – 37°C, with an optimal production at 31°C. *P. verrucosum* grows within the temperature range of 0 – 31°C (optimum 20°C) and (low) water activity of 0.80; ochratoxin A is produced over the whole temperature range (optimum 20°C). Significant quantities of ochratoxin can be produced at a temperature as low as 4°C and a water activity as low as 0.86 (Sweeney and Dobson, 1998).

In the case of fumonisin production, a study using maize showed an optimum temperature between 15 and 25°C using two major fumonisin-producing *Fusarium* species *Fusarium verticillioides* and *Fusarium proliferatum* (Samapundo et al., 2005).

Favorable environmental conditions such as warm temperatures, high rainfall/humidity and high soil fertility raise the prevalence of fungi (e.g.: *Claviceps* sp.) and the production of ergot alkaloids (Strickland et al., 2011). Poisonous alkaloid-containing ergot sclerotia are used for sexual reproduction and as a resting structure to enable survival in unfavorable conditions (e.g.: in temperate zones where they overwinter in the ground or during storage and then sexually fruit the following spring when grass hosts are flowering) (Kren and Cvak, 1999; CAST, 2003). Fungal growth continues until the fungus produces this latent structure. *Claviceps purpurea* occurs in every temperate region and has the widest host range of any other *Claviceps* species (Kren and Cvak, 1999). *Claviceps africana* colonizes sorghum in Southern Africa but also in Southeast Asia, South America and USA (Kren and Cvak, 1999). In a study, *C. Africana* sclerotia could germinate after 1 year of dry storage at ambient temperature (15 – 30°C) (Frederickson et al., 1991). Montes-Belmont et al. (2002) reported optimum climatic conditions for ergot development of a mean day temperature of 25°C, maximum relative humidity of 96 % (maximum temperature 28°C, night relative humidity of 86 %).

1.4. Chemical stability of mycotoxins

Due to their chemical structure and low molecular weight, mycotoxins are chemically stable; they resist high temperatures and several manufacturing processes (Bullerman and Bianchini, 2007). Some review work has been done on the fate of mycotoxins during thermal food processing (Kabak, 2009).

Aflatoxins have a melting point of 268–269ºC and show high stability to dry heat-up temperatures. Temperatures over 150ºC are required to attain partial destruction of the toxin (Samarajeewa et al., 1990).

OTA has a melting point of 169ºC. Dry heating wheat at 100ºC for 40 – 160 min had no effect on OTA. However, wet heating at the same temperature led to a destruction of 50 % OTA after 120 min (Boudra et al., 1995).

FUM may survive most of the commonly used thermal processes as they are also fairly heat stable (up to 100 – 120ºC) (Humpf and Voss, 2004). It is also known that fumonisins can bind to various components of the feed matrix or react with other ingredients of the food, which leads to an underestimation of the potential toxicity of the mycotoxin because of the formation of unknown biologically active decomposition products.

Likewise, DON is a very stable compound, with a melting point of 151 – 153ºC. A significant reduction or destruction of DON was not achieved by thermal processing (CAC, 2003).

ZEN is stable during storage, milling and cooking and possesses a melting point of 164 – 165ºC (EFSA, 2004b). ZEN was shown to survive heat treatments of 140ºC for 4 h (Smith et al., 1994);

a complete reduction of this mycotoxin was observed in aqueous buffer solutions heated at 225ºC for less than 30 min.

Ergot alkaloids were relatively stable during end-use processing of flour into pasta and oriental noodles (Fajardo et al., 1995). Processing flour into pan bread decreased the ergot alkaloid content by approximately 25 % during the baking of a rye roll (Bürk et al., 2006).

It should be kept in mind that in studies carried out regarding the stability of mycotoxins, in general, only the disappearance of the mycotoxin is evaluated. This fact does not necessarily mean that the toxicity risk is reduced. Decomposition or transformation products may be just as dangerous as the parent molecules.

1.5. Masked mycotoxins

The topic of conjugated or masked mycotoxins first became of interest because in some cases of mycotoxicoses, clinical observations in animals did not correlate with the low mycotoxin content determined in the corresponding feed (more interacting factors are explained in the following paragraphs).

Masked mycotoxins are mycotoxins that experienced changes in their chemical structures. Proteins and glucosides, as an example, can be bound to mycotoxins by growing plants in the field to protect themselves from foreign compounds or by microorganisms which may change mycotoxin structures during storage. In rare cases, some mycotoxin conjugates can be excreted directly by fungi (e.g.: 3-acetyldeoxynivalenol and 15-acetyldeoxynivalenol by *Fusarium* sp.) or by mammals (Berthiller et al., 2009).This phenomenon of altered mycotoxin structures may also occur during food/feed processing, as in the case of FUM reaction with reducing sugars (Lu et al., 2002). More than 50 % of the amount of free mycotoxins (especially zearalenone and deoxynivalenol) is considered to exist in commodities in a masked form (Vendl et al., 2010).Unfortunately these conjugated mycotoxins cannot be detected by any routine analysis. However, during digestion the mycotoxin-ligand bond can be released and the mycotoxin acts as a toxin, thus causing its hazardous effects on animals (Berthiller et al., 2003). A recent *in vitro* study indicated that deoxynivalenol-3-glucoside is of toxicological relevance as it may become bioavailable again due to hydrolysis. It was suggested to monitor this toxin together with deoxynivalenol in cereals, especially since the part of the masked toxin might increase in the future due to *Fusarium* resistance breeding efforts (Berthiller et al., 2011). Figure 1 shows scheme representing the example of masked zearalenone-4-glucoside formation and action in the animal.

1.6. Synergistic effects

Because each plant can be contaminated with more than one fungus and each fungus species is able to produce more than one mycotoxin, the probability of co-occurrence of mycotoxins in a feed commodity is very high. On the other hand, the international trading of various feed stuffs also increases this risk. The interaction between mycotoxins often leads to synergistic effects, when the negative effects of one mycotoxin are amplified by the presence of another mycotoxin. Therefore an increase of severity of mycotoxicoses should be taken into account (Speijers and Speijers, 2004).

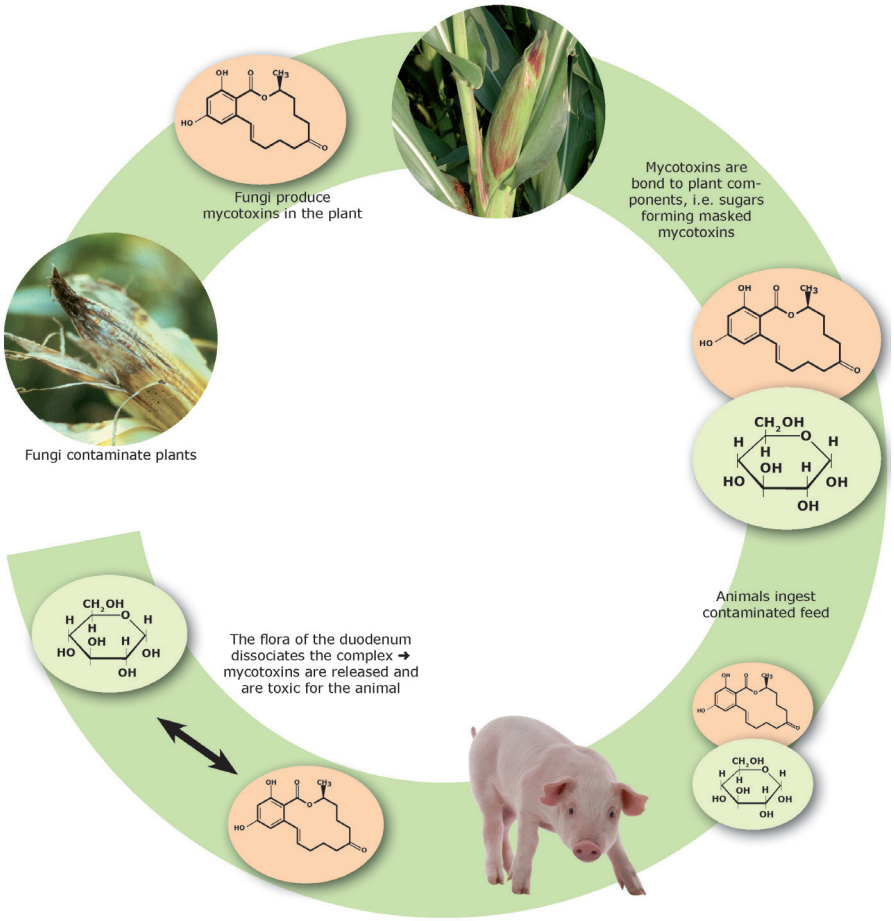


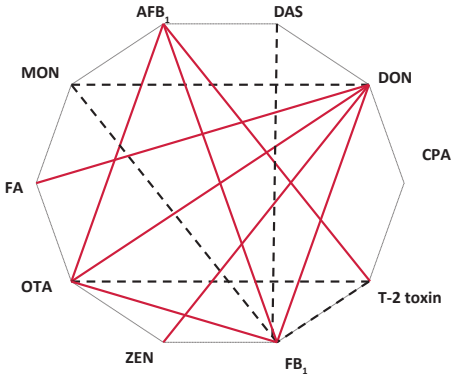
Figure 1- Scheme of the conjugate zearalenone-4-glucoside formation in the plant and subsequent ingestion by the animal followed by hydrolysis and release of the toxic compound.

80 % of pig diseases are related to mis-management due to feed quality, reproduction, housing conditions and biosecurity and only 20 % are due to viral, bacterial or parasitic pathogens. In cases of low levels of mycotoxins in the feed it should be mentioned that interactions between mycotoxins may enhance the toxicological effects. This is especially true in the case of fusariotoxins. *Fusarium graminearum* and *Fusarium culmorum* are known to produce several different fusariotoxins under the same conditions, namely ZEN and DON, which are known to interact synergistically in the animal. Analysis of DON often indicates co-occurrence of other fusariotoxins such as trichothecenes (T-2 toxin, nivalenol, diacetoxyscirpenol), zearalenone and fumonisins.

Trenholm et al. (1994) showed that mycotoxin naturally contaminated feed caused more severe toxicity when compared to the same concentration of purified mycotoxins. Another trial confirmed that fusaric acid is able to increase the toxicity of DON in growing pigs (Smith et al., 1997). Zielonka et al. (2009) reported the difficulties examining histopathological lesions caused by DON intoxication due to the common, often synergistic, reaction of this mycotoxin with other toxins, such as zearalenone. Harvey et al. (1995) fed combinations of aflatoxin B_1 and

fumonisin B_1 to grower pigs and found negative effects on clinical performance, biochemical, hematological and immunological parameters. The toxic reactions could be described as additive or more than additive particularly regarding the initiation of liver disease.

T-2 toxin synergized the activity of DON with respect to several parameters including weight gain (Friend et al., 1992). A summary of synergistic and additive effects of mycotoxins in pigs is presented in Figure 2 and Table 3.



AFB_1 – Aflatoxin B_1; FB_1 – Fumonisin B_1; DON – Deoxynivalenol; OTA –Ochratoxin A; ZEN – Zearalenone; FA – Fusaric acid; DAS – Diacetoxyscirpenol; CPA – Cyclopiazonic acid; MON – Moniliformin

Figure 2 - Synergistic (solid line) and additive (dashed line) effects of mycotoxins in pigs

Table 3 - Mycotoxin combinations in pigs

Mycotoxins	Species tested	Effect	References
AFB_1 + OTA	Pigs	synergistic	D'Mello et al., 1999; Huff et al., 1988a
AFB_1 + FB_1	Growing pigs	synergistic	Harvey et al., 1995; Liu et al., 2002
AFB_1 + T-2 toxin	Pigs	synergistic	D'Mello et al., 1999; Schwarzer, 2009
DON + FA	Pigs	synergistic	Raymond et al., 2005; D'Mello et al., 1999
MON + FB_1	Pigs	additive	D'Mello et al., 1999; Schwarzer, 2009
MON + DON	Pigs	additive	D'Mello et al., 1999; Schwarzer, 2009
OTA + DON	Weaned piglets	synergistic	Speijers and Speijers, 2004
OTA + FB_1	Weaned piglets	synergistic	Creppy et al., 2004 ; Speijers et al., 2004
OTA + T-2 toxin	Weaned piglets	additive	Speijers and Speijers, 2004
DON + ZEN	Weaned piglets	synergistic	Zielonka et al., 2009
FB_1 + DAS	Pigs	additive	D'Mello et al., 1999; Schwarzer, 2009
FB_1 + DON	Pigs	synergistic	D'Mello et al., 1999; Huff et al., 1988a; Speijers and Speijers, 2004
FB_1 + T-2 toxin	Pigs	additive	D'Mello et al., 1999; Schwarzer, 2009
DAS + Afla	Pigs	synergistic	D'Mello et al., 1999

AFB_1 = Aflatoxin B_1; OTA = Ochratoxin A; DAS = Diacetoxyscirpenol; DON = Deoxynivalenol; FB_1 = Fumonisin B_1; CPA = Cyclopiazonic acid; MON = Moniliformin; PCA = Penicillic acid; FA = Fusaric acid; ZEN = Zearalenone

1.7. Mode of action/toxicology/metabolism of mycotoxins

As chemical structures of different mycotoxins vary widely, they cannot be classified as a group according to their modes of action, toxicology or metabolism. Additionally, as previously explained, additive and synergistic effects can occur in the presence of two or more mycotoxins. Nevertheless, in the following pages the most important mycotoxins and their modes of action, toxicology and metabolism as well as main symptoms and target organs will be described.

1.7.1. AFLATOXINS

Aflatoxins were identified in 1960 and represent one of the most studied mycotoxins. They are mainly produced by certain strains of *Aspergillus parasiticus* and *A. flavus* and they occur in agricultural products in tropical and subtropical regions. Aflatoxins are divided into six major toxins (Figure 3) according to their fluorescent properties under ultraviolet light (ca. 365 nm) and their chromatographic mobility (subscripts). Aflatoxin B_1 (AfB_1) and B_2 produce a blue fluorescence while G_1 and G_2 a green one. Two metabolic products - aflatoxin M_1 and M_2 - occur in the milk of lactating mammals which have consumed aflatoxin contaminated feed. Aflatoxin B_1 is the most toxic and the most prevalent among this family.

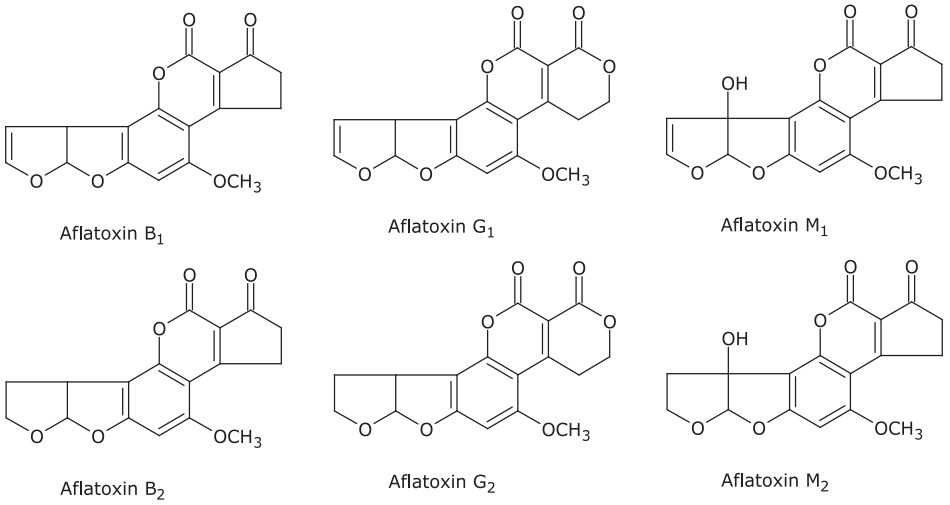


Figure 3 - Chemical structure of the different aflatoxins

1.7.1.1. Exposure and absorption into the organism

Because of aflatoxins' common occurrence in feedstuffs, feeds and milk products, these mycotoxins pose a serious threat to humans and animal species. Although the oral route is the main contamination means, inhalation may also occur as a result of people or animals being exposed to the grains' dust. After respiratory exposure, aflatoxin B_1 may appear in the blood more quickly than after oral exposure. Nevertheless, after 4 hours the plasmatic concentration does not differ between the two routes of contamination. Following ingestion, aflatoxin B_1 is efficiently absorbed in the intestinal tract, of which the duodenum appears to be the major site

of absorption. Due to the particle's low molecular weight, the main mechanism of absorption of this mycotoxin, as suggested by several authors, is passive diffusion, in which no efflux pumps or transporters are involved. From the site of absorption, aflatoxin B_1 enters the blood stream and is transported to the liver, the major site of metabolism (Gratz, 2007).

1.7.1.2. Metabolism

The metabolism of AfB_1 has been extensively reviewed (IARC, 1993; IARC, 2002 and Eaton et al., 2010). AfB_1 in liver and other tissues is metabolized by P450 cytochromes enzymes to aflatoxin P_1, aflatoxin M_1, or aflatoxin Q_1 and AfB_1-8,9-epoxide (Riley and Voss, 2011). These cytochromes P450 are responsible for activation of aflatoxin B_1, aflatoxin M_1 and aflatoxin P_1 which can form nucleic acid adducts or undergo conjunction to glutathione, conversion to dihydriodiols or binding to serum protein and other macromolecules (Figure 4). Variation in the level of the glutathione transferase system and alterations in the cytochrome P450 system are thought to contribute to the differences observed in the susceptibility of an animal to aflatoxins (Bennett and Klich, 2003).

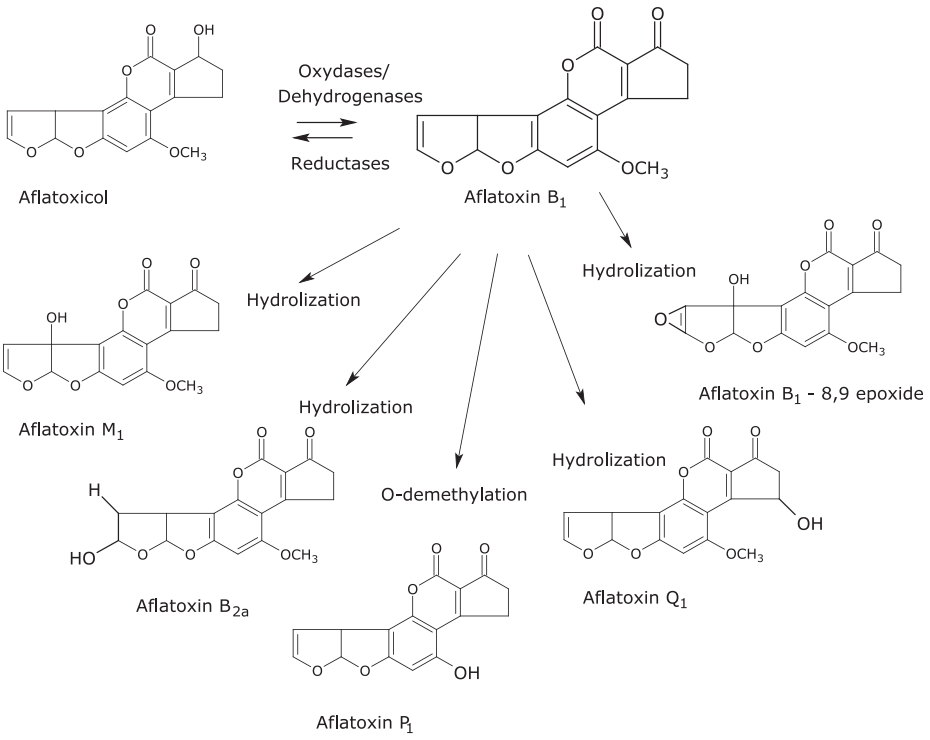


Figure 4 - Aflatoxin B_1 pathways (modified from Leeson et al., 1995)

The metabolism of aflatoxin B_1 can be described in three phases:

I. Bioactivation;
II. Conjugation;
III. Deconjugation.

I. Bioactivation

In order for aflatoxin B_1 to exert its toxic effects, this phase I bioactivation is needed. In this first stage, aflatoxin B_1 is oxidized into several hydroxylated metabolites.

The metabolic pathways for aflatoxin B_1 include o-demethylation to aflatoxin P_1, reduction to aflatoxicol and hydroxylation to AfB_1-8,9-epoxide (acutely toxic, mutagenic, and carcinogenic), aflatoxin M_1 (acutely toxic), aflatoxin Q_1, or aflatoxin B_{2a} (both relatively non-toxic) (Eaton and Gallagher, 1994).

Aflatoxin B_1– 8,9 epoxide is highly unstable, thus several reactions may occur, depending on the second molecule present (Eaton et al., 2010):

- Biological nucleophils (such as nucleic acids) – stable links to RNA and DNA are formed; inducing point mutations and DNA strand breaks. These reactions and the formation of AfB_1-DNA adducts are highly correlated with the carcinogenic effect of AfB_1 in both animal and human cancer cases.
- Water – in the presence of water molecules, aflatoxin B_1– 8,9 epoxide will be hydrolyzed into AfB_1– 8,9- dihydrodiol and becomes available to be linked with serum proteins, such as lysine and albumin. This mechanism may explain the toxic effects of aflatoxin.

II. Conjugation

Phase I metabolites may undergo phase II biotransformation involving the enzymes glutathione S-transferase (GST), ß-glucuronidase, and/or sulfate transferase which produce conjugates of AfB_1-glutathione, AfB_1-glucuronide, and AfB_1-sulfate, respectively. The major conjugate of AfB_1-epoxide identified is the AfB_1-glutathione conjugate. This conjugation is the principal detoxification pathway of activated AfB_1 in many mammals which is essential in the reduction and prevention of AfB_1 induced carcinogenicity. The resulting conjugates are readily excreted via the bile into the intestinal tract. It has been accepted that cytosolic GST activity is inversely correlated to the susceptibility of several animal species to AfB_1 carcinogenicity (Figure 5).

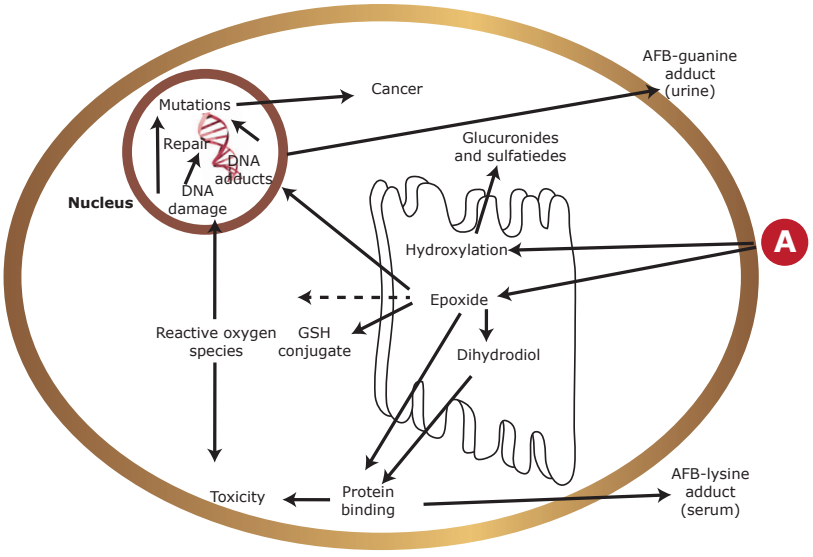


Figure 5 - Simplified mode of action of aflatoxins (A) (adapted from Riley and Voss, 2011).

III. Deconjugation

This phase can occur in the intestinal tract as a result of bacterial activity. Deconjugation is part of the metabolic role of the large intestinal flora, which results in reabsorption and the establishment of an enterohepatic circulation.

1.7.1.3. Excretion and residues in animal products

The excretion of AfB_1 and its metabolites is mainly made through bile and urine. In lactating animals, AfM_1 and other metabolites are excreted in the milk. Many studies exist showing the carry-over of aflatoxin into animal products such as porcine tissue, milk and milk products (Völkel et al., 2011).

Besides AfM_1, AfB_1-DNA adducts and AfB_1-albumin adducts are currently available biomarker for aflatoxin B_1 exposure (Baldwin et al., 2011).

1.7.1.4. Toxicity

AfB_1 belongs to Group 1 of IARC (International Agency for Research on Cancer): carcinogenic to humans and AfM_1 to Group 2b: possibly carcinogenic to humans.

In animals, the effects of aflatoxins are variable depending on sex, age, species and even animal breed. The main target organ for aflatoxins is the liver. Nevertheless, due to the toxins' interference and reactions with nucleic acids, RNA and DNA, proteins and enzymes, their effects on domestic animals are not only hepatotoxic and expressed by toxic hepatitis and jaundice but involve a broad range of organs, tissues and systems (Table 4).

Table 4 - Main systems affected by aflatoxins (all animal species are included in this table. For the effects of mycotoxins in swine, please consult Chapter 2)

Affected system	Effects/Signs/Symptoms
Genes/gene expression	Teratogenic effects - Birth defects of the offspring
	Carcinogenic effects - Higher incidence of cancer in exposed animals
Pathological changes	Weight variation of the internal organs (liver, spleen, kidneys enlargement, fatty liver syndrome), Bursa of Fabricius and thymus reduction, change in the texture and coloration of the organs (liver, gizzard)
Circulatory system	Hematopoietic effects (hemorrhages, anemia)
Immune system	Immunosuppression (decreased resistance to environmental and microbial stressors; increased susceptibility to diseases)
Nervous system	Nervous syndrome (e.g.: abnormal behavior)
Skin	Dermatoxic effects (impaired feathering)
Urinary system	Kidney inflammation

Digestive system	Impaired rumen function, with decreased cellulose digestion, decreased volatile fatty acid formation, decreased proteolysis, decreased rumen motility, diarrhea
Reproductive system	Decreased breeding efficiency (birth of smaller and unhealthy offspring)

1.7.2. TRICHOTHECENES

Trichothecenes are a family of over 200 structurally related compounds mainly produced by several *Fusarium* species (Pestka, 2010) but other fungal species such as *Stachybotrys* and *Myrothecium* are also producers of selected trichothecenes. The trichothecenes produced by the mould genes *Fusarium* are mainly classified into two groups:

- **Type-A** (namely T-2 toxin, HT-2 toxin, diacetoxyscirpenol): characterized by a functional group other than a ketone at C-8 (Figure 6 and Table 5)
- **Type-B** (namely deoxynivalenol, 3- and 15-acetyldeoxynivalenol, nivalenol, fusarenon X): characterized by a carbonyl function at C-8 (Figure 7 and Table 6)

Among this group of mycotoxins deoxynivalenol is the most frequently occurring. The structure of trichothecenes is characterized by a sesquiterpene ring and a C-12,13-epoxide ring. T-2 toxin and diacetoxyscirpenol are soluble in non-polar solvents; deoxynivalenol or nivalenol are soluble in polar solvents like alcohol, but also in water.

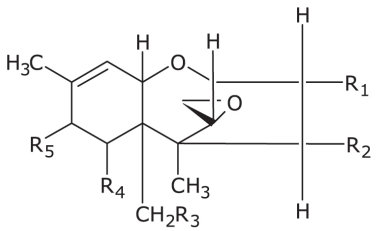


Figure 6 - Structural formula of type-A trichothecenes

Table 5 - Structural formula of type-A trichothecenes

	Molecular formula	R1	R2	R3	R4	R5
Diacetoxyscirpenol	$C_{19}H_{26}O_7$	OH	OAc	OAc	H	H
T-2 toxin	$C_{24}H_{34}O_9$	OH	OAc	OAc	H	$OCOCH_2CH(CH_3)_2$
HT-2 toxin	$C_{22}H_{32}O_8$	OH	OH	OAc	H	$OCOCH_2CH(CH_3)_2$

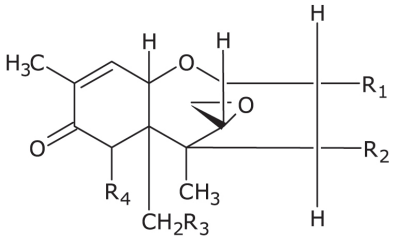


Figure 7 - Structural formula of type-B trichothecenes

Table 6 - Structural formula of type-B trichothecenes

	Molecular formula	R1	R2	R3	R4
Deoxynivalenol	$C_{15}H_{20}O_6$	OH	H	OH	OH
3-Acetyldeoxynivalenol	$C_{17}H_{22}O_6$	OAc	H	OH	OH
15-Acetyldeoxynivalenol	$C_{17}H_{22}O_6$	OH	H	OAc	OH
Nivalenol	$C_{15}H_{20}O_7$	OH	OH	OH	OH
Fusarenon X	$C_{17}H_{22}O_8$	OH	OAc	OH	OH

1.7.2.1. Metabolism

Generally, there are three main metabolic pathways:

I. Conjugation
II. De-epoxidation
III. De-acetylation

The de-epoxidation is the most important step in the detoxification of trichothecenes and can be carried out by microorganisms in the gastrointestinal tract of ruminants.

1.7.2.2. Mechanism of action

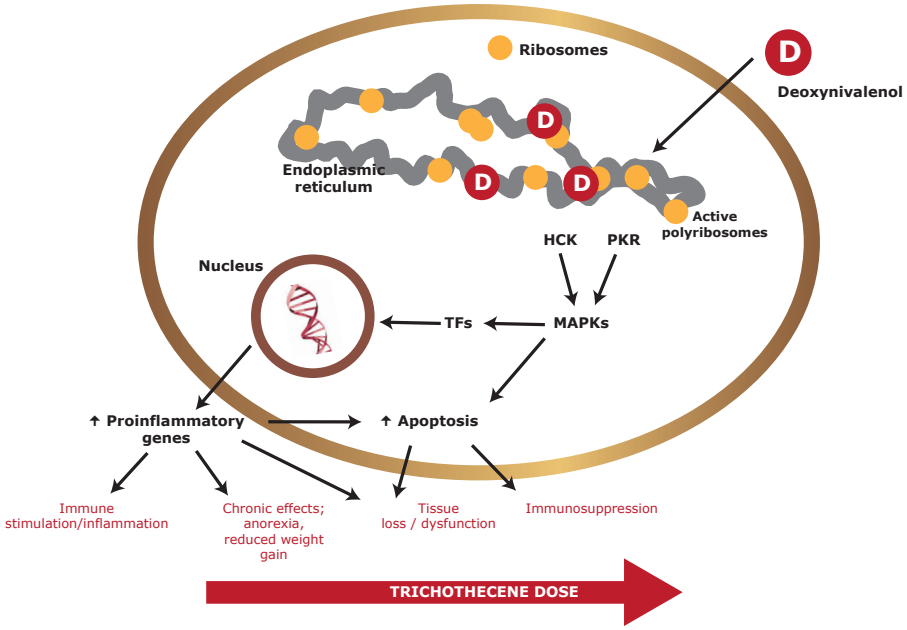
Trichothecenes are potent inhibitors of protein, RNA and DNA synthesis and they can interact with the cell membrane. Trichothecenes bind to active polysomes and ribosomes, peptide linkages are interrupted, initiation and termination sequences are reduced, and the ribosomal cycle is disrupted. Their toxicity is primarily based on the 12,13-epoxyde ring. There are two types of the mechanism of protein inhibition:

- Inhibition of the initial step of protein synthesis (e.g.: T-2, HT-2, DAS)

- Inhibition of the elongation-termination step (e.g.: DON)

Trichothecenes are especially toxic to tissues with a high cell division rate. Additionally, they are very cytotoxic to eukaryotic cells, causing cell lysis and inhibition of mitosis.

Deoxynivalenol enters the cell and binds to active ribosomes which transduce a signal to RNA-activated protein kinase (PKR) and hematopoeitic cell kinase (HCK). Subsequent phosphorylation of mitogen-actived protein kinases drives transcription factor (TF) activation apoptosis and resultant chronic and immunotoxic effects (modified from Pestka, 2007) (Figure 8).



HCK – Haematopletic cell kinase, PKR – RNA-activated protein kinase, MAPKS – Mitogen-activated protein kinase, TFS – Transcription factor

Figure 8 - Mechanism of action of deoxynivalenol (D) (modified from Pestka et al., 2004 and Pestka, 2007)

1.7.2.3. Absorption/Residues

In general, absorption of deoxynivalenol occurs very rapidly within the digestive tract and is widely distributed in many tissues and organs. Residues of DON in pig tissues were reported from several experiments but at very low levels (Döll et al., 2008, Goyarts et al., 2007).

1.7.2.4. Toxicity

Many outbreaks of acute human disease like vomiting, gastrointestinal disorders, diarrhea or headaches have been attributed to consumption of *Fusarium*-contaminated grains. In animals, decrease in feed consumption (anorexia) and vomiting are two characteristic effects of deoxynivalenol contamination (Table 7).

The primary target of T-2 toxin is the immune system and this results for example in changes in the leukocyte count or reduced antibody formation.

Table 7 - Main systems affected by trichothecenes (all animal species are described in this table. For the effects of mycotoxins in swine, please see Chapter 2)

Affected system	Effects/Signs/Symptoms
Circulatory system	Hematopoietic effects (hemorrhages; blood pattern disorders)
Immune system	Immunosuppression (decreased resistance to environmental and microbial stressors; increased susceptibility to diseases)
Digestive system	Gastro-intestinal effects (gastroenteritis/inflammation of the rumen; vomiting; feed refusal)
Reproductive system	Decreased breeding efficiency (birth of smaller and unhealthy offspring)
Nervous system	Neurotoxic effects (restlessness; lack of reflexes; abnormal wings positioning; nervous syndrome)
Skin	Dermotoxicity (oral and dermal lesions; necrosis)
Pathological changes	Necrosis of the lymphoid and hematopoietic tissues; gizzard lesions

1.7.3. OCHRATOXINS

Ochratoxins are metabolites of *Aspergillus ochraceus* and *Penicillium verrucosum* in temperate regions and are present in a large variety of feeds and foods. There are four ochratoxins (A, B, C, and D) and the major mycotoxin among this group is ochratoxin A (OTA). This toxin is a contaminant of cereals, beans and other plant products. The most significant effect of ochratoxins in farm animals is nephrotoxicity (Pfohl-Leszkowicz and Manderville, 2007). Chemically, ochratoxins contain an isocoumarin moiety linked by a peptide bond to phenylalanine.

1.7.3.1. Mechanism of action

OTA primarily affects the enzymes involved in phenylalanine metabolism. It inhibits the enzyme involved in the synthesis of the phenylalanine-tRNA complex. OTA might also interact with other enzymes that use phenylalanine as a substrate, for example, the phenylalanine hydroxylase which catalyzes the irreversible hydroxylation of phenylalanine to tyrosine. In addition, it alters the mitochondrial membrane transportation system; inhibits adenosine triphosphate (ATP) production, enhances membrane lipid peroxidation and superoxide and formation of hydrogen peroxide radicals.

1.7.3.2. Metabolism

The major metabolite of ochratoxin is ochratoxin α, a hydrolysis product by the gut microflora without the phenylalanine moiety. Other metabolites are the hydroxylated derivates 4(R)-, 4(S)- and 10-OH-ochratoxins (Figure 9).

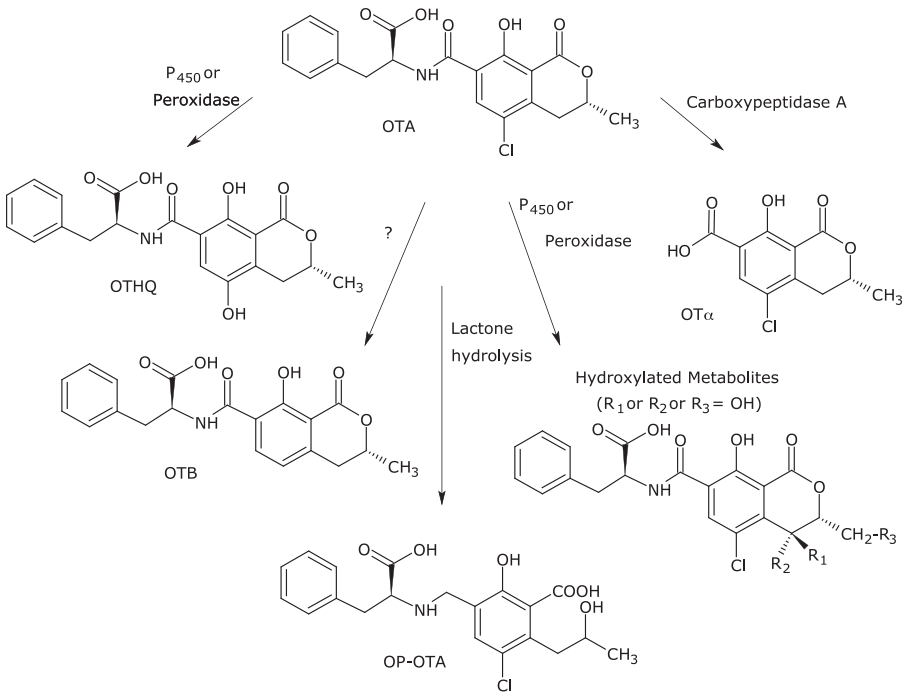


Figure 9 - Metabolism of OTA (modified from Pfohl-Leszkowicz et al., 2007)

1.7.3.3. Absorption/excretion/residues

Between 40 and 66 % of ochratoxin is absorbed from the gastrointestinal tract depending on species. The small intestine has been shown to be the major site of absorption and maximal absorption occurs in the jejunum (Pfohl-Leszkowicz and Manderville, 2007).

OTA binds rapidly to serum albumin and is distributed in the blood mainly in the bound form. Generally, the toxin has a long biological half-life due to its high rate of binding to serum protein but differences exist between species. Therefore the ochratoxin protein adduct in serum can be used as a biomarker for ochratoxins' exposure (Baldwin et al., 2011).

OTA primarily accumulates in the kidneys followed by the liver and muscles but also in whole blood and blood plasma (Battacone et al., 2010).

1.7.3.4. Toxicity

According to IARC ochratoxins are classified as possible human carcinogens (Group 2b).

In animals, toxicity varies widely according to animal species and sex. The kidneys are the main target organs. Ochratoxin has been found to be responsible for porcine nephropathy, an extensively studied disease. Apart from the above mentioned effects ochratoxins may also affect other systems (Table 8).

Table 8 - Main systems affected by ochratoxins (all animal species are described in this table. For the effects of mycotoxins in swine, please see Chapter 2)

Affected system	Effects/Signs/Symptoms
Circulatory system	Hematopoietic effects (hematological disorders, blood in urine and feces)
Nephrotoxic effects	Increased water consumption; kidney and urinary bladder dysfunction
Immune system	Immunosuppression (decreased resistance to environmental and microbial stressors; increased susceptibility to diseases)
Hepatotoxic effects	Liver damage
Digestive system	Gastro-intestinal effects (diarrhea)

1.7.4. FUMONISINS

Fumonisins are a group of mycotoxins mainly produced by *Fusarium verticillioides* and *F. proliferatum*. They were first isolated from cultures of *Fusarium verticillioides* in 1988 in South Africa. The most predominant member among this group is Fumonisin B_1 (FB_1). They mainly occur in maize. Fumonisins are highly polar compounds and soluble in water.

1.7.4.1. Mechanism of action

Fumonisins' toxicity is based on the structural similarity to the sphingoid bases - sphingosine and sphinganine (Figure 10). They are inhibitors of sphinganine (sphingosine) N-acyltransferase (ceramide synthase), a key enzyme in the lipid metabolism, resulting in disruption of this pathway (Figure 11). This enzyme catalyzes the acylation of sphinganine in the biosynthesis of sphingolipids and also the deacylation of dietary sphingosine and the sphingosine that is released by the degradation of complex sphingolipids (ceramid, sphingomyelin and glycosphingolipide) (Wang et al., 1991).

Sphingolipids are important for the membrane and lipoprotein structure and also for cell regulations and communication (second messenger for growth factors) (Berg et al., 2003). Sphingosine is the backbone of sphingolipids (Merrill et al., 2001).

As a consequence of this disruption many bioactive intermediates are elevated, others reduced, as follows: a rapid increase of sphinganine (sometimes sphingosine), an increase of sphinganine degradation products like sphinganine 1-phosphate and a decrease of complex sphingolipids.

Free sphingoid bases are toxic to most cells by affecting cell proliferation and inducing apoptosis or necrotic cell death or by increasing the sphinganine 1-phosphate concentration in kidney and serum. The accumulation of sphinganine is associated with hepato- and nephrotoxic effects. Complex sphingolipids are important for cell growth regulation and also cell-cell interactions. Fumonisin B_1 impairs the barrier function of endothelial cells *in vitro*. These adverse effects on endothelial cells could indirectly contribute to the neurotoxicity and pulmonary edema caused by fumonisins. Sphingosine 1-phosphate activates the endoplasmic reticulum calcium release and also acts as a ligand for extracellular receptors in the vascular system (S1P receptors) (Eaton et al., 2010).

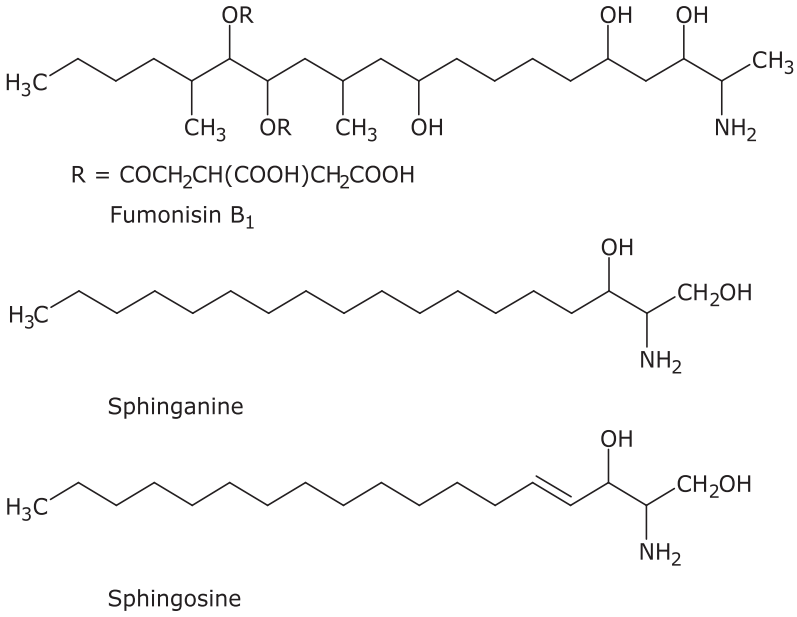


Figure 10 - Structures of fumonisin B_1, sphinganine and sphingosine

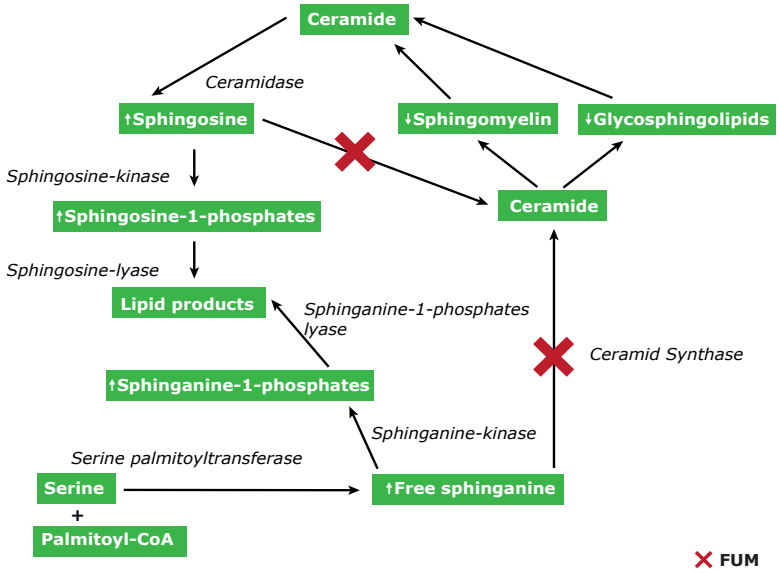


Figure 11 - Lipid metabolism and the inhibition of fumonisins. In order to simplify the graphics, only the main intermediates are described (modified from Merrill et al., 2001 and Voss et al., 2007)

The accumulation of free sphingoid bases (measured by the sphinganine:sphingosine ratio) in the serum and urine is a useful biomarker for the exposure of fumonisins and indicates the degree of sphingolipid metabolism disruption (Riley et al., 1993; Riley et al., 1994).

1.7.4.2. Exposure and absorption into the organism

Several studies indicate that fumonisins are poorly absorbed from the gastrointestinal tract and rapidly cleared from the blood. However, the absorbed fraction seems to undergo a wide distribution, with a high affinity to the liver and kidneys which later on slowly release the toxin (Prelusky et al., 1996). Fumonisins cause apoptosis followed by mitosis in affected tissues (Eaton et al., 2010).

1.7.4.3. Excretion and residues in animal products

Fumonisin carry-over in sow milk and pork meat may only occur after a high level of exposure over a longer period and then this mycotoxin can accumulate in the liver and kidneys (Völkel et al., 2011; Meyer et al., 2003).

1.7.4.4. Toxicity

According to IARC FB_1 is classified as possible human carcinogen (Group 2B).

In the case of animals, horses are the most sensitive species to fumonisin toxicity. The mycotoxin causes a disease syndrome which is called equine leukoencephalomalacia (ELEM) and affects the central nervous system. Several studies have indicated that fumonisins cause porcine pulmonary edema (PPE).

Besides severe lung edema, liver injuries were also found in the exposed animals but fumonisins also cause a broad range of effects on other systems (Table 9).

Table 9 - Main systems affected by fumonisins (all animal species are described in this table. For the effects of mycotoxins in swine, please see Chapter 2)

Affected systems	Effects/Signs/Symptoms
Immune system	Immunosuppression (decreased resistance to environmental and microbial stressors; increased susceptibility to diseases)
Digestive system	Gastro-intestinal effects (diarrhea)
Circulatory system	Hematopoietic effects (hematological disorders; increased concentration of hemoglobulin)
Nervous system	Neurotoxic effects
Hepatotoxic effects	Liver damage
Pathological changes	Kidneys and liver weight increase; liver necrosis; pancreatic necrosis

1.7.5. ZEARALENONE

Zearalenone (Figure 12) is an important mycotoxin occurring in warm and temperate climate regions. It is produced mainly by *Fusarium graminearum* and *Fusarium culmorum* on a variety of cereal crops.

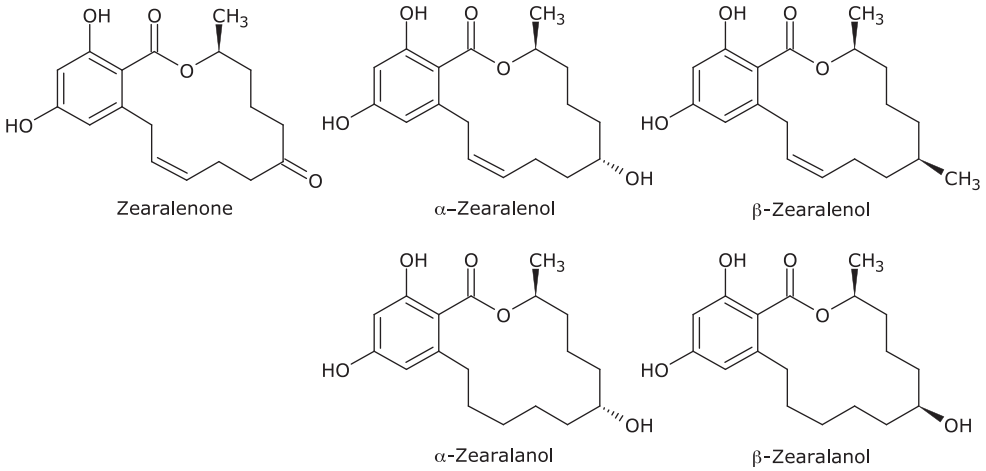


Figure 12 - Chemical structures of ZEN and its derivatives: zearalenone (ZEN), α–zearalenol (α-ZOL), β – zearalenol (β-ZOL), α-zearalanol (α-ZAL) and β-zearalanol (β-ZAL).

1.7.5.1. Metabolism/mechanism of action

Zearalenone is an estrogenic mycotoxin which is often involved in reproductive disorders and hyperestrogenicity in farm animals. The estrogenic effects are based on the structural similarity between zearalenone and estradiol. Estradiol is the most important female sex hormone in the group of estrogens.

The biotransformation of zearalenone takes place in two major pathways (Minervini and Dell'Aquila, 2008):

- Hydroxylation: Formation of α-zearalenol and β-zearalenol, assumed to be catalyzed by 3α- and 3β-hydroxysteroid dehydrogenases.
- Conjugation of zearalenone and its metabolites with glucoronic acid catalyzed by uridine diphosphate glucuronyl transferases.

The reduced form of zearalenone, α–zearalenol, has increased estrogenic effects. Several studies indicated that there are differences in biotransformation of zearalenone in various species, for example, pigs convert zearalenone predominately into α–zearalenol (Malekinejad et al., 2006).

The mycotoxin passes the cell membrane and binds to the estrogen receptor (Figure 13). This complex is transferred into the nucleus and binds there to specific nuclear receptors. Afterwards it generates estrogenic responses via gene activation resulting in the production of mRNAs that code for proteins which are normally expressed by receptor-estrogen complex binding (modified from Riley and Norred, 1996).

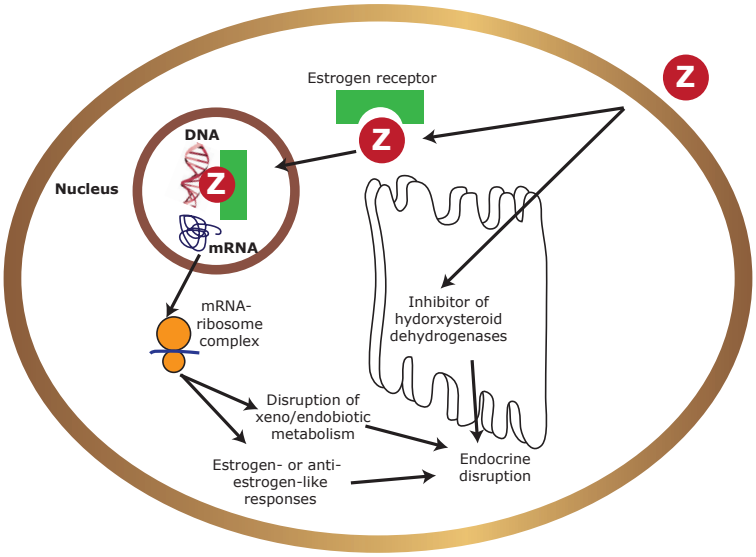


Figure 13 - Simplified view of the mode of action of zearalenone (Z) (reviewed in Zinedine et al., 2008)

1.7.5.2. Exposure and absorption into the organism

Zearalenone is rapidly absorbed and metabolized in intestinal cells. In pigs ZEN and its metabolites show an extensive biliary excretion and enterohepatic cycling (Biehl et al., 1993). Studies investigating the carry-over of ZEN into meat and other edible tissues indicated that there is only limited tissue deposition of this mycotoxin. Transfer of ZEN and its major metabolites into serum was not detected after a administration of 56 µg ZEN per kg feed (Goyarts et al., 2007).

1.7.5.3. Toxicity

Zearalenone poses a relatively low acute toxicity and the oral LD_{50} values are between 2000–20000 mg/kg bodyweight. Effects differ between different species (Table 10). Pigs seem to be more sensitive to the effects of zearalenone than others. Furthermore the toxin has shown haematotoxic effects, namely through changes in blood parameters.

Table 10 - Main systems affected by zearalenone (all animal species are described in this table. For the effects of mycotoxins in swine, please see Chapter 2)

Affected system	Effects/Signs/Symptoms
Digestive system	Gastro-intestinal effects (diarrhea)
Reproduction system	Reproductive effects (feminization; enlargement of mammary glands; impaired semen quality; testicular atrophy; swollen prepuce, reddening and swelling of vulva)
Genes/Gene expression	Teratogenic effects (splay legs)
Pathological changes	Atrophy of ovaries; uterus hypertrophy

1.7.6. ERGOT ALKALOIDS

The term ergot alkaloid refers to a diverse group of approximately forty different toxins which are formed by *Claviceps* sp. on grains such as triticale, corn, wheat, barley, oats, millet, sorghum and rice, and by fungal endophytes such as *Neotyphodium* sp. in grasses, particularly tall fescue and perennial ryegrass (Scott, 2009; Krska and Crews, 2008).

The ergot alkaloids constitute the largest known group of nitrogenous fungal metabolites and all have a common structure, a tetracyclic ergoline ring system of lysergic acid. Clavines are the simplest ergot alkaloids containing only an ergoline ring sytem (Figure 14 - top), whereas the ergopetides have an additional peptide moiety linked to the basic structure (Figure 14 - bottom).

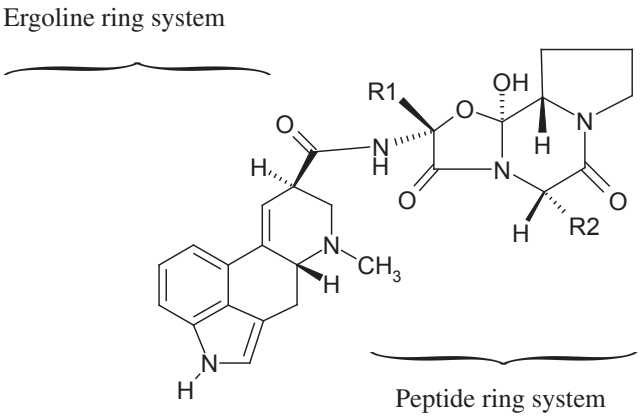


Figure 14 - Structure of ergopeptines (Krska and Crews, 2008)

The main groups of natural ergot alkaloids (CAST, 2003; Panaccione, 2005):

- Clavines – e.g.: agroclavine
- Lysergic acids
- Lysergic acid amides – e.g.: ergonovine (ergometrine, ergobasine), ergine
- Ergopeptines – e.g.: ergovaline, ergotamine, ergocornine, ergocristine, ergosine, ergocryptine

The amount and pattern vary between fungal strains, host plant and climatic and geographical conditions (Hafner et al., 2008).

Ergot alkaloids appear as colorless crystals that are soluble in various organic solvents, but insoluble or only slightly soluble in water (EFSA, 2005). Moreover most alkaloids can form stereoisomers distinguished by the suffix *–inines* that are biologically less active, and easily convert back into the genuine *–ine* form (Flieger et al., 1997).

1.7.6.1. Absorption/excretion/residues

In general, little carry-over of ergot alkaloids into animal tissue has been reported. When a diet of 4 % ergot (containing ergopeptine alkaloids) was fed to pigs there was 90 % absorption but no evidence of alkaloids in tissues (Scott, 2009).

1.7.6.2. Mechanism of action

The biological activity of the ergot alkaloids in animal systems is mainly due to structural similarities of the ergoline ring structure to the biogenic amines serotonin, dopamine, norepinephrine, and epinephrine (Berde, 1980; Weber, 1980). Due to this structural similarity many ergot alkaloids can bind to biogenic amine receptors and elicit such effects as decreased serum prolactin and vasoconstriction. Ergot alkaloids constitute a very diverse class of chemical compounds with widely different toxicological targets and activities, and potential routes of elimination and rates of clearance (Strickland et al., 2011).

1.7.6.3. Toxicity

Animals can be exposed to complex mixtures of alkaloids in many typical animal agriculture production systems. This exposure results from the fact that the kinds of alkaloids present and their levels can vary widely, depending on the fungal strain, the host plant and environmental conditions. Thus it is practically impossible to relate the exposure to individual toxins. Ergot alkaloid toxicoses in livestock due to consumption of ergot infected grains are widespread and result in disruption of several physiological systems (reproduction, growth, cardiovascular) within the body of an animal (Strickland et al., 2011) (Table 11). Ergot alkaloids exert toxic effects on all animal species, and the most prominent toxic signs can be attributed to the interaction of ergot alkaloids with adrenergic, serotinergic and dopaminergic receptors (EFSA, 2005).

Table 11 - Main systems affected by ergot alkaloids (all animal species are described in this table. For the effects of mycotoxins in swine, please see Chapter 2)

Affected system	Effects/Signs/Symptoms
Circulatory system	Vasoconstriction symptoms (elevated body temperatures, increased respiration rate), gangrenous changes in tissue of feet, tail and ear, reduced serum prolactin
Immune system	Immunosuppression (decreased resistance to environmental and microbial stressors; increased susceptibility to diseases)
Digestive system	Gastro-intestinal effects (reduced body weight gain, feed refusal, diarrhea)
Reproductive system	Decreased breeding efficiency (lower conception rates, decreased survival rate of offspring, decreased piglet birth weight, abortions)
Nervous system	Neurotoxic effects (convulsions, hallucination, anorexia,), lameness
Skin	Dermatoxicity (oral and dermal lesions; necrosis), rough hair coat

1.8. Legislation in the European Union (EU)

In the case of EU member states, Commission Directive 2003/100/EC of 31 October 2003 amending Annex I to Directive 2002/32/EC of the European Parliament and of the Council on undesirable substances in animal feed, and the Commission Regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs, are the basic regulations to be applied. Nevertheless, the regulatory agencies within each country are allowed to set higher standard rules. As for the regulations worldwide, the latter is also applicable. Table 12 shows the European regulations applied to feedstuffs destined for animal consumption.

Table 12 - Maximum concentration of aflatoxin B_1 permitted in animal feedstuffs.
Source: Commission Directive 2003/100/EC of 31 October 2003 amending Annex I to Directive 2002/32/EC of the European Parliament and of the Council on undesirable substances in animal feed.

Countries	Products intended for animal feed	Mycotoxin	Maximum content in mg/kg [ppm] relative to a feedingstuff with a moisture content of 12 %
EU member states:	All feed materials	Aflatoxin B_1	0.02
Austria, Belgium , Bulgaria, Cyprus, Czech Republic , Denmark, Estonia, Finland, France, Germany , Greece, Hungary, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, The Netherlands, Poland, Portugal, Romania, Slovakia, Slovenia, Spain, Sweden, United Kingdom	Complementary and complete feed		0.01
	With the exception of:		
	Compound feed for dairy cattle and calves, dairy sheep and lambs, dairy goats and kids, piglets and young poultry animals		0.005
	Compound feed for cattle (except dairy cattle and calves), sheep (except dairy sheep and lambs), goats (except dairy goats and kids), pigs (except piglets) and poultry (except young animals)		0.02
Iceland, Norway and Liechtenstein (following EU legislation)			

In spite of the fact that directives do not exist for other mycotoxins of concern, the European Commission has set up some guidance levels in products intended for animal feeding (Table 13). Recommendations differ between regulations, directives and decisions, in that they are not legally binding for Member States, although they represent an instrument of indirect action aimed at preparation of legislation in Member States.

Table 13 – Guidelines for the concentration of different mycotoxins in animal feedstuffs.

Source: Commission Recommendation (2006/576/EC) of 17 August 2006 on the presence of deoxynivalenol, zearalenone, ochratoxin A, T-2 and HT-2 and fumonisins in products intended for animal feeding.

Mycotoxin	Products intended for animal feed	Guidance value in mg/kg [ppm] relative to a feedingstuff with a moisture content of 12 %
Deoxynivalenol	Feed materials (*)	
	• Cereals and cereal products (**) with the exception of maize by-products	8
	• Maize by-products	12
Deoxynivalenol	Complementary and complete feedingstuffs with the exception of:	5
	• Complementary and complete feedingstuffs for pigs	0.9
	• Complementary and complete feedingstuffs for calves (< 4 months), lambs and kids	2
Zearalenone	Feed materials (*)	
	• Cereals and cereal products (**) with the exception of maize by-products	2
	• Maize by-products	3
	Complementary and complete feedingstuffs	
	• Complementary and complete feedingstuffs for piglets and gilts (young sows)	0.1
	• Complementary and complete feedingstuffs for sows and fattening pigs	0.25
	• Complementary and complete feedingstuffs for calves, dairy cattle, sheep (including lamb) and goats (including kids)	0.5
Ochratoxin A	Feed materials (*)	
	• Cereals and cereal products (**)	0.25
	Complementary and complete feedingstuffs	
	• Complementary and complete feedingstuffs for pigs	0.05

Table 13 Contd.

Mycotoxin	Products intended for animal feed	Guidance value in mg/kg [ppm] relative to a feedingstuff with a moisture content of 12 %
	• Complementary and complete feedingstuffs for poultry	0.1
Fumonisin $B_1 + B_2$	Feed materials (*)	
	• maize and maize products (***)	60
	Complementary and complete feedingstuffs for:	
Fumonisin $B_1 + B_2$	• 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

(*) Particular attention has to be paid to cereals and cereals products fed directly to the animals that their use in a daily ration should not lead to the animal being exposed to a higher level of these mycotoxins than the corresponding levels of exposure where only the complete feedingstuffs are used in a daily ration.

(**) The term 'Cereals and cereal products' includes not only the feed materials listed under heading 1 'Cereal grains, their products and by-products' of the non-exclusive list of main feed materials referred to in part B of the Annex to Council Directive 96/25/EC of 29 April 1996 on the circulation and use of feed materials (OJ L 125, 23.5.1996, p. 35) but also other feed materials derived from cereals in particular cereal forages and roughages.

(***) The term 'Maize and maize products' includes not only the feed materials derived from maize listed under heading 1 'Cereal grains, their products and by-products' of the non-exclusive list of main feed materials referred to in the Annex, part B of Directive 96/25/EC but also other feed materials derived from maize in particular maize forages and roughages.

1.9. Legislation in the United States of America (USA)

In the USA, the Center for Veterinary Medicine of the *Food and Drug Administration* (FDA) also focuses on the same mycotoxins as the EU. There are action, guidance and advisory levels for aflatoxins, fumonisins and deoxynivalenol, respectively (Tables 14, 15 and 16). No action, guidance or advisory levels for ochratoxin A or zearalenone have been established by the FDA in animal feeds (these two mycotoxins are handled on a case-by-case basis).

Table 14 – Action levels for total aflatoxins in livestock feed in mg/kg [ppm].
Source: www.fda.gov (Accessed: 11/01/2013)

Animals	Feedingstuffs	Aflatoxin levels
Finishing (i.e., feedlot) beef cattle	corn and peanut products	0.3
Beef cattle, swine, or poultry	cottonseed meal	0.3
Finishing swine of 100 pounds (45 kg) or heavier	corn or peanut products	0.2
Breeding beef cattle, breeding swine, or mature poultry	corn and peanut products	0.1
Immature animals	corn, peanut products, and other animal feeds and feed ingredients, excluding cottonseed meal	0.02
Dairy animals, for animal species or uses not specified above, or when the intended use is not known	corn, corn products, cottonseed meal, and other animal feeds and feed ingredients	0.02

Table 15 - Guidance levels for total fumonisins in animal feeds in mg/kg [ppm].
Source: www.fda.gov (Accessed: 11/01/2013)

Animal	Feedingstuffs and proportion in the diet	Levels in corn and corn by-products	Levels in complete ration
Equids and rabbits	Corn and corn by-products (no more than 20 % of diet)**	5	1
Swine and catfish	Corn and corn by-products (no more than 50 % of diet)**	20	10
Breeding ruminants breeding poultry and breading mink*	Corn and corn by-products (no more than 50 % of diet)**	30	15
Ruminants ≥ 3 months old being raised for slaughter and mink being raised for pelt production	Corn and corn by-products (no more than 50 % of diet)**	60	30
Poultry being raised for slaughter	Corn and corn by-products (no more than 50 % of diet)**	100	50
All other species or classes of livestock and pet animals	Corn and corn by-products (no more than 50 % of diet)**	10	5

* Includes lactating dairy cows and hens laying eggs for human consumption
** Dry weight basis

Table 16 – Advisory levels for vomitoxin (DON) in livestock feed in mg/kg [ppm].

Source: www.fda.gov (Accessed: 11/01/2013)

Animals	Feedingstuffs and proportion in the diet	DON
Ruminating beef and feedlot cattle older than 4 months and chickens	grains and grain byproducts on an 88 % dry matter basis (not to exceed 50 % of the diet)	10
Swine	grains and grain byproducts on an 88 % dry matter basis (not to exceed 20 % of the diet)	5
All other animals	grains and grain byproducts (not to exceed 40 % of the diet)	5

1.10. Other legislation

In 2002/2003, following the initiative from previous years, the United Nations Food and Agriculture Organization (FAO) conducted an international inquiry and gathered information on the worldwide legislation on mycotoxins. As a result a publication entitled *Worldwide regulations for mycotoxins in food and feed in 2003* was produced. For more information regarding this issue please consult webpage www.fao.org

1.11. Legislation *versus* safe levels of mycotoxins

The development of legislation is crucial to limit the intake of mycotoxins by animals. However, as previously mentioned, animals are often confronted with other interacting factors and challenges which increase their susceptibility to these hazardous substances. That is why in the field it is common to see animals being affected by mycotoxins at levels below those identified in the legislation and in the scientific studies as dangerous. Therefore, with a basis on worldwide practical experience, in the field and in scientific trials, a table was developed by BIOMIN (Table 17) as a practical tool for those involved in the swine industry.

Levels listed refer to complete ration. The table should be considered as a rough recommendation. The negative impact depends not only on the level and type of mycotoxin contamination but also on the general health status of the animal and environmental conditions. All levels of mycotoxins should be considered as unsafe and increased levels carry increased risks to animal health. Low levels of mycotoxin ingestion can have a detrimental effect on the immune system and this is a hindrance for optimum performance.

Table 17 - Risk levels of mycotoxins for swine (ppm = mg/kg).

Risk Levels of Mycotoxins	Low	Medium	High
B-Trichothecenes (DON, AcDON, NIV, FusX) [ppm]			
sow, boar	<0.2	0.2-0.9	>0.9
piglet	<0.15	0.15-0.2	>0.2
grower, finisher	<2.5	0.25-1	>1
A-Trichothecenes (T-2 toxin, HT-2 toxin, DAS) [ppm]			
sow, boar	<0.1	0.1-0.4	>0.4
piglet	<0.05	0.05-0.1	>0.1
grower, finisher	<0.15	0.15-0.4	>0.4
Zearalenone [ppm]			
sow, piglet	<0.05	0.05-0.25	>0.25
grower, finisher	<0.1	0.1-0.25	>0.25
Ochratoxin A [ppm]			
sow, piglet	<0.05	0.05-0.4	>0.4
finisher	<0.08	0.08-0.5	>0.5
Fumonisins [ppm]			
sow, piglet	<1.5	1.5-4	>4
finisher	<2	2-5	>5
Aflatoxin B_1 [ppm]			
sow, piglet, grower, finisher	<0.02	0.02-0.1	>0.1
Ergot Alkaloids [ppm]			
sow, piglet	<0.2	0.2-0.9	0.9
grower, finisher	<0.8	0.8-5	5

1.12. Acknowledgements

A special thank you to Dr. Roman Labuda and Dr. Georg Häubl for carefully revising this chapter and for sharing their knowledge of mycology, microbiology and chemistry with us.

2. MYCOTOXICOSES IN SWINE

INÊS RODRIGUES AND MAXIMILIAN SCHUH

2.1. Mycotoxicoses

Mycotoxicoses are the animal or human diseases caused by ingestion of mycotoxins, inhalation or contact with the skin.

The effects of mycotoxins in animals are diverse, varying from immunosuppression to death in severe cases, depending on toxin-related (type of mycotoxin consumed, level and duration of intake), animal-related (animal species, sex, age, breed, general health, immune status, nutritional standing) and environmental (farm management, biosecurity, hygiene, temperature) factors (Heidler, 2004) (Figure 15). This fact often impedes correct tracking of problems caused by mycotoxins.

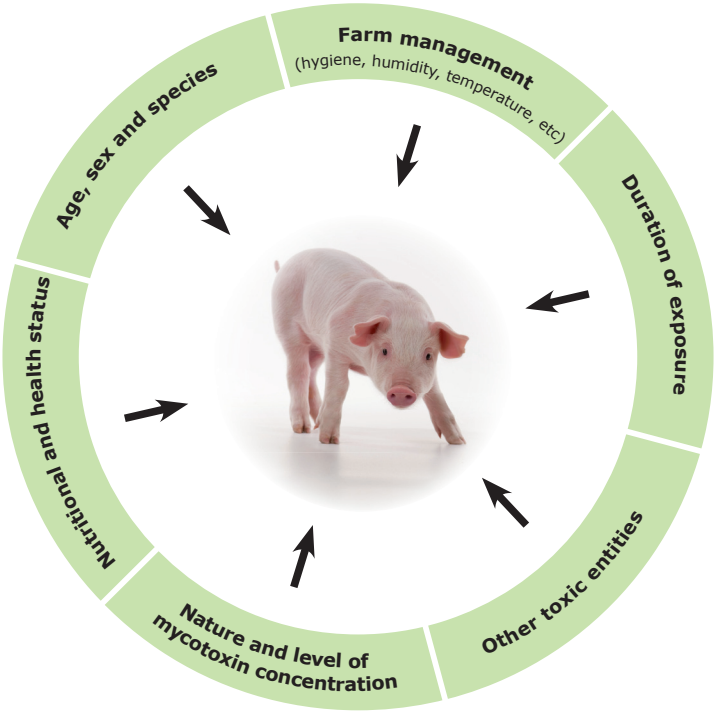


Figure 15 – Interacting factors

In spite of this, many scientific studies report the effects of mycotoxins in pigs at different levels, enabling a better diagnosis of mycotoxin-related ailments (Table 18).

The problem regarding mycotoxins is not only related to their lethal effects on animals but also by decreased animal performance leading to great economic losses.

Table 18 - Effects caused by mycotoxins in swine*

	Effects	Signs/Symptoms
Aflatoxins	Carcinogenic effects	Higher incidence of cancer in exposed animals (CAST, 2003)
	Immunosuppression	Decreased resistance to environmental and microbial stressors Increased susceptibility to diseases (Cysewski et al., 1978; Diekman and Green, 1992)
	Decreased performance	Reduced feed intake Feed refusal Impaired FCR (Diekman and Green, 1992)
	Hepatotoxic effects	Toxic hepatitis (Miller et al., 1981; Miller et al., 1982)
	Nephrotoxic effects	Kidney inflammation (CAST, 2003)
	Hematopoietic effects	Systemic hemorrhages (CAST, 2003)
	Residues	Residues and metabolites in liver and milk (Trucksess et al., 1982; Silvotti et al., 1997)
Trichothecenes	Gastro-intestinal effects	Vomiting Diarrhea Decreased barrier function (Young et al., 1983; CAST, 2003)
	Immunosuppression	Decreased resistance to environmental and microbial stressors Increased susceptibility to diseases Affected immune cells and modified immune response (Sharma, 1991)
	Decreased performance	Feed refusal Decreased weight gain Impaired FCR (Young et al., 1983; Trenholm et al., 1984; Diekman and Green, 1992)
	Hematopoietic effects	Hemorrhages Hematological disorders (Lorenzana et al., 1985; Pang et al., 1987 and 1988; Prelusky et al., 1994; CAST, 2003)

*Adapted from Chapter 2 of Guide to Mycotoxins: Näehrer, 2012)

Table 18 - Contd.

	Effects	Signs/Symptoms
Trichothecenes	Teratogenic effects	Splay legs (Alexopoulos, 2001)
	Dermal effects	Oral and dermal lesions Necrosis (Ueno, 1984; Lawlor and Lynch, 2001; CAST, 2003)
Ochratoxin A	Decreased performance	Reduced weight gain Impaired FCR Increased mortality (Lippold et al., 1992)
	Nephrotoxic effects	Kidney damage (porcine nephropathy) Increased water consumption Kidney and urinary bladder dysfunction Altered urine excretion (wet beds) (Krogh et al., 1976; Rousseau and Van Peteghem, 1989; Hald, 1991; CAST, 2003)
	Hepatotoxic effects	Liver damage (Szczech et al., 1973)
	Gastro-intestinal effects	Diarrhea (Lawlor and Lynch, 2001)
	Immunosuppression	Decreased resistance to environmental and microbial stressors Increased susceptibility to diseases (Harvey et al., 1992)
	Residues	Residues present in the kidneys, liver and meat (Pettersson, 2004)
Fumonisins	Immunosuppression	Decreased resistance to environmental and microbial stressors Increased susceptibility to diseases Decreased vaccine efficacy (Haschek et al., 2001)
	Pulmonary and cardiovascular effects	Porcine pulmonary edema (PPE) (Harrison et al., 1990; Haschek et al., 2001)
	Hepatotoxic effects	Liver damage (Colvin et al., 1993)
	Hematopoietic effects	Hematological disorders Increased serum sphinganine to sphingosine ratio (Riley et al., 1993; Haschek et al., 2001)

Table 18 - Contd.

	Effects	Signs/Symptoms
Fumonisins	Pathological changes	Pancreatic necroses (Voss et al., 2007)
	Residues	Residues in kidneys and liver (Prelusky et al., 1996)
Zearalenone	Reproductive effects	Hyperestrogenism: Feminization in males; enlargement of mammary glands; impaired semen quality; testicular atrophy; swollen prepuce; reddened teats; swelling and reddening of vulva; vaginal prolapse Affected cycling; conception; ovulation and implantation; pseudo pregnancy; abortion; anoestrus; nymphomania; embryonic death; inhibition of fetal development; decreased number of fetuses (reduced litter size) (Ueno, 1984; CAST, 2003; Zinedine et al., 2007)
	Teratogenic effects	Splay legs (Diekman and Green, 1992; Vanyi et al., 1994; Alexopoulos, 2001)
	Pathological changes	Atrophy of ovaries; uterus hypertrophy (CAST, 2003; Cheng et al., 2006)
Ergot alkaloids	Neurotoxic effects	Low prolactin production Agalactia Low colostrum production (Blaney et al., 2000; EFSA, 2005)
	Decreased performance	Reduced weight gain (Blaney et al., 2000; EFSA, 2005)
	Reproductive effects	Shrunken udder Signs of estrus Stillbirths Reduced pregnancy rate Abortion (Blaney et al., 2000; EFSA, 2005)
	Pathological changes	Vasoconstriction of ears Tail and claw necrosis (Blaney et al., 2000; EFSA, 2005)

2.2. Effects of mycotoxins on swine performance

Animal performance is influenced by many factors. The major ones contributing to the profitability of the pig production industry are the numbers of pigs produced per sow and the feed costs of producing those pigs, for which feed intake and feed conversion are determinants. The final objective of the pig producing industry is to maximize the amount of meat (kg) produced per sow per year, while maintaining sow condition and health.

In a feeding herd, there are some parameters that should be measured in order to assess its profitability, as they will influence the variable costs of a farm:

- Mortality rate will have an impact on animal purchase, feed and veterinary costs. An increase in mortality will have a great economic impact as all money invested will generate no return.
- The variation of daily weight gain, directly related to feed conversion, will have an impact on feed costs. Obviously, the more efficient the animal, the lower these costs, and vice-versa.
- The length of the finishing period affects both the feed costs - since a longer stay of animals on the farm is accompanied by increased feed consumption - and veterinary costs arising from any problems which might occur during that extra period.

Having said that, factors which impact on the above mentioned parameters – such as mycotoxins – will certainly reduce the economic turnover of the entrepreneur.

In the next sections, a brief description of the impact of different mycotoxins on animal performance (namely body weight gain, feed conversion and mortality rate, whenever applicable) will be given. Impacts of different mycotoxins on other systems are not discussed in detail here but are described in Table 18.

2.2.1. AFLATOXINS

As in other animal species, aflatoxins have a great impact on performance and health of pigs. Reduced weight gain and lipid digestion, and increased feed conversion were observed when 500, 650 and 800 µg aflatoxin B_1/kg feed were fed to growing and finishing pigs with a body weight between 40 and 140 kg (Bonomi et al., 1992). Likewise, Schell et al. (1993) reported reduced growth rates and depressed feed consumption when 922 µg aflatoxin B_1/kg feed were given to weanling and growing pigs. In another study with starter pigs fed diets contaminated with 500 µg aflatoxin B_1/kg feed for 34 days, the average daily weight gain was reduced by 27.8 % (Lindemann et al., 1997).

In animals as well as humans, all body systems are connected and reflect on general health and performance; therefore there are other factors which cannot be omitted when one describes the effects of aflatoxins in swine performance. As explained in Chapter 1, aflatoxins' main target organ is the liver; therefore hepatotoxicity and liver damage are the main effects to be expected in animals affected by them. Liver damage and the presence of aflatoxin B_1 metabolic products in the liver and tissues may be a reason for discarding of organs and carcasses in some countries. However, it may be that macroscopic lesions are only slight and cannot be observed even though aflatoxin residue levels exist at fairly high levels. This case has been described by Krogh and his team (1973) during a study in bacon pigs in Denmark. From a practical point of

view this would lead to the acceptance of most livers and all other organs and carcasses during meat inspection and the penetration of the aflatoxins into the human food chain.

The above-mentioned impact on swine performance and unwanted introduction of carcinogenic compounds in the food chain, along with the immunosuppressive effects of aflatoxins, which will be discussed later, render aflatoxins a serious threat to pig production.

2.2.2. TRICHOTHECENES

Numerous studies have focused on the impacts of trichothecenes on pig performance. In these studies, DON has been the most studied trichothecene, as it represents the most important trichothecene worldwide which causes enormous economic losses in swine production units.

Figure 16 - Stomach content after vomiting induced by 4.8 mg DON/kg feed
(Courtesy of Prof. Maximilian Schuh, University of Veterinary Medicine, Vienna, Austria)

From all species evaluated to date, pigs seem to be the most susceptible to DON, according to the rank pigs > mice > rats > poultry ≈ ruminants (Pestka, 2007). Although considered to be one of the least lethal trichothecenes, DON's emetic and anorectic potencies are equal to or greater than those reported for the more acutely toxic trichothecenes (Rotter et al., 1996a). In 2004, a comprehensive scientific review on the toxic effects of DON in pigs was part of an opinion paper by the European Food Safety Authority (EFSA). According to this, at low contamination levels, the stimulation of the synthesis of pro-inflammatory cytokines is responsible for the decreased feed intake. However, at high concentrations vomiting is triggered (Figure 16) by the interaction with serotonergic and dopaminergic receptors (EFSA, 2004a). Earlier scientific studies showed negative effects of DON on feed consumption and performance with purified mycotoxin given at extremely high levels, such as 12 mg/kg feed or 20 mg/kg feed (Forsyth et al., 1977; Young et al., 1983). Other studies brought values down to levels which would be more likely to occur in feeds and were performed with naturally contaminated grains. In those, a temporary reduction of feed intake was already observed in pigs given 350 µg DON/kg feed (Friend et al., 1992; Trenholm et al., 1983). Likewise, later experiments performed with 0.6 to 2 mg DON/kg feed reported a decrease in feed consumption and weight gain (Bergsjö et al., 1993; Overnes et al., 1997).

Although the observed reduction of feed intake at these lowest dosages was shown to be temporary, the loss in weight gain during the first growing period was not completely

compensated for during the later periods; therefore animals reached slaughter weight at an older age.

Consistently, in many scientific studies, naturally infected feed exerts a more pronounced negative effect on feed intake and weight gain than pure toxin, which could be explained by the presence in the feed of other toxins produced by the same fungal species. Indeed, in practical on-farm conditions it is not uncommon to find groups of animals with very large heterogeneous weights (Figure 17).

Figure 17 – Heterogeneous weights in growing piglets
(Courtesy of Dr. Guan Shu, BIOMIN Singapore Pte Ltd)

The impact of DON on the barrier function and on the electrical properties of the intestinal mucosa is, to a great extent, related to performance. In poultry, DON inhibited Na^+ transport and Na^+-D-glucose cotransport (Awad et al., 2005). In pigs, low concentrations of DON, T-2 and ZEN probably influenced enterocytes metabolism and evoked inflammation of the mucous membrane of the small intestine (Obremski et al., 2008). The increase of permeability observed in another study can generally be associated with an increased passage of bacteria through the gut epithelium due to the defective epithelial barrier function (Pinton, 2009). These impacts caused by different mycotoxins at an intestinal level might explain a higher incidence of diarrhea in affected animals (Figure 18).

Another toxin of interest in this group of mycotoxins is T-2 toxin which also showed to have an impact on the performance of pigs. When orally administered at levels of 500 µg/kg feed a 10 % reduction in feed intake was observed (Rafai et al., 1995). This result confirmed data gathered in a previous study (Weaver et al., 1978).

2.2.3. OCHRATOXINS

As explained in Chapter 1, the acute course of ochratoxicosis in swine is focused on nephropathy, enteritis and suppression of the immune system (Terao and Ohtsubo, 1991).

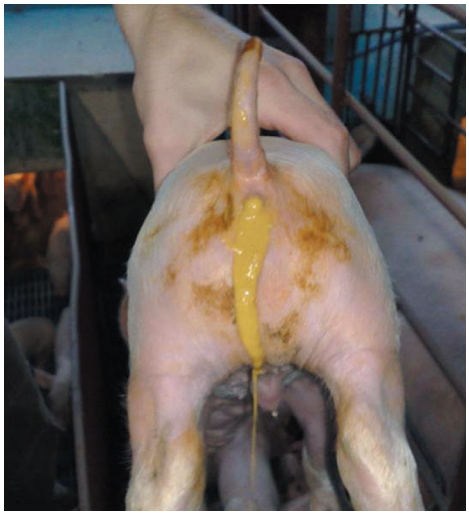


Figure 18 – Piglet with diarrhea (feed analyses revealed the presence of an average of 200 µg ZEN/kg feed, 2000 - 4000 µg FUM/kg feed and 150 µg DON/kg feed)
(Courtesy of DVM Everson Zotti, Sanphar Saúde Animal Ltda, Brazil)

However, impacts on performance have also been described in literature. Lippold et al. (1992) fed up to 2.5 mg OTA/kg feed to 15 kg barrows for 3 weeks. A reduction in the daily weight gain, feed intake and feed efficiency was observed.

As in the case of other mycotoxins, residues of ochratoxin might be found in animal products. This impacts the turnover of farmers in countries were control is done and penalties are enforced.

Several attempts have been made to find a correlation between blood serum concentration and feed levels of OTA. In a western Canadian study involving 1600 pigs, 36 % of serum samples were OTA-positive in a detectable amount (Ominski et al., 1996). In Denmark, visual evaluation of pigs' kidneys has been used since 1978 to indirectly control the level of OTA in pork. Renal OTA residues are used for an effective quality control system to minimize potentially harmful residues in pork products. If residues in the kidney range between 10-25 µg OTA/kg, liver and kidney of slaughtered pigs are discarded. If these values exceed 25 µg OTA/kg, the whole carcass is condemned. This means an enormous economic loss for pig finishing units (Jorgensen and Petersen, 2002).

2.2.4. FUMONISINS

The increasing worldwide occurrence of fumonisins renders this group of mycotoxins an important threat to animal production. Back in the year 2000, a world health organization working group reported that globally 59 % of corn and corn product samples were contaminated with FB_1 (Visconti, 2000). A more recent publication reports even higher values with fumonisin B_1 occurring in 83 % of corn samples and in 81 % of DDGS samples (Rodrigues and Naehrer, 2012). In fact, this 2-year worldwide publication ranks FUM as the second most prevalent mycotoxin worldwide (after DON) and the number one in terms of corn and corn by-products.

In terms of direct impacts on animal performance reports show varying results. Palatability problems have actually been attributed to the presence of this mycotoxin in swine feed (Haschek et al., 1992; Motelin et al., 1994), as well as increased variability of carcass quality (Rotter et al., 1996b; Rotter et al., 1997) which might lead to their lower market value. In terms of food safety, another important fact shown by scientific studies is that, even though FUM are poorly absorbed and rapidly excreted, the absorbed fraction undergoes a wide distribution with a high affinity to the liver and kidney, followed by a slow release of the toxin by these organs. Actually the same study suggests that for pigs exposed to 2-3 mg FUM/kg feed a withdrawal period of at least 2 weeks would be required to ensure that only minimal residue levels remain (Prelusky et al., 1996). Interestingly, fumonisin-induced sphingolipid alterations differ between animal species probably due to the different cell-specific functions of sphingolipids amongst them. In swine, sphingolipids' alteration show a correlation with the accumulation of FUM in the organs, thus more pronounced in the liver and kidney, with reports of renal and liver injury (with a dominant feature of progressive liver disease) and in some cases also pancreatic necrosis (Voss et al., 2007).

In spite of having impacts at many levels and on many systems (Table 18), FUM is well acknowledged as a causative agent for Porcine Pulmonary Edema (PPE) in animals fed high concentrations of FUM (92 mg/kg feed) and in animals fed much lower doses (300 µg/kg feed, 10 and 40 mg/kg) (Zomborszky et al., 1997a,b). The increased respiratory rate reported for animals intoxicated with FUM - and thus the increased oxygen consumption - may be an explanation for the decreased feed efficiency reported for animals with PPE (Smith et al., 1999). In cases of exposure to naturally-contaminated feed for 4 to 7 days, death may follow respiratory distress and pulmonary edema, which appears to be caused by left-side heart failure (Voss et al., 2007).

As a summary, and as stated by Haschek and team (2001), the effects of FUM which create the greatest economic impacts are related to the low-dose effects of this group of fungal metabolites, which include subclinical hepatic disease, altered carcass quality, adverse immune effects with potential predisposition to infectious diseases (to be further discussed in Chapter 3), and potential interactions with other toxins, including mycotoxins and other agents. Potential reproductive effects require further study in the case of swine but, nonetheless, will be discussed later in this chapter.

2.2.5. ERGOT ALKALOIDS

Blaney et al. (2000) reported negative effects of ergot alkaloids on the cardiovascular and central nervous systems due to the rising of blood pressure causing vasoconstriction.

Feed intake, feed conversion and daily weight gain might be impaired in the presence of higher amounts of ergot alkaloids (EFSA, 2005). Interestingly, some papers deny the occurrence of feed refusal to pigs fed up to 8 mg alkaloid/kg feed; however, the use of ingredients to increase palatability of meals, such as full-fat soybean meal, fish meal and flavoring agents is reported as an option to counteract the distastefulness of rye-ergot contaminated grain (Kopinski et al., 2008a).

In litters from sows fed diets containing 3 % sorghum ergot the weight gain observed was 66 % of that observed in the control litters fed the uncontaminated diet (Kopinski et al., 2008b). In a similar study where sows were fed diets containing 1.5 % of ergots, 87 % of piglets died

despite all efforts to supplement them with natural and artificial colostrums, milk replacers and attempts to foster them onto normal lactating sows (Kopinski et al., 2007). From this trial, recommendations were given to limit the sorghum ergot concentration to 0.3 % in diets of multiparous sows before farrowing up to 0.1 % in diets of primiparous sows.

2.3. Effects of mycotoxins on swine fertility

The main aim of the pig industry is to maximize the quantity of quality meat produced per sow per year while maintaining sow condition and health. In the breeding herd, the animal performance is directly related to the reproductive performance of the sows later to be measured by the number of weaned piglets per sow per year. The objective of managers in breeding herds is to increase this number while providing finishing herds piglets with good growth rates and carcass traits.

If it is true that genes are crucial in determining fertility, performance and carcass traits - thus corroborating the importance of genetic breeding programs - one should never forget that environmental factors will influence the way genes are expressed. Environmental factors manipulating gene's expression include diet, physical activity, infectious agents, chemicals, ultraviolet radiation and even emotions. A palatable diet, which guarantees the nutrient intake of animals at their different production stages and which does not pose threats to the animals ingesting them, is crucial for the success of animal production.

Reproductive failure, abortions, poor rearing ability and litter size are the cause of 45 % and 59 % of female pigs culled, in all parities and in gilts and second parities, respectively (Muirhead and Alexander, 1997). A high culling rate related to breeding problems has a large impact on the profitability of pig producers. Because of its multiple origins and high economic impact, reflected by decreased animal performance and increased culling rates, reduced fertility is one of the most complex and puzzling problems dealt by swine producers.

Mycotoxins are included in the toxic substances that often occur in animal feeds, posing a serious menace to animals ingesting them and acting directly and indirectly on pig fertility.

2.3.1. ZEARALENONE AND ITS DIRECT EFFECTS ON FERTILITY

Although zearalenone represents a relatively low toxicity to animals, this mycotoxin is well known due to its interference with the endocrine system. ZEN is the number one regarding its negative effects on fertility and swine are the animals that are most affected by it. As explained in Chapter 1, the resemblance between zearalenone and the primary female hormone, estrogen, misleads the cells' estrogen-receptors causing hyperestrogenism and impaired fertility. Also in Chapter 1 the metabolization of ZEN into the more estrogenic molecule, α–zearalenol, was mentioned, a fact which emphasizes the importance of this compound as estrogenic.

Concentrations of zearalenone ranging from 1 to 30 mg/kg feed are related to infertility and other problems such as anoestrous, pseudo pregnancy, swollen and reddened vulva, mammary gland enlargement and vaginal and rectal prolapse (Figure 19). Moreover, other issues may also occur, such as disrupted placental and piglet development, resulting in decreased litter size and diminished viability of neonates (Miller et al., 1973; Chang et al., 1979; Sundloff and Strickland, 1986).

Figure 19 - Rectal prolapse of sows
(Courtesy of DVM Diego Padoan, BIOMIN GmbH, Austria)

However, as mentioned in the case of deoxynivalenol, scientific experiments performed with pure, crystalline zearalenone may not reflect the practical feeding conditions on a farm as zearalenone often co-occurs with other *Fusarium* toxins, especially deoxynivalenol. More recent data take this fact into consideration. Jadamus and Schneider published in 2002 the results of a long-term study performed in pre-pubertal gilts fed naturally contaminated feed. These animals fed 0.2 mg ZEN/kg feed and 2.6 mg DON/kg feed showed ZEN-related fertility impairments including elevated return to estrus rate, abortions and symptoms of hyper-estrogenism in new-born piglets, even after a short period of exposure during the first reproductive cycle. These clinical signs observed in newborn piglets involving reddened teats, swollen and reddened vulva (Figure 20) and splay legs (Figure 21) suggest placenta and/or milk transfer from the mother sow.

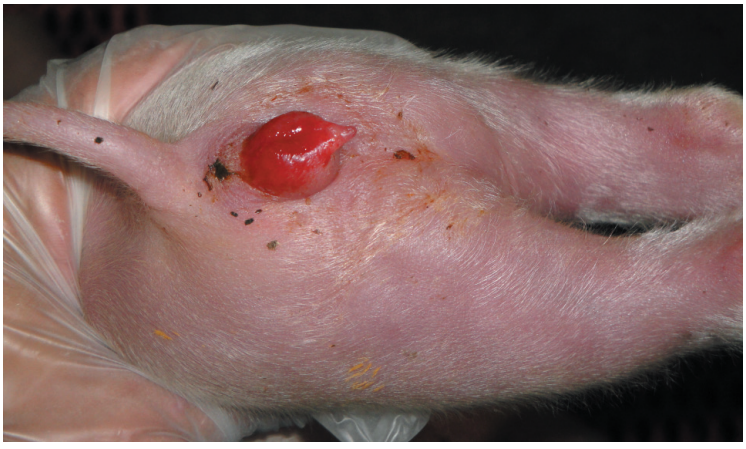


Figure 20 - Suckling piglet: reddening and enlargement of vulva
(Courtesy of Traunkreis Vet Clinic, Ried im Traunkreis, Austria)

Figure 21 - Splay legs of newborn piglets
(Courtesy of Dr. Guan Shu, Biomin Singapore Pte Ltd)

It is important to bear in mind that not only female animals are negatively affected by this mycotoxin, but also pre-pubertal males and boars, even if at higher concentrations (Gaumy et al., 2001). A depression of serum testosterone, weight of testicles and spermatogenesis, along with feminization and suppressed libido can be observed in boars fed ZEN-contaminated diets (Figure 22).

In the field, a direct and important correlation can be made between mycotoxin-contamination and fertility problems. Data collected in five Swedish pig farms showed that 85 % of all sows presented zearalenone in the liquid (Dalin et al., 2006). Furthermore, the level of this mycotoxin in the bile was significantly higher in farms having fertility problems in comparison with control farms.

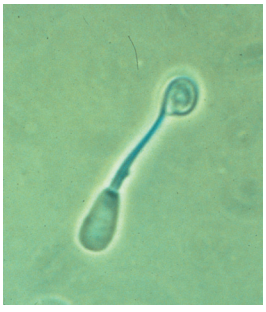


Figure 22 - Breeding boar: pathological forms of spermatozoon after ingestion of 25 mg ZEN/kg feed
(Courtesy of Prof. Maximilian Schuh, University of Veterinary Medicine Vienna, Austria)

2.3.2. ERGOT ALKALOIDS AND THEIR DIRECT EFFECTS ON FERTILITY

As stated by EFSA's opinion paper on the presence of ergots in animal feed, the most prominent clinical sign is agalactia in sows, often leading to starvation of piglets. Other symptoms are feed refusal, reduced weight gain and eventually abortion (EFSA, 2005). Actually, several

scientific studies reiterate this statement and add to the list of negative reproductive effects caused by ergot's presence in swine feed.

Effects of ergot alkaloids were observed in sows fed sorghum grain infected with sorghum ergot (17 % *C. africana* ergot sclerotia) before farrowing. Observed symptoms included shrunken udders, lack of colostrum production, signs of estrus and dead piglets due to starvation (no milk ingestion) (Blaney et al., 2000). Sows fed the grain after farrowing showed a severe reduction in milk production (and in some cases a total cessation of milk production was observed together with a shrinking of the udder) due to low levels of prolactin (Bandyopadhyay et al., 1998; Blaney et al., 2000; Kopinski et al., 2007; Kopinski et al., 2008a). Moreover agalactia (due to interference in the release of prolactin), feed refusal and consequently reduced weight gain are classical signs of poisoning by rye ergot (*C. purpurea*) (Bandyopadhyay et al., 1998; Blaney et al., 2000; Kopinski et al., 2008b). Other symptoms such as strong uterotonic effects, causing stillbirths and reduced pregnancy rate are also frequent (Kopinski et al., 2007; Kopinski et al., 2008a).

2.3.3. OTHER MYCOTOXINS AND THEIR DIRECT EFFECTS ON FERTILITY

Although having a completely different toxicological pattern, studies with T-2 toxin were also carried out to evaluate the impact of this mycotoxin on swine fertility. T-2 caused drastically decreased conception rates, infertility (repeated return to estrus), decreased litter size and reduced weight of piglets (Weaver et al., 1978). This trichothecene is known to inhibit protein synthesis (Ueno, 1991; Bunner and Morris, 1988). Its biological mode of action may be the explanation why this fusariotoxin is responsible for these fertility problems in swine.

Some work has been done on the effects of OTA on male fertility. OTA was given in a concentration of 5 to 10 times the human tolerable daily intake which resulted in a significant reduction of the initial motility and longevity of spermatozoa (Solti et al., 1999). In a more recent experiment, after a 4-week control period, animals were orally given 20 mg OTA daily for 6 weeks, followed by a 9-week withdrawal period (Biro et al., 2003). The report concluded that OTA may affect sperm production and boar semen quality only after a lag period.

FUM on the other hand were reported to cause lesions to fetuses in the uterus in gestating sows (Zomborszky-Kovacs et al., 2002).

2.4. Indirect effects of mycotoxins on swine fertility

Other mycotoxins, although not having direct impacts on swine fertility, are responsible for immunosuppression (which will be dealt with later) and further health problems that will clearly affect the reproductive performance of animals.

2.4.1. OCHRATOXIN A AND ITS INDIRECT EFFECTS ON FERTILITY

Tubular necrosis of the kidneys, porcine nephropathy, urinary bladder and kidney inflammation, enteritis as well as birth-defects and carcinogenic effects (kidney and urinary tract tumors) are some of the pathological consequences observed in animals fed OTA-contaminated diets.

Cystitis and nephritis, inflammation of the bladder and kidney, respectively, are important causes of mortality in all ages of dry sows. The high mortality associated with these conditions affects the herd mortality rate and therefore survival of fetuses during pregnancy, ultimately affecting the herd fertility.

2.4.2. DEOXYNIVALENOL AND ITS INDIRECT EFFECTS ON FERTILITY

Feeding animals with DON-contaminated feeds may lead to digestive disorders such as emesis, diarrhea and feed refusal. Feed refusal is particularly hazardous for female pigs, as the balancing and satisfaction of nutrient requirements - vital for a regular reproductive cycle - will be compromised. Once again, the immunosuppression caused by this mycotoxin can result in an increased susceptibility to other pathogens, namely Candida (Salazar et al., 1980), Listeria (Corrier and Ziprin 1986; Pestka et al., 1987, Tryphonas et al., 1986), Salmonella (Boonchuvit et al., 1975, Tai and Pestka, 1988, 1990; Vidal and Mavet 1989) and Mycobacteria (Kanai and Kondo 1984), which predispose animals to abortions.

2.4.3. FUMONISINS AND THEIR INDIRECT EFFECTS ON FERTILITY

A statistically significant association was found between contamination with fumonisins and the risk of Porcine Reproductive and Respiratory Syndrome (PRRS), previously referred to as “Mystery Swine Disease” (Bane et al., 1992). On the other hand there is a strong association between PRRS and reproductive failure, namely decreased viability of neonates and early farrowing.

2.5. Economic impact of decreased fertility

Whether directly or indirectly it is a fact that the occurrence of mycotoxins in feeds has an effect on swine fertility (Figure 23).

Calculations of the economic impact of a lost cycle must take into account at least the extra semen and artificial insemination costs, as well as the additional local feeding costs. Frequently, veterinary costs and culling costs will also be increased by the occurrence of these problems and obviously the reduction of the litter size and of the number of weaned piglets will be reflected in the profit calculation of farms.

Infertility in sows greatly compromises the breeding herd production and should not be considered in isolation. The existence of toxic agents such as mycotoxins in animal feeds should be monitored and counteracted by professionals in the pig industry as their occurrence may cancel out the effects of work done both by the genetic programs and by the carefully stage-adapted feeding programs, thus impeding the evolution of the whole industry.

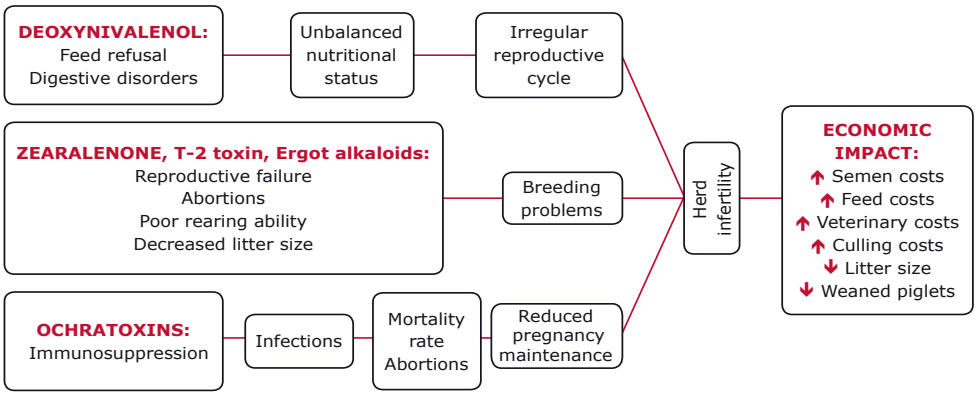


Figure 23 – Direct and indirect effects of mycotoxins in swine fertility and their economic impacts

2.6. The impact of mycotoxins on animal immunity

In modern animal production, the animal's performance boundaries are continuously being challenged in order to provide the highest profitability. Disease outbreaks in farms are known to have economically devastating effects as the whole herd can be affected.

The word "health" means not only free of diseases but also represents a whole new concept of welfare in which the animals are able to fully express their genetic potential, maximizing performance and as a consequence the profit of farmers.

Mycotoxins are toxic substances that impair the physiological function of the immune system of animals, leading to significant negative economic impacts.

The economic viability of modern pig production farms is affected by numerous factors. Amongst them, some exist that cannot be controlled by animal producers, namely fluctuations on the prices of agricultural products and of meat and the stricter policies imposed by agriculture regulatory bodies. Nevertheless, many of them can be improved as a result of the management strategy of farmers.

The occurrence of diseases has a direct negative impact on the economic viability of pig farms as outbreaks represent major cost increases and loss of profit. Disease control and treatment is therefore crucial for the economic viability of pig farms.

For a long time immunosuppression has been described by veterinarians observing animals which consume mycotoxins at levels lower than those causing clinical symptoms of toxicity (Richard et al., 1978; Oswald et al., 2005).

Since the immune system and the impacts of mycotoxins on the immune system are complex topics which have been the subject of much research over the past few years, the next chapter will be solely devoted to them.

3. SWINE IMMUNE SYSTEM

ROGER BERRÍOS AND ISABELLE OSWALD

Humans and animals live in different environments full of microorganisms such as viruses, bacteria, fungi and protozoa. Some of these microorganisms are pathogens, which are able to produce diseases in animals. This microbial invasion is faced using a complex system of defense mechanisms - the immune system - which protects the animal and ensures a healthy body. These defense components are divided into innate immunity and acquired immunity.

The innate (nonspecific) immune system responds immediately once an invader is detected. It controls the infection until the acquired immune system can be activated. However, it lacks any form of memory and therefore, the same invader, when repeatedly encountered, will be treated identically each time. In other words, the innate immune response does not improve with repeated exposure to a specific invader.

The acquired (adaptive) immune system takes longer to be effective after the first exposure to the invader. However, once the foreign invader is recognized and destroyed, it retains the memory of the encounter. If subsequent exposure to the same agent occurs, it responds more rapidly and more effectively. This type of response is the one activated during vaccination. It comprises two types of responses according to the category of the microbial invader. The humoral immune response protects against extracellular invaders such as many bacteria and protozoa, fungi and helminthes, using different antibodies. Specialized cells that destroy infected or abnormal cells are part of the cell-mediated immune response, protecting against intracellular invaders like viruses, intracellular bacteria and protozoa. The immune system has potent mechanisms that ensure survival in the face of a continuing microbial challenge. If appropriate regulation of the immune system is interrupted, clinical disease may result.

The immunomodulating effects of mycotoxins have been evaluated *in vivo* and *in vitro* utilizing numerous animal species, cell culture systems and experimental designs. Their immunosuppressive effects may vary according to dosage, route of administration and animal species. Consumption of low levels of mycotoxins can suppress immune functions and decrease resistance to infectious disease. According to Corrier (1991) the mycotoxin-induced immunosuppression may be manifested as depressed T or B lymphocyte activity, suppressed immunoglobulin and antibody production, reduced complement or interferon activity and impaired macrophage-effector cell function (Oswald et al., 2005; Sharma, 1993). This may eventually decrease resistance to infectious diseases, reactivate chronic infections and/or decrease vaccine and drug efficacy (Oswald et al., 2005; Stoev et al., 2000). Inhibition of DNA, RNA and protein synthesis via a variety of different mechanisms appears to be directly or indirectly responsible for the immune suppressive action of many mycotoxins (Corrier, 1991).

3.1. Organs of the immune system

The immune system of the pig is formed by a group of lymphoid organs classified according to development, production and differentiation of lymphocytes (primary lymphoid organs) and trapping and processing of foreign antigens (secondary lymphoid organs). Primary lymphoid

organs are thymus and bone marrow. Secondary lymphoid organs are, at systemic level, the spleen and lymph nodes; at mucosal level, tonsils and gut associated lymphoid tissue (GALT) - Peyer's patches (Rothkötter, 2009).

3.1.1. BONE MARROW

The bone marrow is distributed in the bones, principally in large bones. Structurally and functionally it is divided in stroma and hematopoietic tissue. The stroma consists of fibers and reticular cells functioning as support for the hematopoietic tissue and it produces growth factors affecting hematopoiesis. The hematopoietic tissue contains hematopoietic stem cells, multipotent stem cells from which all types of blood cells including myeloid (monocytes and macrophages, neutrophils, basophils, eosinophils, erythrocytes, megakaryocytes/platelets, dendritic cells), and lymphoid lines (T cells, B cells, natural killer cells) originate.

3.1.2. THYMUS

The thymus in young pigs extends caudally from the digastric muscle along the carotid arteries at both sides of the neck until the end of the thoracic cavity. The organ is gradually replaced by fat after puberty. It is formed by different lobules covered by a connective tissue capsule. Histologically and functionally it is divided into two zones: the cortex, the outer part, densely infiltrated with lymphocytes (thymocytes) in proliferation; and the medulla, formed by numerous epithelial cells and Hassall's corpuscles that stimulate thymocyte proliferation by secreting growth factors. The main function of thymus is to differentiate T cell precursors, which originate in the bone marrow, into mature T cells.

3.1.3. SPLEEN

The spleen is an elongated, flattened, brownish organ that extends along the posterior part of the ventral stomach to the pancreas. The organ is rounded by a connective tissue capsule comprising muscular fibers and collagen, constituting a support system. The functional part is divided into two forms of tissue: the white pulp, formed by lymphoid follicles (lymphocytes, plasmatic cells, macrophages, dendritic cells) and periarteriolar lymphatic sheaths (mainly T lymphocytes), where immune responses occur, and the red pulp, comprising splenic strings, venous sinuses and sheathed capillaries (periarterial macrophagic sheaths). The immunological function of the spleen is to remove antigens such as blood-born microorganisms, cellular debris and aged blood cells. Additionally, the spleen stores red blood cells and platelets, recycles iron and produces red blood cells in the fetus (hematopoietic function).

3.1.4. LYMPH NODES

Lymph nodes are round or bean-shaped formations of a whitish color surrounded by a capsule. They consist of a reticular network filled with lymphocytes, macrophages and dendritic cells

(DCs) through which lymphatic sinuses penetrate. Its function is to filter and capture antigens from lymph and then proceed to their presentation and antigenic processing. Histologically and functionally the lymph node is divided into medullar and cortical tissue, separated by the paracortex region. The medullary tissue (mainly composed of reticular fiber, collagen, some macrophages and DCs) is distributed peripherically in lymph nodules, and around the efferent hilus. The cortical tissue is located in the central area, together with a large number of lymphoid follicles, surrounding the efferent hilus. It consists of lymphoid follicles (mainly B lymphocytes and several T lymphocytes) and diffuse lymphoid tissue, considered as a T-dependent zone.

3.1.5. PEYER'S PATCHES (PPs)

The PPs are non-encapsulated lymphoid tissue aggregates, located in the gut wall. Almost 80 % of the PPs are found in the ileum forming one continuous structure (up to two meters in length), that extends to the ileocecal junction. Ileal Peyer's patches consist of lymphoid follicles (with great lymphopoetic activity) separated by connective tissue sheaths containing only B cells. In pigs these ileal PPs regress within the first year of life, thus they are considered as primary lymphoid organs. The other 20 % of the PPs are distributed along the jejunum and the proximal portion of the ileum. Jejunal PPs persist for the life of the animal and consist of follicles that mainly contain B and T cells. The PPs function is a site of rapid B cell proliferation.

3.1.6. TONSILS

Tonsils consist of either a solitary node or an aggregation of nodes and diffuse lymphoid tissue. They are located in the soft palate between the respiratory and digestive tract. The surface of the tonsils may be relatively smooth or it may have surface invaginations which allow a high concentration of lymphatic tissue in a given area. Histologically, the tonsils exhibit crypts and lymphoid follicles (consisting mainly of B cells) which are surrounded by diffuse lymphoid tissue (formed by T cells).

3.2. Cells of the immune system

The cells of the immune system originate from pluripotent stem cell (descendants of totipotent cells) and can differentiate into almost any cell. Two lines of stem cells originating from pluripotent stem cells are important in the immune system: myeloid stem cells and lymphoid stem cells. Myeloid stem cells produce monocytes (myeloid dendritic cells, follicular dendritic cells and Langerhans cells), neutrophils, eosinophils and basophils. Lymphoid stem cells produce lymphocytes (B and T), natural killer cells and plasmacytic cells (plasmacytoid dendritic cells). The leukocytes (white cells formed in the bone marrow) are the defensive cells of the body which circulate in the bloodstream.

3.2.1. MYELOID CELLS

Myeloid cells, granulocytes and monocytes/macrophages, play important roles in inflammation and host innate and adaptive immune responses (Ezquerra et al., 2009). Granulocytes act as the

first line of defense against invading pathogens and are classified into neutrophils, basophils or eosinophils according to the staining characteristics of their predominant granules.

3.2.2. NEUTROPHILS

The principal function of neutrophils is the phagocytosis and destruction of microorganisms. Through the production of chemokines, pro-inflammatory cytokines and cell–cell contacts, neutrophils recruit and activate monocytes, dendritic cells (DCs) and lymphocytes (Ezquerra et al., 2009). This contributes to the clearance of the microbes and infected cells, and leads ultimately to the initiation of an adaptive immune response.

3.2.3. BASOPHILS

Basophils act as effector cells in IgE-associated type I hypersensitivity reactions. They also produce cytokines (IL-4, IL-3) which mediate immunoregulatory functions (Ezquerra et al., 2009).

3.2.4. EOSINOPHILS

Eosinophils are important in host defense against parasites and other infections, especially against RNA viruses (Ezquerra et al., 2009). They also release a range of cytokines and lipid mediators which modulate innate and adaptive immunity.

3.2.5. MONOCYTES/MACROPHAGES

Monocytes (in blood) or macrophages (in tissue) and their precursors, constitute the so-called mononuclear phagocyte system (Ezquerra et al., 2009). They adopt different names according to the organ in which they are located (Histiocytes in connective tissue, Kupffer cells in liver, Microglia in neural tissue). Macrophages have important regulatory and effector functions in the specific immune response and in the maintenance of tissue homeostasis. Macrophages are responsible for beginning the healing process in damaged tissue. They contribute to the resolution of inflammation, being involved in cell migration, matrix remodeling and angiogenesis. Under specific conditions monocytes can also differentiate into dendritic cells (DCs).

3.2.6. DENDRITIC CELLS (DCS)

Dendritic cells are a heterogeneous group of potent antigen-presenting cells with the unique capacity to prime naive T-cell responses (Summerfield and McCullough, 2009). They express several families of specialized pattern recognition receptors for pathogen-associated molecular patterns (PAMPs). DCs are the main cellular element controlling T lymphocyte activation and regulation. They are involved in B cell responses by production of B cell stimulatory factors

which are important for B cell proliferation, differentiation and isotype switching. Moreover, DCs play a functional role for natural killer cell activation and are in a two-way communication with neutrophils. DCs show a high level of heterogeneity, particularly in the specialized roles of various DC subsets depending on their tissue localization and local immunological environment, which guides their function.

3.2.7. LYMPHOID CELLS

3.2.7.1. Natural killer cells

Natural killer cells represent an important cell population of the innate (natural) immune system. They have the capability to spontaneously attack pathogen-infected and malignant body cells and to produce immune-regulatory cytokines (Gerner et al., 2009).

3.2.7.2. T lymphocytes (T cells)

T lymphocytes belong to the adaptive (acquired) immune system and perform a wide variety of functions in immune regulation, inflammation and protective immune responses (Gerner et al., 2009). They are produced in the spleen, and act in the cellular response. Porcine T cells can be divided into a number of subpopulations, including a prominent fraction of T cells expressing T cell receptors (TCR) with γδ-chains (TCR-γδ) and αβ-chains (TCR-αβ) . TCR-γδ T cells can express cluster of differentiation 8a (CD8a) and major histocompatibility complex (MHC) class II, two molecules which in swine seem to be correlated with an activation status of T cells. Functional properties of these cells seem to include cytolytic activity as well as antigen presentation. TCR-αβ T cells in swine comprise MHC class I restricted cytolytic T cells (an important part of the cell mediated immune response to virus infections and tumor), T helper cells – Th cells (including subpopulations Th1, Th2 and Th17) and recently identified regulatory T cells (Gerner et al., 2009).

3.2.7.3. B lymphocytes (B cells) and Immunoglobulins (Igs)

B lymphocytes are produced in the bone marrow, and are responsible for the humoral immune response (Bianchi and van der Heijden, 1994). On their surface, B cells contain receptors called immunoglobulins (Igs). B lymphocytes, equipped with their surface Ig receptor, will recognize native unprocessed antigens. Any B lymphocyte, stimulated either by α T- dependent antigen or by α T- independent antigen, generates a plasma cell clone, which will produce and secrete a large quantity of specific antibodies (Igs) for the epitope (part of an antigen that is recognized by antibodies) that finally induces the immune response.

All immunoglobulins originate as B cell antigen receptors, shed into body fluid where they act as antibodies. There are five class of immunoglobulins in mammals (IgG, IgM, IgA, IgE, IgD), each of them adapted to a specific environment. Ig G is predominant in serum and responsible for systemic defense. IgM is produced mainly during primary immune response. IgA is predominant in secretions and present in the intestinal and respiratory tracts. IgE is responsible for immunity to parasitic worms and for allergies, and finally IgD is found on the surface of lymphocyte but its further functions are not completely clear (Butler et al., 2009).

3.3. Other components of the immune system

3.3.1. THE COMPLEMENT SYSTEM

The complement system is a defense mechanism that comprises an enzyme cascade system for several proteins activated by mechanisms of innate and acquired immunity. The complement system has three activation pathways (Tizard, 2008): the alternative, the lectin- or mannan-binding (innate defense mechanism) and the classical pathway (associated with acquired defense mechanism). The first two pathways are activated by PAMPs and the last one by antigen-antibody complexes. Activated pathways start the formation of membrane attack complexes, opsonization of pathogens (to increase the uptake by phagocytic cells), chemotaxis (attraction of phagocytic cells) and contribute to inflammatory processes and immune regulation (regulate antibody formation).

3.3.2. HOST DEFENSE PEPTIDES (HDPs)

Host defense peptides are a large group of innate immune effectors that are also termed antimicrobial peptides (Sang and Blecha, 2009). In pigs, about 30 HDPs have been identified and partially characterized relative to their structure and function. Antimicrobial activity of porcine HDPs has been extensively evaluated for their protective role against a broad spectrum of microorganisms *in vitro* and *in vivo*. Porcine HDPs, represented prominently by 11 cathelicidins and 13 β-defensins, comprise a group of critical effectors in the porcine immune system (Sang and Blecha, 2009).

3.3.3. CYTOKINES

Cytokines are small proteins secreted by cell macrophages, DC and mast cells once PAMPs or alarmins are bound to their receptors. Cytokines serve as intercellular signaling molecules and trigger inflammation while starting acquired immunity. They can be categorized into four groups based on their functions (Murtaugh, 1994; Tizard, 2008). Group one involves cytokines mediating innate immunity, including type I interferon (INF-α and INF-β) and pro-inflammatory cytokines (interleukin-1 (IL-1), IL-6 and tumor necrosis factor-α (TNF-α)). Group two includes cytokines regulating lymphocyte activation, growth and differentiation (IL-2, IL-4, IL-12 and transforming growth factor-β (TGF- β)). Group three includes cytokines regulating immune mediated inflammation (INF-γ, TNF-β, IL-5 and IL-10). Group four includes cytokines that stimulate hematopoiesis by expansion and differentiation of bone marrow progenitors. They are called colony stimulating factors – CSFs (IL-3, granulocyte-macrophage CSF, granulocyte CSF).

3.3.4. MAJOR HISTOCOMPATIBILITY COMPLEX (MHC)

Antigen-presenting cells use receptors called major histocompatibility complex (MHC) molecules to bind and present antigens. Antigen fragments can trigger an immune response only when they are bound to MHC molecules and these molecules can bind T cell antigen

receptors. The MHC consists of three major gene clusters - class I, II and III (Lunney et al., 2009). Class I molecules are found in all nucleated cells where their function is to present endogenous antigens to CD8+ T cells. MHC class II molecules are present on dendritic cells, macrophages and B cells. Their expression on various capillary endothelia in pigs has also been documented. MHC class III region contains a mixture of genes some of which encode complement components.

3.4. Other cells involved in immune response

Fibrocytes are a blood-derived cell population with fibroblastoid morphology, which is distinct from DC. Fibrocytes are very effective antigen-presenting cells due to their expression of MHC class II, CD1 and CD80/CD86, as well as their endocytic activity. They also produce cytokines and chemokines (IL-6, IL-10, macrophage-colony-stimulating factor, macrophage inflammatory protein-1α, MIP-1b and monocyte chemoattractant protein-1) which are important for T lymphocyte and DC development (Summerfield and McCullough, 2009). Vascular endothelial cells and skin keratinocytes can synthesize and secrete IL-1 and express MHC class II molecules and present antigens to T cells. Some smooth muscle cells, epithelial cells, thymic epithelial cells and corneal cells can act as antigen-presenting cells to activate T cells (Tizard, 2008). Normal microflora of skin, mouth and intestine among others, can help to inhibit the growth of pathogenic bacteria.

3.5. Immune system: main mechanisms of action

3.5.1. PATHOGEN RECOGNITION

A key function of the immune system is the ability to recognize damaged tissues and invading microorganisms by PAMPs. Many cells of the immune system have a set of specialized receptors to recognize these patterns, located on the cell surface (to extracellular invaders) or within the cytoplasm (to intracellular invaders). The receptors include: toll-like receptors (single-chain membrane glycoproteins located on cell surfaces and within the cells which recognize microbial proteins, lipoproteins, lipopolysaccharides (LPS) and viral nucleic acid), nucleotide-binding oligomerization domain-like receptors (found inside the cell, where they detect pathogens and trigger host defense signaling pathways) and peptidoglycan-recognition proteins (Tizard, 2008). The latter are expressed in pigs in the skin, bone marrow, intestine, liver, kidney and spleen. Once bound to peptidoglycans they induce the production of antimicrobial peptides. Retinoic acid inducible gene-like receptors located within the cell bind viral RNA which triggers the production of interferons. The most important cells expressing these receptors are the macrophages, dendritic cells and mast cells.

Any damaged or dying cell releases molecules - so-called alarmins - which activate cells of the innate and acquired immune system. Once PAMPs or alarmins bind to pattern-recognized receptors, they activate cells for innate and aquired immune system to synthesize and to release cytokines, such as TNF-α, IL-1 and IL-6 among others, chemokines and other specific peptides and lipids. which activate the process of innate and acquired immune system.

3.5.2. THE INFLAMMATORY RESPONSE

The inflammatory process is a mechanism to ensure that defensive cells and molecules are focused and concentrated at sites of microbial invasion and tissue damage. Defensive cells and molecules can attack and destroy invaders or damaged cells. The process begins when PAMPs or alarmins are bound to receptors on DCs, macrophages and mast cells. These cells become active and begin to synthesize and secrete a mixture of cytokines and other molecules such as chemokines and vasoactive factors that trigger inflammation and start to activate acquired immunity.

The major pro-inflammatory cytokines released by sentinel cells are IL-1, TNF-α, IL-6 as well as IL-12 and IL-18 (Murtaugh, 1994). TNF-α triggers local release of chemokines and cytokines, promotes adherence, migration, attraction and activation of leukocytes, and facilitates the transition from innate to acquired immunity. IL-1, in combination with TNF- α, acts on vascular endothelial cells to make them adhesive for neutrophils. Furthermore, they stimulate the synthesis of specific enzymes which generate oxidants, such as nitric oxide, and inflammatory lipids such as prostaglandins (substances that participate in the dilation and constriction of blood vessels, control of blood pressure and modulation of inflammation) and leukotrienes (fatty molecules of the immune system that contribute to inflammation) which further promote inflammation. IL-1 also induces the liver cells to produce acute-phase protein, a class of proteins whose plasma concentrations increase - positive acute-phase proteins - or decrease - negative acute-phase proteins - in response to inflammation. IL-6 regulates the transition from a neutrophil-regulated process to a macrophage-regulated process. Several types of chemokines, which regulate the composition of the inflammatory cell populations attracting different types of cells to the site of inflammation, are released. Other released components include several vasoactive molecules such as histamine, vasoactive lipids, four groups of pro-inflammatory prostaglandins, vasoactive peptide enzymes, more cytokines and chemokines. All these components and cells contribute to the expression of the five main signs of inflammation: heat, redness, swelling, pain and loss of function.

3.6. Mycotoxins and cellular immunity

Mycotoxins can impair the immune system at several levels. This section describes the effects of a variety of mycotoxins on cellular immunity. The rate of lymphocyte proliferation provides an indication for general lymphocyte activity or responsiveness to specific antigens. The lymphoproliferation assay is the classic method for assessing lymphocyte activity or responsiveness. This method uses a mitogen, which stimulates mitosis and lymphocyte transformation. The most efficient lymphocyte mitogens used are proteins called lectins, isolated from plants. These include phytohemagglutinin (PHA) obtained from *Phaseolus vulgaris* (common bean), concanavalin A (ConA) obtained from *Canavalia ensiformis* (jack-beans) and pokeweed mitogen (PWM) obtained from *Phytolacca Americana* (American pokeweed plant). The lectins bind to sugar residues on cell surface glycoproteins and trigger signal transduction pathways leading to cell division (mitosis). Not all lymphocytes respond equally to all lectins: PHA and ConA stimulate T cells, PHA also has a slight effect on B cells, PWM stimulates both T and B cells.

3.6.1. AFLATOXINS

Weaning piglets were fed a control diet or diets contaminated with either 385, 867 or 1807 µg pure aflatoxin B_1/kg feed (Meissonnier et al., 2008b) for 28 days. On day 4 and 15, pigs were vaccinated with ovalbumin. Toxin exposure did not impair the mitogenic response of lymphocytes but delayed and decreased their specific proliferation in response to the vaccine antigen, suggesting impaired lymphocyte activation in pigs exposed to aflatoxin B_1. A significant up-regulation of pro-inflammatory (TNF-α, IL-1β, IL-6, IFN-γ) and regulatory (IL-10) cytokines was observed in spleen from pigs exposed to the highest dose of aflatoxin B_1. These results indicate that, during inflammatory response, dietary exposure to aflatoxins decreases cell-mediated immunity. In this particular case aflatoxin caused a delay and diminished vaccination response to ovalbumin.

Pigs in the field might be unprotected despite vaccination as a result of aflatoxin contamination in the feed. For that reason, when a failure in the vaccination program is observed on a pig farm, mycotoxins also have to be taken into consideration as a possible cause. On the other hand, if a chronic up-regulation of pro-inflammatory and regulatory cytokines is maintained, the animal has to use part of the ingested nutrients to maintain this process (representing a waste of energy and nutrients), instead of using the energy to perform.

Mycotoxins can be transmitted through milk from mother sows to the suckling piglets and its toxic metabolites may also cross the placental barrier. According to Silvotti et al. (1997) aflatoxins reach the suckling piglet at a concentration approximately 1000-fold lower than it was in the feed that was fed to the sow. To test the effect of aflatoxins transmitted via milk, blood samples from 25-day-old piglets from sows exposed to aflatoxin B_1 and G_1 during gestation and lactation were collected and cellular populations were separated for immunological measurements. The lymphoproliferative response to mitogens was reduced, and monocyte-derived macrophages failed to efficiently produce superoxide anions (a compound used to destroy pathogens in oxygen-dependent mechanisms – like oxidative burst) after oxidative burst stimulation *in vitro*. Neutrophils showed a reduction of chemotactic response *in vitro* to chemoattractant bacteria factor and casein (Silvotti et al., 1997). Piglets showed alterations of immunocompetent cells. The authors suggest that these alterations are due to the effect of aflatoxins transmitted via milk from the affected mother sows; however, it is possible that they also resulted from exposure to aflatoxins in the uterus. The first days of life are critical, not only for the future productive performance of the animal, but also for animal health and welfare. The descendants of sows affected by aflatoxins may have a weak immune system, reducing the viability of the piglets to face pathogens.

Mocchegiani et al. (1998) also showed that thymus weights in piglets of aflatoxin B_1 + G_1-exposed sows (800 µg/kg feed) were reduced either as absolute or as relative weight values when compared to controls. Thymus histopathology showed a marked depletion of the thymus cortical lymphocytes in piglets from aflatoxin B_1 and G_1-exposed sows compared with healthy controls (Mocchegiani et al., 1998). Furthermore, piglets showed decreased thymic endocrine activity and zinc deficiency (due to intestinal malabsorption caused by aflatoxins) compared to the healthy control. Zinc is required for the activation of the thymic hormone which is responsible for the performance of cell-mediated immunity. The authors concluded that zinc may be crucial for thymic involution in piglets from aflatoxin-exposed sows. *In vitro* experiments carried out with primary cultures of swine alveolar macrophages (SAM) containing 5 x 10^5 cells, showed that exposure to 1.5 µg aflatoxin B_1/ml for 24 h led to a reduction of

viable macrophages to 41 % of the control levels (Liu et al., 2002). Furthermore, a reduction in phagocytic ability to 36 % of the control level was shown in SAM treated with 0.1 μg/ml aflatoxin B_1 for 24 h. However, aflatoxin B_1 (0.1 and 0.5 μg/ml) did not affect the expression of IL-1β and TNF-α mRNA.

Alveolar macrophages act at respiratory surface levels (alveolus – air sac in the lungs) where they clean off particles such as dust or microorganisms. A reduction of the number of viable macrophages in the alveolus and a decrease in the phagocytic ability can affect the capacity of animals to counteract colonization of pathogenic bacteria in the lung. Thus, it increases the susceptibility to respiratory diseases and the number of acute outbreaks followed by more severe respiratory diseases.

Marked reduction of phytohemagglutinin M (PHA-M) mitogen responsiveness in piglets from aflatoxin exposed sows was observed when compared with healthy controls (Mocchegiani et al., 1998; Silvotti et al., 1997; van Heugten et al., 1994). Previous experiments in pigs have shown that aflatoxins decrease lymphocyte proliferation (Harvey et al., 1995; van Heugten E. et al., 1994). Cytokine mRNA expression in PHA-stimulated blood cells was tested in piglets fed with low doses of aflatoxins over 4 weeks. The results showed on the one hand a marked and slight decrease in proinflammatory cytokine mRNA expression of IL-1β and TNF-α respectively, and on the other hand an increase in anti-inflammatory cytokine mRNA expression of IL-10 (Marin et al., 2002).

IL-10 acts as an immunosuppressive or anti-inflammatory cytokine. Considering this fact, the authors suggest that the reduction of the other cytokines, IL-1β and TNF-α, can be explained due to an increase of IL-10 observed in this study, or due to a direct effect of aflatoxin. The total effect is a reduction in the capacity of involved immune cells to induce an anti-inflammatory response in the case of pathogen invasion. The increase of pro-inflammatory cytokines is a way of signaling – to focus or limit the infection during the inflammatory process. This attracts more components of the immune system to participate in the inflammatory process which is important to eliminate the pathogen. Since mycotoxins affect this process they limit the communication with other cells of the immune system and reduce the inflammatory response in case of an invasion. Consequently pathogens can easily invade, colonize and damage animal tissues and furthermore trigger diseases.

3.6.2. TRICHOTHECENES

Topical exposure of swine to a sublethal dose of T-2 toxin (15 mg/kg body weight (BW)) caused a significantly lower response of enriched peripheral blood mononuclear cells to mitogens ConA, PHA, and pokeweed (Pang et al., 1987). Pigs that inhaled T-2 toxin showed a decline in total leukocyte count mainly due to a decrease in lymphocyte count (Pang et al., 1988). Similar to the studies performed with aflatoxin and fumonisin, T-2 toxin also produced a reduced lymphoproliferative response against mitogens as well as a lower lymphocyte content in blood which could be caused by the cytotoxic effect of T-2 toxin.

There was no sign of disturbed functional capacity of lymphocytes (proliferation capacity and ability to produce IL-4 and IFN-γ) described in weaning piglets fed a diet naturally contaminated with low concentrations of DON (0, 0.28, 0.56 or 0.84 mg DON/kg feed) (Accensi et al., 2006). In a different experiment the effect of DON on proliferation of ConA stimulated porcine peripheral blood lymphocytes (PBL) was evaluated *in vitro* and *in vivo* (Goyarts et al.,

2006). A dose-response-dependent decrease of MTT and BrdU values in ConA stimulated PBL was observed when pure DON (70, 140, 280 and 560 ng DON/ml) was added to a lymphocyte culture, reaching significance at 280 ng DON/ml. The results *in vivo* after acute (one dose 5.7 mg DON/kg feed) and chronic (5.7 mg DON/kg feed over 4 weeks) exposure showed a significant inhibition of lymphocyte proliferation only in the DON acute group using the MTT assay, but values tended to be decreased in the BrdU assay and after chronic DON exposure.

In the study of Goyarts et al. (2006) blood samples were extracted and lymphocytes were isolated and incubated for 12 hours with the mitogen ConA and different concentrations of pure DON. The supernatant was incubated with BrdU or MTT for 4 hours and analyzed using a microplate photometer (BrdU) and ELISA reader (MTT). BrdU is a synthetic nucleoside, analogous to thymidine. In the S phase of cell replication, cells synthesize new DNA using nucleotides (such as thymidine). Culture cells incorporate BrdU (serves as chemical marker) instead of thymidine into the new DNA. MTT (yellow tetrazolium salt) is used to measure the activity of an enzyme (NAD-dependant dehydrogenase), present in the active mitochondria (cell organells), that degrades MTT to form a dark blue formazan product. Comparing the obtained reactions in the supernatant of the lymphocyte culture treated or not with DON, allows evaluation of viability and proliferation capability of the lymphocytes against ConA. In both experiments the proliferative response of the lymphocytes was dose-dependent inhibited, producing a more cytostatic than cytotoxic effect.

Twenty-four pigs were fed either control feed or feed naturally contaminated with 2.2-2.5 mg DON/kg feed for 9 weeks (Pinton et al., 2008). In the mesenteric lymph node, significantly lower TGF-β and IFN-γ mRNA expression levels were observed in animals fed with DON when compared with control piglets. IFN-γ is a cytokine that is important in viral and intracellular bacterial infections as well as in control of tumour cells. In viral infections this cytokine has the capacity to inhibit the viral replication, avoiding transmission of the virus. IFN-γ also stimulates the growth of T-lymphocytes. The reduction of IFN-γ due to exposition to DON can impair or weaken the immune response against viral infections or intracellular bacteria.

In vitro concentrations of DON higher than 0.1 μg/ml had a significant cytotoxic effect, increased apoptosis and necrosis of porcine macrophages after 24h of exposure (Vandenbroucke et al., 2009). Low concentrations of DON (0.025 μg/ml) could modulate the morphology of macrophages through ERK1/2 F-actin reorganization, resulting in an enhanced uptake of *Salmonella typhimurium* in porcine macrophages. These results suggest that low but relevant concentrations of DON modulate the innate immune system and could increase the susceptibility of pigs to infections with *S. typhimurium*. Macrophages eliminate bacteria which could cause damage or disease in the animal. In this study a direct impact of DON on macrophages was observed - strongly impairing the capacity of the animal to counteract pathogenic infections such as *S. typhimurium*. Even low DON concentrations were sufficient to modify macrophages. A higher number of *S. typhimurium* was found inside the macrophages exposed to DON compared to those macrophages that were not exposed to DON. This has implications as bacteria can survive within the affected macrophage. Therefore, affected animals can develop the respective disease or convert into asymptomatic pigs that carry the bacteria and act as a source of contamination for the environment and for other pigs (Vandenbroucke et al., 2009). In the case of inappropriate management of the slaughter house, meat of asymptomatic pigs can pose a risk of infection to final consumers.

In a different experiment monocyte-derived dendritic cells (MoDC) from pigs receiving a control diet or DON-contaminated diet were treated with DON *in vitro* (Bimczok et al.,

2007). *In vitro* treatment of porcine MoDC with DON interfered with phenotypic maturation of the DC. Porcine MoDC which were not exposed to DON presented a pleomorphic shape with cytoplasmic veils, whereas affected porcine MoDC presented a rounded shape without cytoplasmic veils and showed a minor secretion of IL-10 compared to control, leading to a decrease of endocytic activity associated with delayed maturation. DON disrupts porcine DC function *in vitro* and *in vivo*, which might contribute to the immunosuppressive effects of this mycotoxin.

Eighteen young pigs were fed with different diets containing 0, 2.5, or 5 mg/kg feed of purified nivalenol (NIV) over 3 weeks (Hedman et al., 1997). There were no differences between groups either in total and differential leukocytes from blood samples collected at 0, 1 or 3 weeks or in the number of thymocytes (thymus derived monocytes). After 3 weeks, the number of splenocytes (spleen derived monocytes) showed a dose-dependent decrease which was significant in pigs fed 5 mg NIV/kg feed. Flow cytometric analysis of lymphocytes revealed decreased numbers of both the CD4+ and CD8+ subpopulations in the spleen.

Nivalenol and other trichothecenes can be immunostimulatory or immunosuppressive depending on dose, exposure frequency and timing of functional immune assay (Pestka et al., 2004). Low dose trichothecene exposure transcriptionally and post-transcriptionally upregulates the expression of cytokines, chemokines and inflammatory genes with concurrent immune stimulation, whereas high dose exposure promotes leukocyte apoptosis with concomitant immunosuppression (Pestka et al., 2004; Rotter et al., 1996a).

3.6.3. OCHRATOXIN A

Cell-mediated immunity was measured in 18 growing gilts (20.1 kg) randomly assigned to two dietary treatments, 0 or 2.5 mg OTA/kg feed for 35 days (Harvey et al., 1992). To determine delayed hypersensitivity and cutaneous basophil hypersensitivity, all gilts were injected intradermally on day 35 with 0.1 ml of tuberculin, 1 cm lateral to the cranial left teat and 0.1 ml of PHA, 1 cm lateral to the second left teat. An additional intradermally injection with 0.1 ml physiological saline solution was applied as a control in the same location of the corresponding right teat. The results in animals fed with OTA where compared with results of animals in control group. When an antigen such as tuberculin is injected into the skin it induces a slow development of the inflammatory response at the site of injection (noted as increase of skin thickness). This so-called delayed hypersensitivity is mediated by T cells and NK cells and activated by antigen-presenting cells.

The results showed reduced cutaneous basophil hypersensitivity to PHA injection at 24 hours with 14.73 and 11.33 mm skin thickness for the control and ochratoxin group (OTA) respectively. A diminished delayed hypersensitivity response to tuberculin was seen after 48 hours in the OTA group (14.07 mm skin thickness) compared to the control group. *In vitro* response of macrophages and lymphocytes showed a reduced macrophage activity (control 91 %, OTA 73 %) confirmed also by a reduced number of phagocytized red blood cells in the OTA group (1.28 RBC/macro) compared to the control (2.29 RBC/macro). The stimulation index of lymphocytes to PHA was reduced in the OTA group (8.33) compared to the control (13.17). Also, the IL-2 production after stimulation with ConA was significantly decreased in the OTA group compared to the control. Reduced macrophage activity, lower

lymphoproliferative response to mitogens and reduced production of IL-2 were also observed in previously mentioned experiments with other mycotoxins. These data suggest that OTA may suppress cell-mediated immune response in growing pigs.

3.6.4. FUMONISINS

Chronic exposure to fumonisin B_1 (FB_1) prevented the establishment of the trans-epithelial electrical resistance – an indicator of the epithelial integrity – and altered the resistance of an already established monolayer, composed of the porcine intestinal epithelial cell line IPEC-1. Also, FB_1 decreased the proliferation of undifferentiated porcine epithelial intestinal cells (Bouhet and Oswald, 2005). This alters the integrity of the intestinal epithelium and facilitates pathogens to enter the body.

Bouhet et al. (2006) observed that weaned piglets, which orally received 0.5 mg of purified FB_1/kg body weight per day for 7 days, showed a decrease in expression and synthesis of mRNA IL-8 in the ileum. However, no difference was observed for IL-1β, IL-6, IL-12 and TNF-β mRNA levels compared to controls. Similar results were observed *in vitro* with the porcine intestinal epithelial cell line IPEC-1. The authors suggested that this may contribute to the increased intestinal colonization by pathogenic *Escherichia coli* that were observed in FB_1-treated pigs.

The intestinal epithelium is the organ most exposed to different microorganisms and pathogens such as *E. coli*, which at low concentrations are normally part of the intestinal flora. An increase in the number of *E. coli* also increases the possibility that those bacteria attack endothelial cells, causing their damage. In this case, endothelial cells start to produce IL-8, which furthermore chemoattracts neutrophils and/or macrophages to the site of damage. The reduction of IL-8 in the ileum observed in the study of Bouhet et al., 2006 may contribute to a minor attraction of neutrophils and macrophages to the site of infection reducing the capacity to eliminate *E. coli.* This furthermore allows the bacteria to invade the intestinal epithelia and possibly causes E. *coli* related disease.

An experiment was conducted in which weaned piglets were orally exposed to 1 mg FB_1/kg body weight for 10 days. Animals exposed to FB_1 showed a longer shedding of $F4^+$ enterotoxigenic *E. coli* following infection and a lower induction of the antigen-specific immune response following oral immunization when compared to controls (Devriendt et al., 2009). This suggests that consumption of FB_1 contaminated feed could lead to a prolonged intestinal infection. The analysis of cytokine mRNA revealed a significant reduction of interleukin IL-12p40 expression in the ileal Peyer's patches and IL-6 in jejunal lamina propria of FB_1 exposed animals. Oral exposure of piglets to FB_1 impaired the ability of $CD11R1^+$, a jejunal lamina propria antigen-presenting cell, to mature in response to the mucosal antigens F4 and flagellin. A decreased upregulation of MHC class II molecule and reduced T cell stimulatory capacity upon stimulation were observed.

The interleukin IL-12p40 is the principal activator of T helper 1 cells (Th1) and NK cells for more effective lysis of pathogens. IL-6 stimulates the acute-phase reaction, which enhances the innate immune system and protects against tissue damage. IL-6 increases the synthesis of major acute-phase proteins, such as the C-reactive protein, which increases the rate of phagocytosis of bacteria. Additionally, it induces B and T cell growth and differentiation. In the study of

Devriendt et al. (2009) the FB_1 consumption led to a prolonged *E. coli* infection compared to the control group. The enterocytes, neutrophils and macrophages produce cytokines in reaction to damage and/or presence of pathogens (in this case *E. coli*) to stimulate the immune response. However, in these animals the production of IL-12p40 (in ileal Peyer's patches) and IL-6 (in jejunal lamina propria) was reduced. This also reduces the activation of Th1 cells and NK cells, and the production of acute phase proteins and IgA. At the same time the antigen-presenting cell $CD11R1^+$ is also negatively affected in its function (to process and present the antigen to the effector cells of the immune system). Step by step, FB_1 deteriorates the immune system of hosts. First it affects recognition and processing of pathogens by the antigen-presenting cells. Secondly, it avoids signaling (by lowering cytokine production) and thus the attraction of effector cells for pathogen elimination. Ultimately this leads to prolonged intestinal infection.

Cell-mediated immune response was evaluated in pigs chronically exposed to a sublethal dose of FB_1 (Haschek et al., 2001). Pigs were fed 2 mg FB_1/kg per day as culture material for 5 weeks and then vaccinated with a killed pseudorabies virus vaccine. There were no significant changes in response to phytohemagglutinin, total lymphocyte numbers, numbers of CD3+, CD4+, CD8+, or CD4+/8+ subpopulations. Chronic ingestion of low doses of fumonisin did not appear to affect cell-mediated immune response of pigs. Pigs fed a sublethal dose of fumonisin from culture material for 7 days had decreased clearance of *Pseudomonas aeruginosa* and particulate material (copper phthalocyanine as monastral blue) from the pulmonary circulation.

In an *in vitro* study the phagocytosis by alveolar macrophages was evaluated using flow cytometry of fluorescein isothiocyanate labeled opsonized *Salmonella typhimurium* (Haschek et al., 2001). A fumonisin dose-dependent inhibition of *S. typhimurium* phagocytosis using a murine monocyte/macrophage (RAW264) cell line exposed to FB_1 at ≥ 25 µM for 24 hours was observed. In addition, phagocytosis of *S. typhimurium* was decreased in alveolar macrophages from pigs fed FB_1 and isolated before pulmonary edema developed. Consequently, it can be stated that FB_1 exposure can suppress non-specific immunity in pigs. As observed in the study, fumonisins can affect the phagocytic ability of alveolar macrophages to eliminate *S. typhimurium* and *Pseudomonas aeruginosa*, which leads to an increase in the susceptibility of pigs to diseases caused by the above mentioned pathogens. Similar results were observed with aflatoxin B_1 and fumonisin B_1 in swine alveolar macrophages (Liu et al., 2002).

Furthermore, *in vitro* effects of different concentrations of FB_1 on cell proliferation, cell cycle progression and IL-2 production were investigated using cultures of swine peripheral blood mononuclear cells (PBMC) (Marin et al., 2007). The incubation with 10 µM of FB_1 induced a decrease in PBMC proliferation. Treatment with 200 µM of FB_1 induced a high blockade of the cell cycle, with 92.4 % of cells in G0/G1 phase. A significant decrease of IL-2 production was also observed in supernatants of ConA stimulated PBMC treated with 100 and 200 µM FB_1.

The cell cycle includes a series of actions in the cell that lead to cellular division and replication. This process can be divided into two periods: the first is the interphase in which the cell grows, accumulates nutrients and duplicates its DNA preparing for the following step (mitosis). It includes G1, S and G2. G1 is the initial phase of the cell in which the cell increases its size and controls the necessary metabolic processes to initiate DNA synthesis. S stands for the DNA synthesis and replication. G2 is the second phase of growth, in which the cell continues growing and controls all processes to start mitosis.

The second period is the mitosis in which the cell stops growing and divides its genetic material to produce two identical cells. The phase G0 is a period in which the cell stops the preparation for its division and remains in a state of quiescence. The cells need to develop from G0 to G1 to enter the cell cycle. The proliferation of lymphocytes is critical for the development of the immune response. If a lymphocyte (T or B) encounters a pathogen they start to proliferate and to divide, triggering a normal immune response. However, in the study of Marin et al. (2007) FB_1 interfered with the normal immune response which caused a lack of production of IL-2. IL-2, as T-cell growth factor, plays an important role in the cellular transition from G0 to G1. The deficiency of IL- 2 or the alteration of sphingosin metabolism (due to FB_1) can inhibit the proliferation of lymphocytes.

Exposure of 5 x 10^5 swine alveolar macrophages (SAM) to 5 µg FB_1/ml for 72 hours led to a reduction of viable cells to 65 % of the control levels (Liu et al., 2002). A reduction in phagocytic ability to 55 % of the control level was shown in SAM treated with 50 ng FB_1/ml for 24 hours. FB_1 induced the apoptosis of SAM with evidence of laddering and nuclear fragmentation. SAM incubated with 2 and 10 g FB_1/ml for 24 hours decreased the mRNA levels of interleukin IL-1β and tumor necrosis factor TNF-α. As can be seen in this study, exposition of swine alveolar macrophages to concentrations of FB_1 affects their phagocytic capacity and number of viable macrophages depending on the concentration. FB_1 acted as an extrinsic factor, inducing apoptosis (programmed cell-death) of alveolar macrophages and reducing the viability of the remaining macrophages which were no longer able to produce IL-1β and TNF-α. This again leads to higher general disease susceptibility.

In vitro investigations on pig PBMC indicated that FB_1 decreased IL-4 and increased interferon-gamma (IFN-γ) synthesis at both the protein and mRNA levels (Taranu et al., 2005). Similar results were obtained in weaning piglets fed 1.5 mg purified FB_1/kg BW showing impaired cytokine balance in mesenteric lymph nodes and spleen. Feeding weaning piglets with 8 mg FB_1/kg over 28 days led to a significant decrease in the expression of IL-4 mRNA by porcine whole blood cells indicating a reduced cellular response.

3.6.5. MULTIPLE MYCOTOXINS

So far only studies regarding the immune response in pigs performed with a single mycotoxin have been described. However, in the field it is more probable to deal with multiple mycotoxin contamination since one fungus is capable of producing a variety of mycotoxins. Mycotoxins can synergistically affect the immune system even if they occur at low analytical levels.

In vitro studies evaluating the effect of different concentrations of *Fusarium*-toxins (FB_1, ZEN, NIV, DON; individually and in combination) on the lymphocyte proliferation were performed (Luongo et al., 2008). Results showed a strong inhibitory effect with increasing concentrations of ZEN starting at concentration of 5 µM. NIV and DON inhibited lymphocyte proliferation starting from a concentration of 0.125 µM for NIV and 0.25 µM for DON. When cells were incubated with a combination of FB_1 + ZEN, an inhibitory effect on cellular proliferation higher than that induced by ZEN alone was observed. Similarly, enhancement of inhibition of cellular proliferation was found in co-incubation with NIV and DON. Likewise, histopathological evidence of spleen dysfunction was seen in two groups of pigs fed 6100 µg DON and 235 µg ZEN/kg feed, and 9570 µg DON and 358 µg ZEN/kg feed (Tiemann et

al., 2006) even in the absence of clinical signs of mycotoxicosis. The proliferation rate of splenocytes was inhibited in pigs fed 9570 and 358 µg DON and ZEN/kg feed. *In vivo*, no inhibitory effects were detected after ConA stimulation of blood lymphocytes. *In vitro* studies also showed lower proliferation rates of blood lymphocytes and splenocytes pre-exposed to DON.

The immune response of weaning pigs fed a diet containing 1 mg DON/kg feed and 250 µg ZEN/kg feed over 6 weeks was evaluated (Cheng et al., 2006). Results of alveolar macrophage activity of the pigs showed a significant decrease in chemotactic activity to 49 % of the value found in the control group and a reduction in percentage of phagocytic macrophages. The results of tested cytokine gene expression in the spleen of pigs showed a reduction in the expression levels of cytokines IFN-γ, TNF-α, IL-1β, IL-2 and IL-6. Histopathological studies showed a necrosis of lymphocytes and depletion in the spleen, peripheral hemorrhage and focal necrosis in lymph nodes.

A more recent study with twenty-four 4-week-old weaned castrated male pigs (Pietrain/Duroc/Large-white) were randomly assigned to 4 dietary treatment groups during a 35-day experimental period to evaluate the individual or combined effects of DON and FUM on their immune response (Grenier et al., 2011). The dietary treatments included a control group and three experimental groups: 2 mono-contaminated either with 2.8 mg DON/kg feed or with 5.9 mg FUM/kg feed (4.1 mg FB_1/kg + 1.8 mg FB_2/kg feed) and 1 co-contaminated with 3.1 mg DON and 6.5 mg FUM/kg feed (4.5 mg FB_1/kg + 2.0 mg FB_2/kg feed). Histological analysis of the lungs revealed a depletion of bronchiole-associated lymphoid tissue (BALT). These lesions were only observed in animals fed FUM or FB_1 + DON-contaminated diets, with higher intensity in the latter. The expression of mRNA encoding cytokines (IL-12p40, IL-8, IL-1β, IL-6 and MIP-1β) was determinated in spleen samples collected at the end of the experiment using real-time PCR. DON and FUM contaminated groups showed a significant decrease of mRNA encoding IL-8 and IL-1β, respectively. Furthermore, a significant decrease in mRNA for all tested mycotoxins was found in animals fed diets co-contaminated with both mycotoxins.

3.7. Mycotoxins and humoral immunity

A great variety of infectious agents/pathogens can produce diseases in pigs which are difficult to control once established on the farm. Louis Pasteur was the first who demonstrated that immunity to infectious agents can be produced by vaccination. A satisfying vaccination response in pigs is characterized by production of a certain level of antibodies, specific for one pathogen which consequently protects the body against this specific disease. B lymphocytes are responsible for the production of specific antibodies. They need to produce the antibody levels necessary for full protection against the pathogen. The capacity of mycotoxins to decrease the level of specific antibodies might be a consequence of their previously described impact on the lymphoproliferative response or on the appropriate synthesis of antibodies. There are several studies which show the effect of mycotoxins on humoral immune response. These will be described below.

3.7.1. AFLATOXINS

Weaning piglets fed 140 or 280 µg Afla/kg feed showed a decreased primary and secondary immune response expressed as numerically lower serum antibody levels against *Mycoplasma agalactiae* in aflatoxin-fed animals compared to immunized control piglets (Marin et al., 2002). However, increased γ-globulin in the serum has been reported in pigs exposed to aflatoxins. This increase may have been the result of impairment of liver function and, therefore, decreased immunoglobulin catabolism (Marin et al., 2002; Miller et al., 1981; Panangala et al., 1986).

3.7.2. TRICHOTHECENES

During a 28-day period pigs were fed either a control diet or diets contaminated with 540, 1324 or 2102 µg pure T-2 toxin/kg feed (Meissonnier et al., 2008a). Pigs were immunized with ovalbumin and subsequently humoral immune response was measured. Pigs fed 1324 or 2102 µg T-2 toxin/kg feed exhibited reduced anti-ovalbumin antibody production. Also for DON, the response to ovalbumin was measured in 24 pigs fed for 9 weeks either control feed or feed naturally contaminated with 2.2-2.5 mg DON/kg feed (Pinton et al., 2008). On day 4 and 15 of the experiment, the animals were subcutaneously immunized with ovalbumin. The results showed a significant increase in the concentration of ovalbumin-specific IgA and a numerical increase for ovalbumin-specific IgG. Reduced antibody titers and delayed development after intra-abdominal immunization with sheep red blood cells (SRBC) were found in groups of young pigs fed 3 mg DON/kg feed over 28 days of the experiment (Rotter et al., 1994). Similarly, pigs exposed to T-2 toxin inhalation showed a reduced antibody titer to SRBC (Pang et al., 1988).

In a different experiment, antibody titers to tetanus toxoid, SRBC, *Mycobacterium paratuberculosis* (MPT) and diphtheria toxoid (DT) after feeding pigs naturally DON-contaminated diets at 0.6, 1.8 and 4.7 mg DON/kg feed, were evaluated. A significant dose-dependent reduction in secondary (9 weeks) antibody response to tetanus toxoid with increasing DON-levels was observed whereas the antibody titers to SRBC, MPT or DT were not significantly influenced (Overnes et al., 1997).

Further research on the effect of DON on Ig (A, G, M) concentrations *in vitro* and *in vivo* (Goyarts et al., 2006). Results *in vitro* showed that 70, 140, 280 and 560 ng DON/ml declined immunoglobulin levels to 80 %, 43 %, 24 % and 23 % (IgA); 62 %, 23 %, 9 % and 9 % (IgM) and 59 %, 39 %, 34 % and 46 % (IgG) compared to the stimulated control (=100 %). *In vivo* serum IgA of pigs showed no significant differences between acute (one dose 5.7 mg DON/kg feed) and chronic (same dose over 4 weeks) groups, whereas IgM and IgG were significantly increased in the DON acute group. Eighteen young pigs (51 days of age) were fed with different diets containing 0, 2.5, or 5 mg purified NIV/kg feed over 3 weeks (Hedman et al., 1997). Analysis of IgG and IgA in plasma showed a time-dependent tendency of increasing plasma concentration of IgA and decreasing concentration of IgG in the 2.5 mg/kg group.

3.7.3. FUMONISINS

Feeding weanling piglets 8 mg FB_1/kg feed over 28 days led to a significant decrease in antibody titer after vaccination against *Mycoplasma agalactiae*. However, no effect on the serum concentration of the immunoglobulin subset (IgG, IgA, and IgM) was seen (Taranu et al., 2005).

In further experiments, specific antibody levels after vaccination with *Mycoplasma agalactiae* in females and castrated males fed 8 mg FB_1/kg feed (Marin et al., 2006) over 28 days were compared. Specific antibody levels in castrated males were significantly decreased compared to control levels (fed mycotoxin-free diets). In females, the toxin had no effect on the production of specific antibodies. In males, ingestion of FB_1-contaminated feed significantly decreased mRNA expression level of IL-10, IL-6 and IL-4. In females, the toxin had no effect on cytokine mRNA levels. In this study in particular, males were more susceptible to FB_1 than females. The reduction of the cytokines (IL-10, IL-6 and IL-4) affected humoral immune response since they participate in different processes of the immune system, activating T and B lymphocytes (growth and differentiation) and stimulating synthesis and regulation of antibodies. In this study FB_1 diminished the synthesis of these cytokines, which affected the appropriate synthesis of specific antibodies – as it was observed in the decreased level of antibodies against *Mycoplasma* (post vaccination), but not in the level of total antibodies. Feeding FUM-contaminated feed might lead to inappropriate vaccination response, reducing the level of specific antibodies and reducing the period of protection through the vaccine or just leaving unprotected animals against this specific disease.

3.7.4. MULTIPLE MYCOTOXIN CONTAMINATION

The immune response of weaning pigs fed a diet containing 1 mg DON/kg feed and 250 μg ZEN/kg feed over 6 weeks was evaluated (by Cheng et al., 2006). Pseudorabies antibody titers were significantly decreased on day 28 post vaccination in pigs fed the contaminated diet compared to the control.

In the same experiment mentioned in section 3.6.5., at day 4 and 16 all piglets were immunized by subcutaneous inoculation with 1 and 2 mg of ovalbumin (OVA), respectively. Total plasmatic concentration of the immunoglobulin subsets and titers of specific antibody anti-OVA were measured by ELISA. Animals fed mycotoxin-contaminated diets showed a reduced anti-OVA IgG in their plasma and this decrease was even more pronounced in the animals fed both mycotoxins.

3.8. Mycotoxins, susceptibility to diseases and vaccine efficacy

The effects of mycotoxins on different levels of the pig immune system are proven and it can be concluded that mycotoxins are generally immunosuppressive agents affecting pigs. Their net effect is making pigs defenseless and more susceptible to develop diseases. The following studies demonstrate the susceptibility to specific diseases in pigs consuming mycotoxins.

3.8.1. AFLATOXINS

Pigs fed a diet that contained aflatoxin B_1 (70 – 140 µg/kg) showed an enhanced susceptibility to infection with *Brachyspira* (former *Serpulina, Treponema) hyodysenteriae* (Joens et al., 1981). The incubation period of *B. hyodysenteriae* in pigs exposed to aflatoxin B_1 was shorter (7.7 days) than in pigs that were only exposed to *B. hyodysenteriae* and not to aflatoxin B_1 (17.1 days). In this experiment, the group given *B. hyodysenteriae* and aflatoxin B_1 (Group II) showed a longer period of diarrhea and dysentery than the group given *B. hyodysenteriae* alone (Group III). Deaths occurred in 4 out of 8 pigs in Group II and only in 1 out of 8 pigs in Group III. However, there was no statistical significance regarding the last 2 parameters.

Similarly, 24 pigs were divided into 4 groups of 6 pigs each (initial weight 15 – 19.1 kg). Two groups were fed a normal diet and the other two were fed the same diet supplemented with aflatoxin B_1 (1.3 mg/pig per day). One group of each treatment was vaccinated with erysipelas bacterin. Twenty-one days after vaccination pigs were challenged with an inoculum of virulent *Erysipelothrix rhusiopathiae* (Cysewski et al., 1978). On the basis of the response to the challenge, pigs were classified as immune, partially immune, or susceptible. Three of the vaccinated pigs fed the normal diet were immune, two were partially immune and one was susceptible, whereas none of the vaccinated pigs given the aflatoxin diet was immune, only one was partially immune, and the remainders were susceptible. Two of the non-vaccinated pigs fed the normal diet were partially immune and four were susceptible; all of the non-vaccinated pigs fed the aflatoxin diet were susceptible. The authors concluded that aflatoxin consumption interfered with the development of acquired immunity and apparently increased the severity of the *E. rhusiopathiae* infection in unvaccinated pigs (Cysewski et al., 1978).

3.8.2. FUMONISINS

Weaned pigs received 0.5 mg FB_1/kg of BW (equivalent to 5 to 8 mg/kg feed) daily for 6 days (Oswald et al., 2003). On day 6, weaned pigs were challenged by oral inoculation with an extraintestinal pathogenic *E. coli* strain. The colonization of the small and large intestines was 400 to 700 fold higher (in colony forming units of the inoculated strain per gram of tissue) in the FB_1-treated animals compared to the control. These results lead to the assumption that the immune system of the FB_1-treated animals was weakened by the toxin permitting an increased bacterial colonization. This can predispose animals to development of infectious diseases.

A fumonisin B_1 experiment with 20 piglets (9.6 ± 2.1 kg), randomly assigned to four groups, was conducted (Halloy et al., 2005). One of the groups received orally 0.5 mg FB_1 (as crude extract)/kg BW per day (corresponding to feed contaminated with 5-8 mg FB_1/kg), for 7 days. The next day piglets were treated intratracheally with 5 ml of an overnight culture of *Pasteurella multocida* (>2.109 cfu/ml) for 14 consecutive days. The second and third group received either the dose of FB_1 or *P. multocida*. The fourth group was the control group. The following parameters were measured: body weight and cough frequency throughout the experiment, lung lesions, bronchoalveolar lavage fluid (BALF) cell composition and the expression of inflammatory cytokines. The main finding of this study was observed in the group receiving the FB_1-containing culture extracts and *P. multocida.* This combination reduced the growth rate of animals, induced coughing, enhanced the extension of lung lesions presenting a sub-acute interstitial pneumonia, increased the total number of cells present in the BALF as well as the

number of macrophages and lymphocytes, and increased the expression of TNF-α, IFN-α and IL-18 mRNA. The exposure to fumonisin significantly increased the expression of IL-8, IL-18 and IFN-α mRNA compared with *P. multocida* infection that only increased the expression of TNF-α. Based on the findings of this study the authors suggested that the consumption of FB_1 may predispose piglets to the development of pneumonia induced by *P. multocida*.

3.8.3. OCHRATOXINS

Eighteen young pigs (Landrace x Bulgarian, 9 male and 9 female; 14-15 kg) were used for an experimental study (Stoev et al., 2000). The animals were assigned to three groups: control group received a commercial ration; the other 2 groups received the same ration artificially contaminated with either 3 mg OTA/kg feed (3 ppm group) or 1 mg OTA/kg feed (1 ppm group) during 21 days. On days 15 and 17 all six animals in the 3 ppm group and two animals in the 1 ppm group died with clinical and pathological symptoms of salmonellosis and renal ochratoxicosis. The disease was confirmed by an isolation of *Salmonella choleraesuis* in feces and liver. The rest of the animals and the animals of the control group were not affected. Apparently, in this case the OTA-predisposed animals developed salmonellosis. In a further experiment, based on the above results, twelve young pigs (6 male and 6 female, approx. 17 kg) were divided into equal control and treated groups and fed a commercial pig starter diet without mycotoxins and with 1 mg OTA/kg feed (Stoev et al., 2000). On day 14 all pigs were weighed and immunized against salmonellosis by subcutaneous injection (3 ml) of a 48 h broth culture of *S. choleraesuis* treated with formalin (0.5 %) and alum (0.5 %). The humoral antibody titer against *S. choleraesuis* was measured by a serum agglutination test 21 days after immunisation. The highest dilution producing agglutination determined the final titer. The mean values of antibody titer in the treated animals were statistically significantly reduced compared to the control 21 days after inoculation. There was no enteric disease in the controls, but in the ochratoxin treated group haemorrhagic diarrhoea occurred in two pigs on day 47. However, this was associated with *Brachyspira hyodysenteriae* and *Campylobacter coli.* The results of these experiments suggest that ochratoxin can induce immunosuppression and predispose, in some circumstances, to development of infectious disease in pigs fed this mycotoxin.

3.9. Conclusions

This chapter provides a scientific overview of the effects of mycotoxins on the immune system of pigs. It is evident that there is an impact of these toxic metabolites on the humoral and cellular immunity of pigs. As can be observed from the mentioned studies, different mycotoxins contained in pig feed can affect the immune system of pigs at different levels. Some of them can alter physical barriers (integrity of epithelial cells) opening the door for invasion of opportunistic and pathogenic microorganisms. Others can alter the inflammatory process and cell mediated immune response. Mycotoxins reduce the number and viability of antigen-presenting cells and impair the chemotactic and phagocytic ability of neutrophils and macrophages to phagocyte pathogens. They impede cells to release signaling molecules (cytokines) to positively regulate the immune response (inflammatory process and development

of acquired immunity) and reduce the lymphoproliferative response (T and B cells) to pathogens. Furthermore, they can affect the humoral immune response due to a deficiency in the appropriate production of antibodies, provoking failure of the vaccination program. Individually and/or together (synergistic effects), they can weaken the immune system of pigs, predisposing animals to pathogens and consequently developing the disease related to the pathogen. Even though these effects are often neglected as they are asymptomatic, they may account for a great economic loss for the farm. On the one hand the animals are more susceptible to diseases and infections, and on the other hand, treatments will not be as effective as expected. This is especially true in cases where animals have to face different challenges. In spite of the effects encountered, several of the mechanisms by which the mycotoxins impair the immune system are still unclear. Therefore, further studies are necessary to elucidate the immunosuppressive mechanisms of these silent toxic agents.

4. ANALYZING MYCOTOXIN CONTENT IN COMMODITIES/ FEEDS

INÊS RODRIGUES AND ELISABETH PICHLER

Since the visual diagnosis of mycotoxicoses in animals is complex and often erroneous as the same symptoms can be caused by different etiologic agents, the best way to identify a problem involving mycotoxins is by analyzing commodities or finished feed for their presence.

Nonetheless, analyzing samples for the occurrence of mycotoxins is not a simple task. A sampling procedure is a multistage process and consists of three distinct phases: sampling, sample preparation and analysis (Cheli et al., 2009). Procedures of handling feed samples comprise the collection of the largest sample size possible and testing samples soon after sampling to avoid changes in respect of quality and contamination.

4.1. Sampling

Sampling of commodities and/or feed is the first critical step concerning chemical analysis of mycotoxins and well known as largest source of error (80 %) in terms of mycotoxin detection. This is explained by the fact that fungal development and mycotoxin production are "spot processes" significantly affected by crop variety, agronomic practices, weather conditions during growing and harvest, storage and processing conditions and toxigenic potential of the different mould species (Cheli et al., 2009). As these "hot spots" of heavily contaminated material are randomly distributed within a lot, an underestimation of mycotoxin content is possible if too small a sample size without contaminated portions is analyzed or, conversely, an overestimation of mycotoxin load may occur if a small sample featuring one or more contaminated spots is tested.

As an attempt to overcome these problems, several sampling methods/proposals are available for cereals and cereal products, depending on the country.

In the European Union, Commission Regulation (EC) No 401/2006 of February 2006, lays down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs. The objective of this regulation is to fix general criteria which the sampling method should comply with. The proposal of the EC will be generally explained below. This can be used as a guideline for managers in the animal production industry with adaptations to individual cases, depending on the available infrastructures.

The method of sampling may be applied to all the different forms in which the commodities are put on the market as commodities may be traded in bulk, containers, or individual packaging such as sacks, bags and retail packages. For the sampling of lots traded in individual packs, such as sacks, bags and retail packages, the following formula may be used as a guide (EC, 2006):

$$\text{Sampling frequency} = \frac{\text{weight of lot} \times \text{weight of incremental sample}}{\text{weight of aggregate sample} \times \text{weight of individual packaging}}$$

where weight is given in kg

sampling frequency: every n^{th} sack or bag from which an incremental sample must be taken (decimal figures should be rounded to the nearest whole number).

In general, incremental samples of around 100 g should be taken from the lot. The number of samples may vary according to the lot weight (Table 19).

Table 19 – Subdivision of lots into sublots depending on product and lot weight
(Source: Commission Regulation (EC) No 401/2006 of February 2006)

Commodity	Lot weight (Metric ton (MT))	Weight or number of sublots	Number of incremental samples	Aggregate sample weight (kg)
Cereals and cereal products	≥ 1,500	500 MT	100	10
	> 300 and < 1,500	3 sublots	100	10
	≥ 50 and ≤ 300	100 MT	100	10
	< 50	-	3-100 (*)	1-10

(*) Depending on the lot weight – see Table 20

'Lot' means an identifiable quantity of a food commodity delivered at one time and determined by the official to have common characteristics, such as origin, variety, type of packing, packer, consignor or markings.

'Sublot' means a designated part of a large lot in order to apply the sampling method on that designated part. Each sublot must be physically separate and identifiable.

'Incremental sample' means a quantity of material taken from a single place in the lot or sublot.

'Aggregate sample' means the combined total of all the incremental samples taken from the lot or sublot.

In case the lots weight less than 50 tons, the sampling plan is to be adapted with 3 to 100 incremental samples, depending on the lot weight (Table 20)

Table 20 – Number of incremental samples to be taken depending on the weight of the lot of cereals and cereal products
(Source: Commission Regulation (EC) No 401/2006 of February 2006)

Lot weight (MT)	Number of incremental samples	Aggregate sample weight (kg)
≤ 0.05	3	1
> 0.05 and ≤ 0.5	5	1
> 0.5 and ≤ 1	10	1
> 1 and ≤ 3	20	2
> 3 and ≤ 10	40	4
> 10 and ≤ 20	60	6
> 20 and ≤ 50	100	10

Other crucial parameters to be taken into account in regard to sampling are:

- Sampling raw materials or mixtures of commodities: When deciding whether to test single commodities or mixtures of commodities (e.g. finished feed) one should always bear in mind that the first option enables an easier way to obtain reliable results and represents an easier and faster problem solution, as contaminated component can be exchanged or reduced.
- Granulometry or particles and/or seed size: Smaller grain particle sizes usually reflect a better distribution of mycotoxins, so in this case, a smaller subsample may be taken.
- Techniques used to physically collect samples: In case sampling is done in a static manner, i.e. the samples are removed from a static lot (storage bins, rail cars, bags etc), a probe should be used. It is important that the lot has been well mixed prior to the collection of the sample. As far as possible these incremental samples should be taken at various places at both surface and depth throughout the lot or sublot, so that every part of the feedlot has an equal chance of selection. Dynamic sampling, though, should be the preferred way of sampling. In this case, samples are removed from a moving stream of product, while this is transferred (e.g. from a conveyor belt). Automatic equipment can be used in this method, to ease the sampling method.
- Tools used for collecting samples: A higher variability of results has been associated if a smaller probe is used (Park et al., 2000), therefore the use of a long probe which will not discriminate the material to be sampled (by taking only big or small particles, for example) is recommended.

4.2. Sample preparation

Sample preparation represents the second crucial step in a proper sampling procedure. It consists of the careful combination of the incremental samples in order to achieve an aggregate sample which is then usually reduced to obtain a laboratory sample. For this step, two approaches can be used:

- Dry milling: In which samples are milled without application of water. This process might lead to clogging of samples with high oil content.
- Slurry mixing: In which samples are milled together with an appropriate amount of water at high speed in a slurry mixer, resulting in a homogeneous paste.

In general, the application of slurry mixing technique leads to smaller particle size and a better homogenization of the sample, which will reduce the subsampling variability and enable a better estimation of the true mycotoxin content of a lot (Cheli et al., 2009).

If samples are to be analyzed by an external laboratory, these should be placed in an appropriate container. Freezing or airtight packing is necessary for feed samples with high moisture content (liquid feed, silage).

Figure 24 shows a simplified procedure prior to analytical testing.

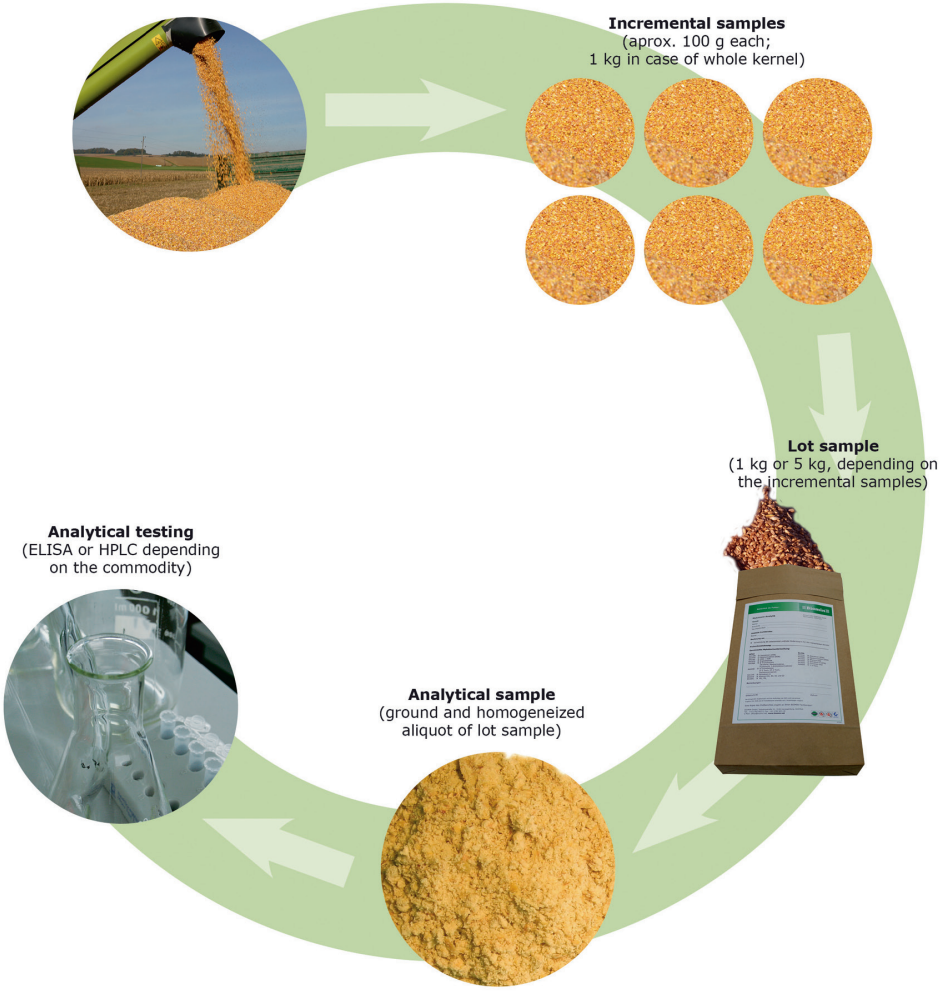


Figure 24 – Schematic of the sampling process

4.3. Analytical methods

The analytical process follows the sampling procedure. There are many considerations that could be of relevance, but only the more practical ones will be discussed here.

Before analyzing a commodity/feed sample one should ask: "Which result am I expecting from the analysis?" If a yes/no response or a semi-quantitative response is considered satisfactory, then rapid tests (section 4.3.1) serve the purpose and enable an increase in knowledge of the presence and distribution of mycotoxins in feedstuffs. If a more accurate result is needed, then reference methods (section 4.3.2) are to be used. At the end of this section an overview on the advantages and disadvantages of rapid methods vs. reference testing methods is given (Table 21).

4.3.1. RAPID TESTS

Rapid tests provide quantitative results in the calibration range and for validated commodities. They require minimal lab equipment and basic personnel training. These are suitable for screening in quality control laboratories in feed and food industries and are commonly used by veterinarians and for on-site testing.

Rapid tests are packaged systems of the principal or key components of an analytical method used to determine the presence of a specific analyte(s) in a given matrix(es). Rapid test kits include directions for their use and are often self-contained, complete analytical systems; but they may require supporting supplies and equipment. Rapid tests can detect a specific analyte(s) in given matrix(es) in significantly less time than reference methods (source www.aoac.org).

For a rapid detection of mycotoxins the following technologies are widely accepted by the industry and recognized by the scientific community: enzyme linked immunosorbent assay (ELISA), lateral flow test and fluorometry.

4.3.1.1. Enzyme linked immunosorbent assay (ELISA)

This is one of the most popular immunologically-based methods used in test kits for the analysis of mycotoxins in foods and feeds. A commonly used approach is a competitive assay where a known amount of labeled (enzyme) toxin competes with any possible toxin in the sample for the specific antibodies attached to the reaction vessel. Any unbound toxin is then washed from the vessel. The quantification is dependent upon the amount of enzyme labelled toxin remaining in the vessel to react with the substrate for the enzyme. This reaction results in a colored product that can be measured optically (Figure 25).

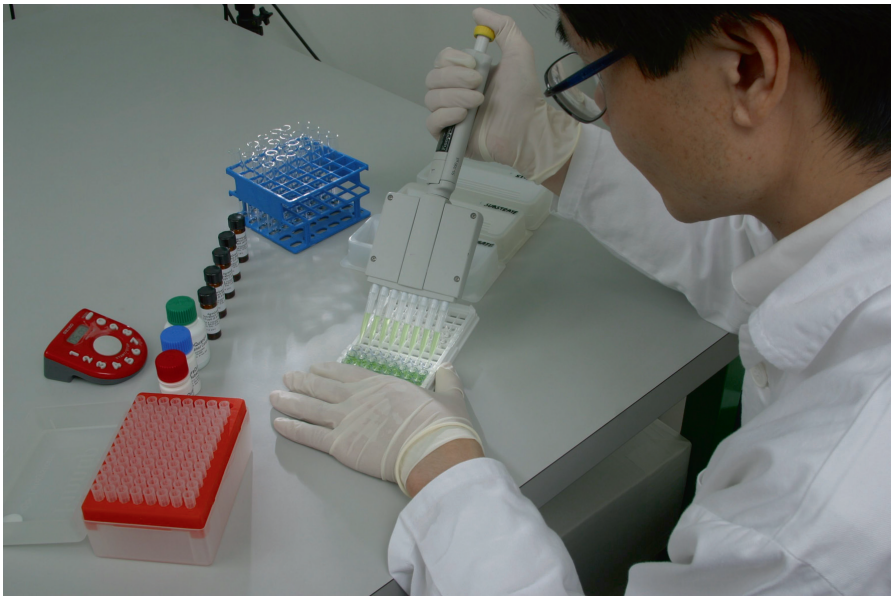


Figure 25 – ELISA test kit

4.3.1.2. Lateral flow test

The lateral flow test is a one-step lateral flow immunochromatographic concentration assay based on an inhibition immunoassay format. A typical immunochromatography test strip is composed of a sample pad, a conjugate pad, a membrane, an adsorbent pad and an adhesive backing. A sample extract is placed on the sample pad or the test strip is dipped directly into the sample extract. Any mycotoxin present binds to the anti-mycotoxin antibody gold particle complex in the conjugate pad and they migrate together with the anti-2nd antibody gold particle complex along the membrane. The membrane contains a test zone and a control zone, onto which a mycotoxin-protein conjugate and a 2nd antibody are dried. The mycotoxin-protein conjugate in the test zone can capture any free anti-mycotoxin antibody gold particle complex, allowing color particles to concentrate and form a visible line. Hence, a positive sample will result in no visible line in the test zone, the control zone will always be visible indicating the validity of the performed test.

4.3.1.3. Fluorometry

The basis of fluorometry is the quantification of compounds by measuring their fluorescence using a fluorometer. In some cases the compounds may be innately fluorescent, and in others the compounds are rendered fluorescent through some chemical derivatization. Tests for compounds using fluorometry include the extraction of the compound from the specific matrix, followed by a cleanup process using either immunoaffinity columns or solid phase cleanup columns, and then a derivatization (if necessary) and measurement of the fluorescence.

4.3.2. REFERENCE TESTING

A reference method is an internationally or nationally recognized standard analytical method applicable to the particular analyte being tested (source: "Association of Analytical Communities," AOAC INTERNATIONAL at www.aoac.org).

Reference testing is more widely used in legal issues, as in the case of governmental control and enforcement of legislation, and in the food industry. It requires expensive lab equipment and well trained personnel. The advantages of using this quantitative method is the achievement of low detection limits, the possibility of testing very complex commodities (for instance finished feed and silage) and the analysis of several substances at once. ISO 17025 is the main standard used by testing and calibration laboratories. ISO 17025 accreditation ensures that a laboratory is audited at least once a year by strict auditors who check not only whether processes are in place but that people are competent and that constant improvement is attained.

The most common analytical reference methods for the detection of mycotoxins are described below.

4.3.2.1. Thin layer chromatography (TLC)

Thin layer chromatography is the separation of compounds in a mixture that are spotted near one end of a plate coated with a thin layer of an adsorbent matrix such as silica gel. Separation

occurs when the end of the plate nearest the spotted mixture is placed in a solvent system in the bottom of a closed vessel and the solvent is allowed to migrate through the adsorbent matrix moving toward the top of the plate. As this occurs the mixture of compounds separate based on the interactions between the solvent system (mobile phase) and the matrix (stationary phase). If the mycotoxin of interest is present in the mixture it will be identified by comparison with standard spots of this mycotoxin that are spotted on the plate in a similar manner as the mixture.

4.3.2.2. Gas Chromatography (GC)

Gas chromatography uses sophisticated equipment in which compounds are separated by a gas flowing through a heated glass column coated with a stationary nonvolatile liquid. Samples injected into the system are separated into the specific components on the column and the separated analytes coming off the column are detected by a chemical or physical detection system.

4.3.2.3. High performance liquid chromatography (HPLC)

HPLC is similar to TLC in principle but is a much more sophisticated system. A small portion of a sample to be analyzed is injected into a stream of solvent being pumped through a column of an adsorptive matrix. As the sample moves through the column the compounds are separated by basically the same principle as described for TLC. The sample components elute off the column as separate entities, and the flow of solvent with the respective compounds passes through a detector to measure the response of the specific compound. The measurements are determined based on detector response which are compared to selected concentrations of standard that were injected into the instrument as part of the analysis sequence.

HPLC can be coupled with a variety of detectors, e.g. spectrophotometric (UV-VIS) detectors, refractometers (RI), fluorescence detectors (FLD), electrochemical detectors, radioactivity detectors and mass spectrometers.

4.3.2.4. Liquid chromatography- mass spectrometry (LC/MS)

The technology of liquid chromatography-mass spectrometry (LC/MS) or LC coupled to tandem mass spectrometry (LC/MS/MS) allows efficient spectrometric assays in a routine laboratory setting. This technique can be used for a wide range of potential analytes to be measured, with no limitations by molecular mass, a very straightforward sample preparation, not requiring chemical derivatization and has only limited maintenance needs. The main advantages include low detection limits, the ability to generate structural information, the requirement of minimal sample treatment and the possibility to cover a wide range of analytes differing in their polarities. Sometimes co-eluting matrix components influence the ionization efficiency of the analyte positively or negatively, impairing the repeatability and accuracy of the analytical method. Therefore a sample clean-up prior to liquid chromatography will be necessary. In order to overcome matrix effects, stable isotope labeled internal standards are used.

Table 21 – Overview on the advantages and disadvantages of rapid methods vs. reference testing methods

	Rapid methods	Reference testing
Advantages	• Fast • Inexpensive • Very reliable for raw materials (corn, wheat) • Quantitative for the validated commodities	• Reliable and quantitative for most commodities • Result refers to the single toxins • Necessary for legal issues
Disadvantages	• Matrix problems can occur in e.g. mixed feedstuffs • Result can be a sum of similar toxins e.g. all Type-B trichothecenes	• More time consuming • Relatively expensive

5. FIGHTING MYCOTOXINS

INÊS RODRIGUES
(Adapted from Chapter 5 of Guide to Mycotoxins: Rodrigues, 2012)

5.1. Prevention methods

As explained in Chapter 1, despite the fact that proper conditions for growth of fungi can occur at all times during crop growth, harvest and storage, fungal species are roughly divided into field fungi and storage fungi. Field fungi are those which in general require higher moisture to grow and produce mycotoxins (> 0.9 water activity), infecting seeds and plants in the field, namely *Fusarium* sp. Storage fungi are those which require lower water activity, thus being more prominent after harvest and during storage, such as *Aspergillus* and *Penicillium* sp.

Therefore, prevention of mycotoxin production must start in the field prior to seeding and should continue up until the animal intakes the feed.

5.1.1. AVOIDING CONTAMINATION IN THE FIELD AND DURING HARVEST

Although many *Fusarium* species can infect cereal grains, *Fusarium graminearum* is the major causal agent of head scab of small grains and of red ear rot in maize. There are two typical routes of entry of *Fusarium graminearum* infection in cereals (Reid et al., 1999): 1) The spores

Figure 26 - The typical entry routes of *Fusarium graminearum* infection in cereals

are already on the field at the time of silks' emergence thus infecting the silk channel; 2) Birds, insects or extreme weather conditions are able to cause damage to the kernels before their hardening thus providing an opportunity for fungal invasion and damage as well (Figure 26). There are many factors that can influence the growth of *Fusarium* fungi and the occurrence of fusariotoxins in the field. Some prevention measures are available which are based on the knowledge of predisposing factors (Jouany, 2007):

- Land management/crop rotation: Cropping systems in which maize is rotated with wheat or in which wheat is grown each year in the same field appear to increase the disease epidemic. Thus, avoiding this type of rotation can have a positive impact on the prevention of the development of these fungi.
- Plant variety: A lot of work has been invested in plant breeding over the past few years. Plant breeding can be considered as the best solution for *Fusarium* control in susceptible crops. However, quantitative trait loci for mycotoxin resistance are often linked with genes responsible for morphological plant characteristics; therefore an improvement to the first trait will usually lead to adverse effects on the agronomic properties of the plant.
- Tillage procedures: Many *Fusarium* fungi species are soil-borne and survive in crop residues as saprophytes, breaking down plant residues. The spore formed as they multiply is very resistant to temperature and to adverse weather conditions, therefore assuring their survival in the crop residues. It is consequently advisable to eliminate residues from the field by means of deep tillage.
- Plant stress: Stress factors, such as high temperatures, drought, poor fertilization and high competition for nutrients are some of the aspects known to increase mycotoxin production in the field. The choice of a specific variety of seed for a certain location, irrigation in critical periods and a balanced fertilization are some of the measures that can be used to avoid mycotoxin contamination during plant growth.
- Crop damage: Mechanical, insect or bird damage of grains provide a good opportunity for fungal invasion and development, thus their prevention is of major importance.
- Harvesting: An adequate harvesting date is extremely important to avoid fungal growth and subsequent mycotoxin contamination as unstable weather conditions such as late rain will exponentially increase its occurrence.

These measures, however, are limited in their ability to avoid mycotoxins. Moreover, after harvest, other mycotoxin producing fungi will develop and produce mycotoxins depending on the storage conditions.

5.1.2. AVOIDING CONTAMINATION DURING STORAGE

During storage, some precautions can be taken in order to avoid fungal growth and further mycotoxin production (Table 22).

Table 22 – Factors which influence mycotoxin occurrence during storage and preventive measures

Factor	Mode of interaction	Preventive measures
Moisture and temperature	The interaction between moisture level and temperature is the most important physico-chemical factor affecting preservation of commodities and feeds during storage. Ideally, both should be kept as low as practicable. Nonetheless, even with low temperatures (5 to 10°C) if the moisture level is sufficiently high, fungi can still develop (Smith and Henderson, 1991). Temperature fluctuations (rise of 2-3°C) may also be a sign of microbial growth and/or insect infestation.	Make sure storage facilities are dry and present minimum temperature fluctuations.
Aeration	Mould growth in grains usually occurs heterogeneously, therefore the development of "hot spots" (areas in which the concentration of mycotoxins is higher) is common. This happens because warm air within the grains and feed comes into contact with cooler air, promoting an interchange of moisture and increasing the odds of condensation occurrence. Once again, these high-moisture spots are an incentive for fungi germination (Smith and Henderson, 1991). The aim of aeration is to cool the grain, but maintaining it in constant movement will increase the efficiency of storage. However, note that dust coming from the outside of the grain is hygroscopic – and often has higher moisture content – and carries a higher proportion of fungal spores than whole clean grain.	When possible, aerate the stored goods by circulation of air through the storage area to maintain proper and uniform temperature levels throughout the storage area.
Sanitation	Fungal development is likely to occur at several points of the storage to feeding pathway, including storage bins, the feed mill, mixed feed bins, pipelines of the feeding system and ultimately in animal feeders.	Cleaning of equipment on a regular basis is highly recommended.
Pests	Pests are on the one hand a general source of injurious or unwanted organisms. The metabolic activity of insects and arthropods causes an increase of both moisture content and temperature of infested material. On the other hand, they also have an important contribution to the physical damage of grains and commodities. Arthropods also act as carriers of mould spores and their fecal material can be utilized as food source by moulds (FAO, 2003).	Use good housekeeping procedures to minimize the levels of insects and fungi in storage facilities. Chemicals used should not interfere with the intended end use of the goods.

Factor	Mode of interaction	Preventive measures
Pests (contd).	Use of fungistatic agents is a common management practice which can be very efficient in reducing mold growth and further mycotoxin production from the time of application (Leeson and Summers, 1991). Nevertheless, it should be emphasized that once the mould has already damaged grain and/or produced mycotoxins, the effectiveness of this practice is limited. Also important to bear in mind is the fact that sub-lethal applications of fungicides are known to stimulate mycotoxin formation. This is likely to occur because fungi are stressed, but not killed (Benbrook, 2005). So, if fungicides are being used, the product's instructions should be carefully followed to avoid this occurrence.	
Physical damage	Although most mould pathogens can directly penetrate plant tissues, it is important to avoid mechanical and insect damage. Broken kernels caused by general handling and/or insect damage, provide additional entry sites for mould pathogens, facilitate infection and promote distribution throughout the grain mass (Santin, 2005).	Effective post-harvest insect management together with correct equipment calibration is highly recommended.

5.2. Elimination of mycotoxins

Due to the fact that preventive methods during crop growth, harvesting and storage only reduce the potential risk of mycotoxin contamination, detoxification procedures after harvest are necessary. These processes should deactivate, destroy or remove the toxin and the fungal spores while retaining the nutrient value and acceptability of the feed by the animal. Also, the deposition of toxic substances, metabolites or toxic byproducts in the feed is to be avoided as well as significant alterations in the product's technological properties.

Furthermore, the process should be readily available, easily utilized, inexpensive and effects on the environment should also be considered. Detoxification procedures are divided into three categories: 1) Physical; 2) Chemical and 3) Biological methods (adsorption and biotransformation).

5.2.1. PHYSICAL PROCESSES

Many of the physical processes discussed below have been used with the purpose of decreasing the mycotoxin contamination of commodities, such as cleaning, mechanical sorting and separation, washing, density segregation and thermal inactivation.

The efficacy of these physical treatments depends on the level of contamination and the distribution of mycotoxins throughout the grain. Results obtained are often uncertain and

connected with high feed losses. Furthermore these methods represent a high investment of money, thus their practical application is very limited. Nonetheless, they will be briefly referred to below.

5.2.1.1. Cleaning

Broken kernels are more easily infected by fungi and therefore mycotoxins than intact kernels. Also, as mentioned before, grain dust is likely to transport fungal spores which will increase the odds of mycotoxin production. The main objective of the cleaning process is to remove contaminated grain dust, husks, hair and small particles by aspiration or scouring.

5.2.1.2. Mechanical sorting and separation

In this process the clean product is separated from mycotoxin-contaminated grains. High feed losses are possible due to incomplete and uncertain separation. Therefore mechanical sorting and separation is not cost-efficient.

5.2.1.3. Washing

Washing procedures using water or sodium carbonate solution are reported to result in some reduction of mycotoxins in grains (Wilson et al., 2004). This process however may only be used in feeds or commodities which will undergo wet milling or ethanol fermentation otherwise costs of drying would be prohibitive.

5.2.1.4. Density segregation

For the segregation of contaminated grain, flotation can be used. This method can reduce mycotoxin contamination but it should be noted that appearance and weight of a particular kernel does not necessarily indicate mycotoxin contamination.

5.2.1.5. Thermal inactivation

As explained in section 1.4, mycotoxins are very heat stable therefore heat treatments such as boiling water, roasting, pelleting or even autoclaving cannot adequately destroy them.

5.2.1.6. Irradiation

Some experiments have been carried out involving the use of irradiation to reduce the mycotoxin load of commodities (Ritieni et al., 1999; Kottapalli et al., 2003; Aziz and Moussa, 2004). However, results showed a reduction in fungal spore contamination but not in terms of mycotoxins already present in the material.

5.2.2. CHEMICAL PROCESSES

Various chemicals (many acids, bases, aldehydes, bisulfite, oxidizing agents and different gases) have been tested for detoxification of mycotoxins but only a limited number of chemical methods are effective and might be used in practice without forming toxic residues or without

having a negative effect on nutrient content, flavor, color, texture, and/or functional properties of the feedstuff/feed.

For achieving adequate decontamination results several parameters such as reaction time, temperature and moisture have to be monitored. Chemical methods also need additional cleaning treatments and are therefore very expensive and time consuming. Furthermore toxic by-products may be produced. Treatment of contaminated feed with ammonia was the most common method used in the past. Although early studies showed this technique to be safe and effective, ammoniation is only permitted in certain countries to decrease aflatoxin level in specific commodities.

5.2.3. BIOLOGICAL METHODS - ADSORPTION

The use of adsorbent materials is a very common method employed to prevent mycotoxicoses, in particular aflatoxicosis. These compounds are added to the feed to bind the toxin during the digestive process in the gastrointestinal tract resulting in a reduction of toxin bioavailability.

The efficacy of binding mycotoxins is dependent on the molecular structure and physical properties of the sorbent as well as on the physical and chemical properties of the mycotoxins (Dakovic et al., 2005). Substances scientifically investigated as potential mycotoxin-binding agents include, among others, bentonites, zeolites, organophilic clays, activated charcoal and yeast cell walls.

5.2.3.1. Bentonites

Bentonites, the montmorillonite-rich clays, are phyllosilicates characterized by alternating layers of tetrahedral silicon $[SiO_4]^{-4}$ and octahedral aluminum connected in a 2:1 arrangement. They are available throughout the world.

Various studies have been conducted in order to understand the adsorption mechanism of aflatoxins by these phyllosilicate minerals (Phillips et al., 2002; Kannewischer et al., 2006). The conclusions of these studies vary from proposing that adsorption occurs only on the external or the edge surfaces, to proposing that the interlayer space is accessible as well, or that aflatoxins are bound at edges, within the interlayer, and at the basal surfaces of smectite by selective chemisorption. More recently, Deng and co-workers (2010) showed evidences that multi-layer adsorption on external surface of the smectite was unlikely. Rather, they proposed that aflatoxin molecules occupy the interlayer space together with exchange cations and water molecules. In the same experiment, the intensity of the bonding was shown to be very high, suggesting high stability of interlayer-adsorbed aflatoxin. Bentonites reported the most promising results as far as aflatoxin B_1 adsorption is concerned (Vekiru et al., 2007).

5.2.3.2. Zeolites

Zeolites are porous tectoaluminosilicates that possess an infinite three-dimensional cage-like structure. These minerals are characterized by their ability to lose and absorb water without damaging their structures, commonly used as molecular sieves, thus allowing the retention of molecules which are smaller than the pores. However, as stated by Daković et al. (2005), aflatoxin B_1 is a molecule too large to enter the zeolite channels, so the adsorption is limited only to the external surface of the zeolite particle.

5.2.3.3. Organoclays

The permanent negative charge in the above-mentioned minerals' (bentonites and zeolites) structure make them suitable for modification using long-chain organic cations (surfactants) which results in increased hydrophobicity of the mineral surface and a potential higher affinity for hydrophobic mycotoxins (Daković et al., 2007).

Organophilization is a process which enables the obtainment of organoclays. Organoclays are manufactured by modification of bentonites (or zeolites) with quaternary amines. Quaternary amines are surfactants, which have a hydrophilic (water loving) and a lipophilic (oil loving) end. A wide variety of surfactant molecules can be used to modify clays. These compounds range in number and in size of the alkyl chain groups and identity of the polar head (Lemke et al., 1998). In simple terms, these molecules are placed between the clay layers (in the case of bentonites) to increase the lipophilicity and the distance between layers thus increasing the adsorption capacity of the clay. In the case of zeolites, however, the surfactants are too large to enter zeolite channels or to access internal cation exchange positions and their adsorption is limited only to the external surface of the zeolite.

Organoclays are used in the treatment of wastewater, for example, because of their high adsorbing capacity. The problem with these substances is that, along with mycotoxins, they adsorb other components of the feeds (e.g. vitamins, nutrients, trace elements, pigments) since they do not have any preference for mycotoxins. Due to this specificity, their efficacy is lowered in complex media by more than 50 %.

Lemke and co-workers (1998) studied the interactions of zearalenone with organoclays and agreed with previous studies showing that the greater the hydrophobicity of the clay, the higher the affinity for binding zearalenone. In other words, adsorption of zearalenone steadily increases as the alkyl chain length of the exchanged surfactant increases (and the cation exchange capacity decreases). Besides high desorption rates associated with high cation exchange capacities of organoclays with longer alkyl chains, the behavior and specificity of adsorption of these compounds in complex media (with other nutrients) is still to be fully addressed in scientific studies.

In studies performed at the Department for Agrobiotechnology (IFA-Tulln) of the University of Natural Resources and Life Sciences (Vienna, Austria) the adsorption of fumonisins and ochratoxin A was reduced by approximately 80 % and 40 % at pH 3 and pH 6, respectively when complex media were used instead of buffer solutions (Tables 23 and 24). A reason that might explain this is the non-specificity of the binding between mycotoxins and organoclays.

Table 23 - Reduction of fumonisins adsorption caused by unspecific binding of feed ingredients (e.g. nutrients, vitamins)

Fumonisin-adsorption [%]			
in buffer solution		in complex medium[1]	
pH 3.0	pH 6.0	pH 3.0	pH 6.0
94.7	43.8	14.8	6

[1]containing essential nutrients, trace elements and vitamins (simulation of the feed)

Table 24 - Reduction of ochratoxin A adsorption caused by unspecific binding of feed ingredients (e.g. nutrients, vitamins)

Ochratoxin-adsorption [%]			
in buffer solution		in complex medium[1]	
pH 3.0	pH 6.0	pH 3.0	pH 6.0
83.8	57.1	68.0	22.9

[1]containing essential nutrients, trace elements and vitamins (simulation of the feed)

A later study on the adsorption of aflatoxins to these modified minerals concluded that the fact that aflatoxin B_1 is almost planar and not very flexible, allied to the presence of functional groups, might be the reason why this mycotoxin is more highly adsorbed by unmodified zeolite than by the organic version of this mineral (Daković et al., 2005).

As for safety, organoclays were shown to be toxic to hydra, a well-established and sensitive *in vivo* indicator of toxicity (Marroquín-Cardona et al., 2009). These results reinforced previous studies in mice (Lemke et al., 2001; Afriyie-Gyawu et al., 2005). Furthermore, according to IPCS INCHEM (International Programme on Chemical Safety - Chemical Safety Information from Intergovernmental Organizations) quaternary ammonium compounds can cause toxic effects by all routes of exposure including inhalation, ingestion, dermal application and irrigation of body cavities.

5.2.3.4. Activated charcoal

Activated charcoal is formed by a pyrolysis of organic materials. It is a very porous non-soluble powder with a high surface to mass ratio (500-3500 m^2/g). Although it has been shown to work quite effectively in *in vitro* experiments, by adsorbing high percentage of mycotoxins *in vivo*, it has less or even no effect against mycotoxicoses. This might be due to the non-specificity of the binding promoted by this material, in which essential nutrients are also adsorbed, particularly if they exist in the feed at higher concentration than that of mycotoxins (Huwig et al., 2001). This statement was further confirmed as aflatoxin B_1 adsorption by charcoal was greatly affected in real gastric juice when compared to the results obtained in buffer solutions and adsorption of vitamin H by activated charcoal reached 99 % (Vekiru et al., 2007).

5.2.3.5. Yeast and yeast cell wall derived products

Yeast and yeast cell wall derived products have also been used as adsorbents for mycotoxins. These are usually constituted by the inner or outer portion of the yeast cell wall - glucomannans and mannanooligosaccharides - respectively. The adsorption of aflatoxin B_1 by glucomannan-based products was found to be the lowest at pH 2 and 6.5, as represented by the sorption isotherms (Maroquín-Cardona et al., 2009). Recent reports show that the main forces involved in the molecular mechanism of the binding of aflatoxin B_1 by glucomannans are Van der Waals attractions and hydrogen bonds (Yiannikouris et al., 2006). However, it is well established that these bonding forces are reversible, largely dependent on the orientation of the molecules (Maroquín-Cardona et al., 2009) and weaker than the chemisorption bonding mechanisms

which are involved in the binding of aflatoxin B_1 to smectite (Grant and Phillips, 1998). Furthermore, toxicity of these kinds of products was observed in hydra toxicity bioassay, which may be due to the growth of intact yeast and microorganisms in them (Maroquín-Cardona et al., 2009; Kannewischer et al., 2006).

5.2.4. TESTING MYCOTOXIN BINDERS

It is still difficult to know precisely which mineral properties influence mycotoxin adsorption. Aflatoxin adsorption values neither correlated with the amount of smectite in bentonites nor with their cation exchange capacity (Vekiru et al., 2007). Therefore thorough tests of potential mycotoxin binders are crucial. Lemke and co-workers (2001) developed a multi-tiered approach for the *in vitro* prescreening of clay-based enterosorbents. Later on, Vekiru et al. (2007) investigated various adsorbents for their ability to bind aflatoxin B_1 using a similar protocol. This includes the following various tests:

- Adsorption test for screening at different pH-values – to understand the efficacy of the materials for mycotoxin binding at different pH conditions.
- Chemisorptions test – to evaluate the binding efficacy, given by the chemisorptions index ($C\alpha$). A $C\alpha=1$ indicates total binding, with no desorption of aflatoxin B_1 from the binder material.
- Comparison of adsorption in buffer, artificial gastric juice and real gastric juice – to ascertain the influence of incubation medium on aflatoxin B_1 adsorption.
- Isothermal analysis – in order to evaluate the affinity and capacity of materials.

These tests prove whether the material enables chemisorption (which indicates strong binding and no or low desorption of the already adsorbed mycotoxins), if it has a high adsorption capacity and a high mycotoxin affinity (with no or low adsorption of essential nutrients). However, the suitability of binding materials to be included in animal diets has to be further investigated in regard to the absence of toxicity (nonexistence of heavy metals, dioxins, amongst other toxic substances), their low effective inclusion rate in feed, their rapid and uniform dispersion in the feed during mixing and their heat stability during pelleting, extrusion and storage.

5.2.5. BIOLOGICAL METHODS - BIOTRANSFORMATION

Further literature research confirms that materials promoting the adsorption of mycotoxins might be satisfactory with respect to aflatoxins, but they are not effective in preventing toxic effects of *Fusarium* mycotoxins, such as trichothecenes or zearalenone (Avantaggiato et al., 2005; Huwig et al., 2001).

In order to fill the void which cannot be addressed by mycotoxin binders, i.e. the elimination of the negative effects of less or non-adsorbable mycotoxins, biotransformation methods have been developed.

Biotransformation is the conversion of mycotoxins into less or non-toxic molecules by enzymes or microorganisms. As in the case of physical adsorption, this degradation takes place in the gastro-intestinal tract of the animal consuming mycotoxin contaminated feed (Rodrigues et al., 2009).

Biotransformation concept and studies go back to 1960's when the first bacterial strain was found to degrade aflatoxins (Ciegler et al., 1966). Many other scientific studies are available regarding microorganisms with detoxification capabilities (Wegst and Lingens, 1983; Yoshizawa *et al*., 1983; Varga et al., 2000). However, for their use in feed additives, mycotoxin-detoxifying microorganisms and enzymes have to meet several demands:

- Metabolites formed have to be non-toxic
- Detoxification process has to take place very rapidly in the digestive tract
- Used microorganisms and enzymes have to be safe
- Microbial and enzyme additives have to be stable during storage and must be able to act in a complex environment such as the gastrointestinal tract
- Mycotoxin-detoxifying microorganisms and enzymes have to prove their efficacy in feeding trials with target animals.

When these requirements are met microorganisms represent an innovative way to counteract mycotoxins in animal feeds.

5.2.5.1. Microbial strains with practical applications

A lot of experiments have been performed on trichothecenes biotransformation over the past 20 to 30 years using rumen fluid and intestinal contents. The use of these media is justified by the well known higher resistance of ruminants to the negative effects of trichothecenes when in comparison with, for example, monogastric animals.

The first experiments showing the degradation of DON into de-epoxy-deoxynivalenol (DOM-1) were performed by Yoshizawa and co-workers in 1983. Likewise, many other researchers successfully conducted *in vitro* trichothecene transformation experiments with ruminal or gut microflora resulting in DOM-1 (King et al., 1984; Swanson et al., 1987; He et al., 1992; Kollarczik et al., 1994). However, no pure culture of the DON-biotransforming strain could be isolated. Due to the variation of medium components (energy source, minerals, antibiotics) and subsequent subcultivation in dilution series and highly active enriched cultures, Binder and co-workers were the first to isolate a pure bacterial strain, BBSH 797, that is able to biotransform DON to DOM-1 (Figure 27) (Schatzmayr et al., 2006a). The toxicity of the compound formed by biotransformation of DON (DOM-1) was tested using a chicken lymphocyte proliferation assay (LPA) (Schatzmayr et al., 2006b). At a concentration of 0.15 µg DON/mL proliferation of lymphocytes was lower in comparison with the control. After adding 0.3 µg/mL to the cells, only one third of them could proliferate whereas at a concentration of 0.63 µg DON/mL the growth of lymphocytes stopped. In the case of DOM-1 only a concentration of 116 µg/mL inhibited proliferation of lymphocyte cells completely.

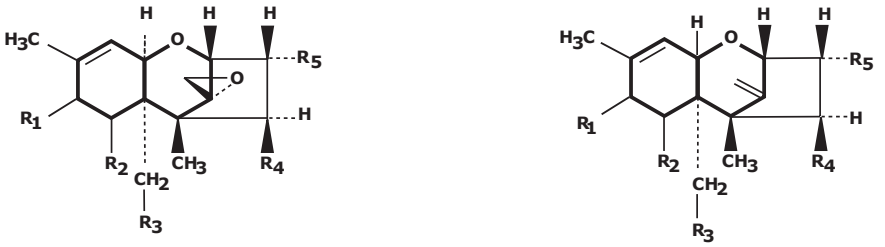


Figure 27 – Trichothecenes molecular scheme before (left) and after being detoxified (right) by BBSH 797

For the use of BBSH 797 as a feed additive, the fermentation and stabilization processes were optimized with respect to fast growth of the microbe and high biotransformation activity of the resulting product. For enhancement of stability during storage and within the gastro-intestinal tract, a three-step encapsulation process was implemented. So far, strain BBSH 797 is the only microorganism that has been developed into a commercial product designed for detoxifying trichothecenes in animal feed (EFSA, 2009).

While investigating yeasts associated with termites and recognizing the ability of a particular yeast strain to assimilate inulin and galacticol, one new species of basidiomycete yeast of the *Trichosporon* genus was found. The yeast strain isolated from the hindgut of lower termites – *Trichosporon mycotoxinivorans* MTV (MTV) - was found suitable to detoxify both ochratoxin A and zearalenone after incubation studies (Schatzmayr et al., 2003; Molnar et al., 2004; Schatzmayr et al., 2006b). Since *T. mycotoxinivorans* can be fermented, concentrated, freeze-dried and stabilized without losing its deactivating abilities, its utilization as a feed additive for mycotoxin detoxification seems practicable (Vekiru et al., 2010).

MTV detoxifies OTA by the cleavage of its amide bond resulting in phenylalanine and the non-toxic ochratoxin alpha (OTα) (Figure 28). Regarding the toxicity of OTA, studies were developed concerning the toxicity of the degradation metabolite, OTα (Schatzmayr et al., 2006b). On the one hand, growth of macrophages was depressed from 0.741 to 2.222 µg OTA/mL. At concentrations above 6.667 µg OTA/mL their growth was completely inhibited. On the other hand, concentrations up to 20 µg OTα/mL did not affect macrophages growth (Schatzmayr et al., 2006b). These results were in accordance with those of other scientific studies where OTα was shown to be non-toxic or at least 500 times less toxic than OTA (Chu et al., 1972; Bruinink et al., 1998).

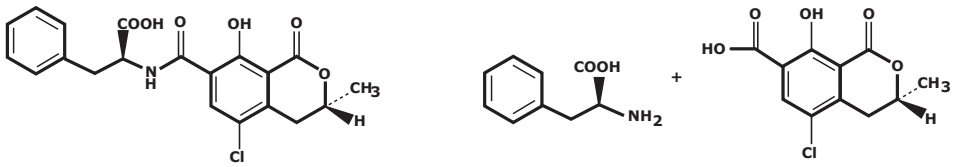


Figure 28 – Ochratoxins (left) are transformed into the less toxic metabolite ochratoxin α and phenylalanine moiety (right) by the yeast Trichosporon mycotoxinivorans MTV

As for the reaction between MTV and ZEN, a nonestrogenic ZEN metabolite (ZOM-1) was the main product of the degradation (Figure 29) (Vekiru et al., 2010). The sample resulting from the ZEN incubation with MTV did lack the ability to induce any response indicating an estrogenic

activity (remaining at baseline values) when tested by an E-screen assay, a commonly used system for evaluating the ability of chemicals to induce a hormonal response. This confirmed the stable degradation of zearalenone into a non-estrogenic metabolite (Schatzmayr et al., 2006b; Vekiru et al., 2010).

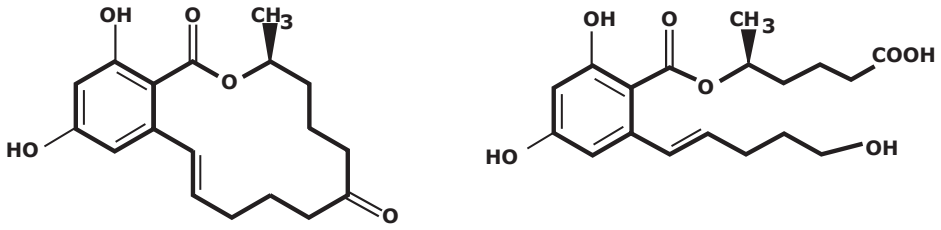


Figure 29 – Zearalenone (left) is transformed into the non-estrogenic metabolite ZOM-1 (right) by the yeast *Trichosporon mycotoxinivorans* MTV (adapted from Vekiru et al., 2010)

As for the deactivation of FUM, *Sphingopyxis* MTA 144 is a bacterium which is capable of biotransforming fumonisin B_1 into non-toxic metabolite 2-keto-HFB_1 (Hartinger et al., 2011). Heinl and co-workers (2010) provided a basis for the development of an enzymatic detoxification process for fumonisin B_1 in food and animal feed by isolation of two genes of *Sphingopyxis* sp. MTA14 *fumD* and *fumI*. FUM*zyme*® is encoded as a part of a gene cluster of *Sphingopyxis* sp. MTA 144 and enables the bacterial strain to degrade fumonisin B_1 into the metabolite hydrolyzed FB_1 (HFB_1) (Hartinger and Moll, 2011). Development of a novel feed additive for fumonisin detoxification based on this enzyme - which efficacy was proven *in vitro* and *in vivo* - has been ongoing for several years and is now on its final stages. The heterologously expressed aminotransferase *fumI* was shown to deaminate hydrolyzed fumonisin B_1 (Heinl et al., 2010) (Figure 30). Feeding experiments reported strongly reduced toxicity of HFB_1 with respect to sphingolipid metabolism, neural tube defects and at hepatic and intestinal level (Voss et al., 2009; Grenier et al., 2012). A recently published study demonstrated that HFB_1 does not cause intestinal or hepatic toxicity in the sensitive pig model and only slightly disrupts sphingolipids metabolism. This suggests that conversion to HFB_1 could be a good strategy to reduce FB_1 exposure (Grenier et al., 2012).

5.2.5.2. Other considerations for mycotoxin degrading microorganisms

When considering a microorganism as a potential mycotoxin biotransforming agent, the ability to degrade the mycotoxin into a non-toxic metabolite should be taken into account. The safety and non-pathogenicity of microorganisms must also be thoroughly studied.

A high demand has to be made on the detoxification velocity of the mycotoxin degraders for use as animal feed additive as there may be a great variation between microorganisms. In some cases, their degradation velocity is not fast enough for an effective application in animal nutrition (Wegst and Lingens, 1983).

Finally, the inactivation must be proven successful in complex media rather than only in buffer solutions.

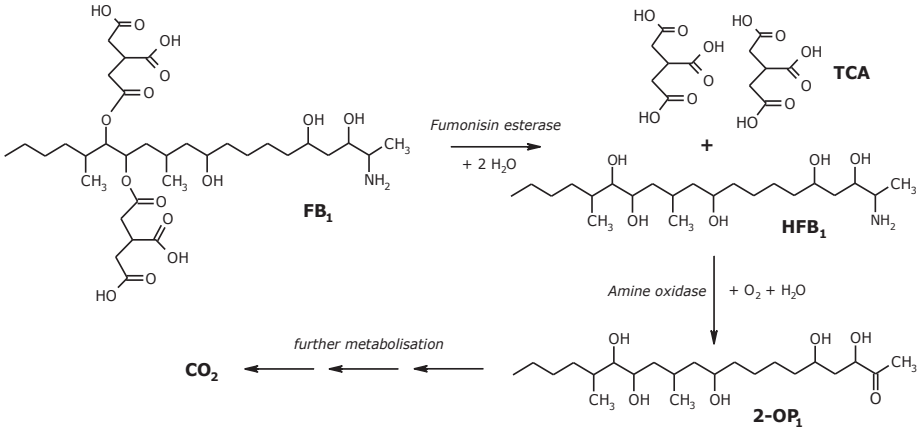


Figure 30 - Microbial degradation of fumonisin B_1

5.2.5.3. Field trials

Although *in vitro* studies represent the basis of research and product development, successful *in vivo* experiments are crucial to support the effectiveness of any creation in the field. Unfortunately, trials with mycotoxins are very difficult to run and often in controlled experiments animals show high tolerance to contaminated feeds (Fink-Gremmels, 2008).

Nonetheless, the efficacy of the above-mentioned bacteria and yeast strains has been shown in the field on various occasions under controlled conditions.

As an example of successful *in vivo* trials involving swine, highly significant ($P<0.001$) results were obtained in a piglet trial where feeds were contaminated with 2500 µg DON/kg (Binder et al., 2000). Animals supplemented with 1.24 x 10^6 CFU/g BBSH 797 had a feed conversion ratio (FCR) of 1.6 against the 2.0 shown by non-supplemented animals.

More recently, the multi-organ toxicity of a combination of 1000 µg DON/kg feed and 250 µg ZEN/kg feed in pigs was partially or completely compensated when animals were supplemented with these microorganisms incorporated in 1.5 kg (0.15 %) of the commercial product Mycofix® (Cheng et al., 2006).

At the University of Santa Maria (Brazil) gilts fed 2000 µg ZEN/kg feed presented an enlargement of vulva and an increase in the relative weight and size of the reproductive tract. The presence of 0.5 % of Mycofix® showed a protective effect and the reproductive tracts were comparable to those of the control group (data not published).

At the University of Bologna (Italy) ninety-six weaned piglets underwent a challenge trial and were given feed contaminated with 2500 µg DON/kg feed. Animals supplemented 0.25 % of Mycofix® showed improved daily weight gain, final weight and feed conversion rate in comparison to those given the mycotoxin challenge alone.

At the International Institute of Animal Investigation (Mexico) young piglets fed 500 µg OTA/kg feed and 250 µg ZEN/kg feed showed a statistically significant lower final body weight and daily weight gain in comparison with the control group. Besides these performance findings, clinical problems in mycotoxin challenged groups included swollen vulva and prepuce, rectum prolapse, vomiting, diarrhea and frequent urination. Treatment groups fed 0.05 % and

0.1 % of Mycofix® showed lower incidence of these occurrences and presented statistically improved performance data.

At the Institute for Animal Nutrition at the University of Berlin (Germany), 200 µg ZEN/kg feed and 2500 µg DON/kg feed were continuously fed to sows over a period of 3 reproductive cycles. Fertility problems due to ZEN, shown in higher non-conception rates, abortions and symptoms of hyperestrogenism, occurred after a very short exposure time during the first reproductive cycle. Visible, trichothecene-related negative effects on piglet health were lesions, necroses and edema (skin, ear, tail), as well as deformations of extremities, mainly during the 2nd and 3rd reproductive cycle. Sow losses in groups fed fusariotoxin-contaminated feed were due to bacterial infections of kidney, ureter, uterus, teats, lung, liver and intestinal tract, indicating a suppressed immune system of animals. However, Mycofix® successfully reduced non-conception rate, suppression of feed consumption, rearing losses and organ infections as well as pathological changes of ovaries and mucous membranes of uteri. Moreover, symptoms of hyperestrogenism, malformation of extremities, necroses on ears, tails and teats as well as lesions and edema of skin of piglets occurred less often and less severely in the presence of this commercial additive.

Prepubertal gilts fed 500 µg/kg OTA and 250 µg/kg ZEN presented small ovaries and underdeveloped uterus along with histopathologic changes such as lesions of ovarian atrophy and regression of endometrial growth (Acda et al., 2008). Treatment groups fed the same concentration of toxins and supplemented with 0.05 %, 0.1% and 0.2 % of Mycofix® not only did not show these alterations but also did not present the nephotoxicosis observed in the toxin group. Moreover, animals supplemented with the highest dosage of the commercial product obtained a better FCR and a better average daily weight gain.

Trials involving prototype products, such as the previously mentioned fumonisin esterase *fumD* (FUM*zyme*®) showed promising results when piglets were challenged with 50 mg fumonisin B_1/kg feed. Groups treated with an inclusion rate of 0.25 % and 0.5 % of this enzyme on a bentonite carrier improved their average daily feed intake (0.25 %: +52.4 %; 0.50 %: +46.8 %); their average weight (0.25 %: +54.7 %; 0.50 %: +48.3 %); their average daily weight gain (0.25 %: +91.5 %; 0.50 %: +80.5 %) and as for the biomarker for fumonisins' exposure, the sphinganine - shingosine ratio, it was improved in a statistically significant manner (0.25 %: +19.4 %; 0.50 %: +25.1 %). More recently, a great step forward in the direction of a successful application of FUM*zyme*® as a feed additive, was taken. In a study performed by Schwartz and team (2012) the capability of different units of the enzyme mixed into feed to hydrolyze 30 mg FB_1/kg in growing piglets was assessed. In the course of this study the different biomarkers for assessing the extent of hydrolysis were also investigated (Schwartz et al., 2012). Several biomarkers were found to be suitable for monitoring *in vivo* fumonisin hydrolysis and detoxification: fumonisin concentration in feces, fumonisin concentration in urine, and sphinganine - shingosine ratio in serum. Research results showed that the recommended dose of FUM*zyme*® required for complete gastrointestinal hydrolysis of 30 mg FB_1/kg feed is in the range of 10 to 100 U/kg.

When products developed to counteract mycotoxins are tested under laboratory or under controlled conditions in scientific studies, many parameters are usually measured. Trials can focus simply on performance but the ideal and usual situation is that these parameters are broadened to include the measurement of biochemical, immunological and pathological

parameters (namely growth performance, serum biochemistry parameters, alveolar macrophage activities, antibody titers, cytokine secretion profile and histopathological findings). The best sign of product efficiency is found when all tested parameters are improved with its inclusion. Often, only some parameters reflect a positive response to the treatment. In some cases whilst some of the parameters are improved, others are negatively affected. Above all, it is important that people interested in the topic consult the original data of all scientific work which are mentioned in this and all other books or summaries.

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Karin Nährer

Karin Nährer is working for BIOMIN Holding GmbH in Herzogenburg as a Product Manager in the Competence Center Mycotoxins with the main focus on Mycotoxin Risk Management including the BIOMIN mycotoxin survey program.

During her study at the University of Applied Science in Tulln with the master program "Biotechnical Processes" she joined BIOMIN Research Center as a Master student working on the technological potential of the fumonisin B1 degrading enzyme Fumonisinesterase as a biological feed additive. After obtaining her degree in biotechnology she joined the Product Management of BIOMIN Holding GmbH in July 2007.

Isabelle Oswald

Dr. Isabelle Oswald qualified as an engineer in agricultural sciences and received her PhD from Rennes University in 1980. After a post-doctoral training in immunology at the NIH (Bethesda, Maryland, USA), she got a position at INRA (French National Institute of Agricultural Research) in Toulouse. She is currently Research director at the INRA Reseach Center in Food Toxicology (ToxAlim). She is leading a research team with 7 scientists, 4 technicians and 9 doctoral and post-doctoral fellows. This team has two main goals (1) to determine the toxic effects of

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Elisabeth Pichler

Dr. Elisabeth Pichler recieved her PhD in Chemistry from the Technical University of Vienna in 2002. During her diploma and PhD thesis she studied the microbial degradation of certain mycotoxins like deoxynivalenol or ochratoxin A. Her work on ochratoxin A resulted in Patent, WO 03/053161; 2003. In 2002 she started to work for BIOMIN as laboratory manager. After changing to Romer Labs she worked in several positions: product manager for chromatography products, head of the analytical services lab in Europe and – at the moment - director of regulatory affairs.

Inês Rodrigues

Inês Rodrigues studied Zootechnical Engineering (focused on animal production) at Évora University, Évora, Portugal, from 2000 to 2005. After having different experiences such as working at the Veterinary University as a Laboratory Research Assistant and being a trainee at the Directorate-General for Agriculture and Rural Development (European Commission) she found a perfect balance between contact with people, science and regulations. Ever since 2007, she has been working in BIOMIN as a Technical Manager giving support on mycotoxins-related issues. Her passion for writing together with her thirst for knowledge resulted in several publications throughout these years, from technical articles to peer-reviewed papers and participation in books related with the mycotoxin problematic in animal husbandry.

Maximilian Schuh

Prof. Schuh received his doctoral degree of veterinary medicine in 1972 and his PhD in 1982 from the University of Veterinary Medicine Vienna. His PhD thesis was concerned to the chronic effect of deoxynivalenol (vomitoxin) in fattening pigs. His research career began in 1968 at the Institute of Medical Chemistry investigating toxicological cases from field outbreaks of pigs and ruminants as well as experiments regarding effects of antibiotics and their residues in animal tissues. After a one and a half year stay (1977-1978) at the Veterinary Diagnostic Laboratory of

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