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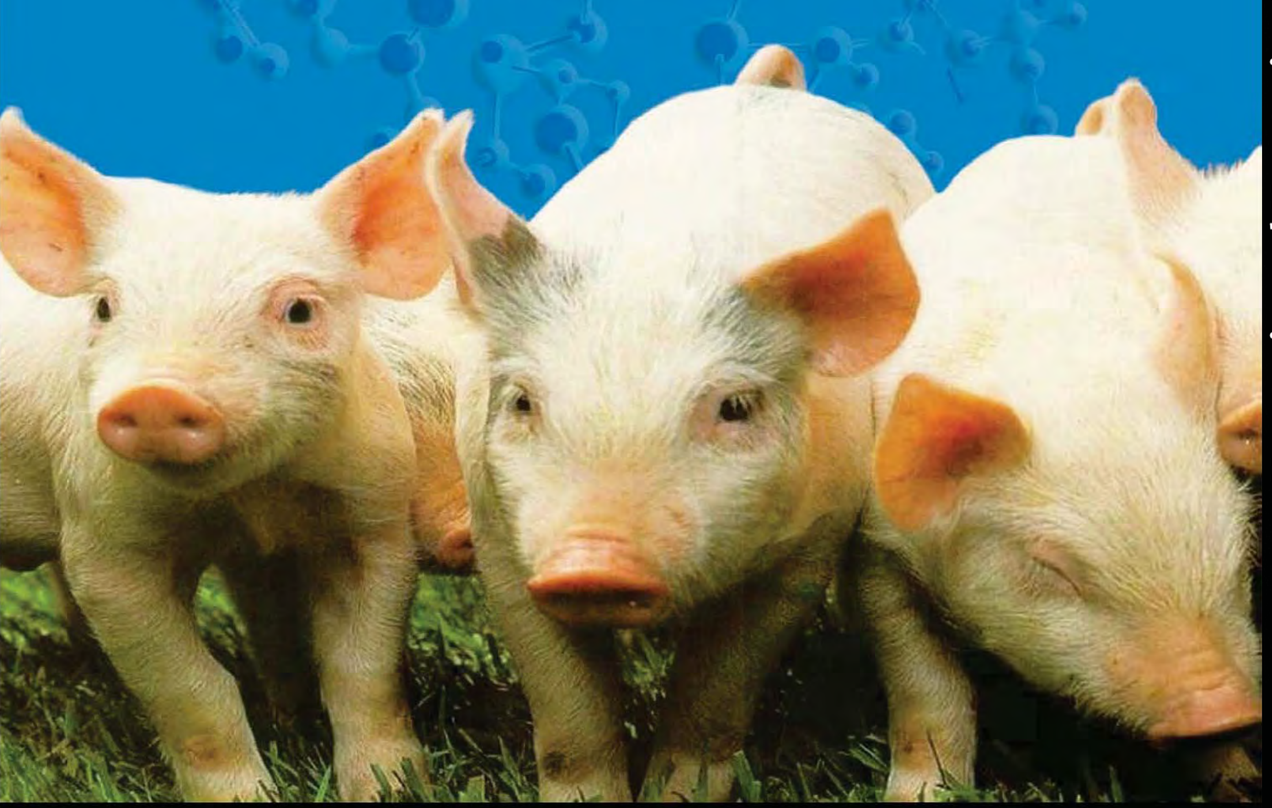

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Mycotoxins and Mycotoxicosis in Swine

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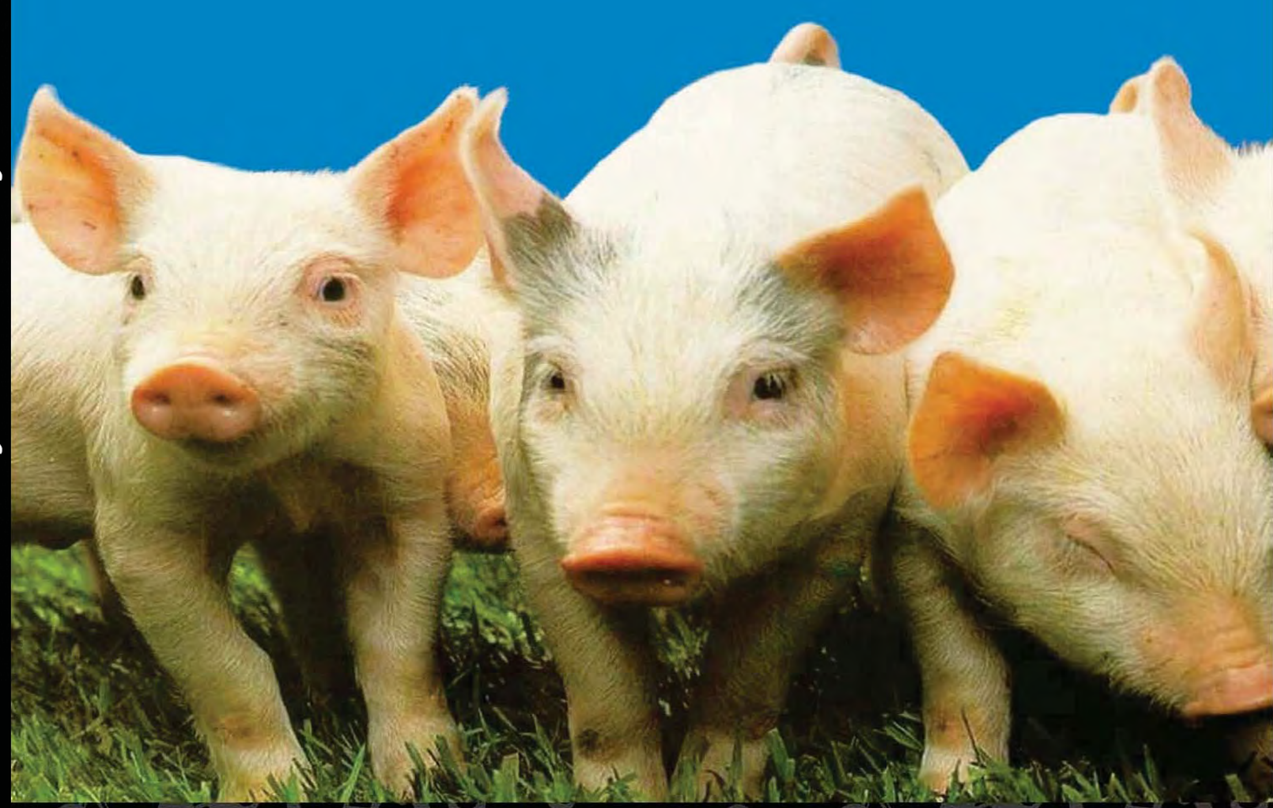

Carlos A. Mallmann
Paulo Dilkin

LAMIC
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Mycotoxins and Mycotoxicosis in Swine

Carlos A. Mallmann

Paulo Dilkin

Translated by **Gabriela Zaviezo, M. A.**

Edited by **Douglas Zaviezo, Ph. D.**

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PRESENTATION

Special Nutrients, a U.S. based company, as a sponsor of this book and as a world pioneer in the applied science of mycotoxin detoxification, would like to take this opportunity to thank the authors, Dr. Carlos Agusto Mallmann and Dr. Paulo Dilkin of the Department of Preventive Veterinary Medicine at the Universidade Federal de Santa Maria, RS, Brazil, for allowing us to translate this excellent book, which provides the technical community around the world knowledge regarding mycotoxins, their dangerous effects, and technical recommendations to maintain the optimal productivity of swine. It is our pleasure to briefly introduce the authors of this book.

Carlos A. Mallmann

Dr. Carlos A. Mallmann received his Doctorate in Veterinary Medicine and Masters of Science degrees from the Universidade Federal de Santa Maria (UFSM) RS, Brazil. He received his Doctorate in Mycotoxicology from the Free University of Berlin and the Superior School of Veterinary Medicine of Hannover in Germany. He is currently a professor of Public Health and Epidemiology at the Department of Preventive Veterinary Medicine (UFSM) and general coordinator of the Laboratory of Mycotoxicological Analysis (LAMIC). Dr. Mallmann is also a consultant for official institutions, as well as some of the most important animal nutrition and feed manufacturing companies in Brazil and other countries.

Paulo Dilkin

Paulo Dilkin is Doctor in Veterinary Medicine, specialized in animal toxicology. He has a Masters in preventive veterinary medicine and Doctorate in Microbiology (mycotoxicology). He is currently a professor in the Department of Preventive Veterinary Medicine at the Universidade Federal de Santa Maria (UFSM), RS, Brazil. Dr. Dilkin is a scientific adviser for LAMIC/UFSM, developing analytical procedures, experimental mycotoxicological animal tests and production of mycotoxin standards. He is also a legal expert in mycotoxin involvements, and a consultant in implementing quality control programs for monitoring mycotoxins in feed and feed ingredients, as well as the development and utilization of antimycotoxin additives.

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FOREWORD

Mycotoxins constitute a highly dynamic field of study; everyday new information is generated, creating greater challenges. Therefore, we are constantly emerged in this fascinating and dynamic professional field. We believe that this book could be a significant contribution to the swine industry, which has already reached a high level of productivity and performance through new technologies in the areas of genetics, nutrition, management and biosecurity. Mycotoxins are becoming increasingly more important because of how they limit improvements in efficiency and productivity that have already been implemented. Therefore, we think that mycotoxicology will be one of the strategic areas in the near future, not only in Brazil, but also around the world, because of mycotoxins' repercussions on the deleterious effects on the safety of human and animal nutrition and its economical implications.

This book was written using practical knowledge acquired during our professional lives, but was fundamentally based on results of research published by the international scientific community. However, this is not a finished project, since not all the knowledge generated until today could be presented in this book. There is also new research published on this topic every day. Nonetheless, we hope to present some important basic knowledge, addressed mainly to veterinarians, researchers, students, professionals, and swine producers.

We begin this book by defining some basic concepts concerning mycotoxicological dynamics. Then, we detail concepts concerning aflatoxins, zearalenone, fumonisins, trichothecenes, ochratoxin A, and other less prevalent and less well known mycotoxins. Finally, we highlight the actions that should be taken or the way to manage mycotoxins, proposing different tools that are approved and widely used in mycotoxicological monitoring. This chapter also contains a great part of our professional experience and the most frequently ratified as the existing solutions in this field of study. Although this book revises mycotoxicological concepts of the commercial swine industry, it also deals with some historical aspects, mold that produce mycotoxins, physical and chemical characteristics of mycotoxins, toxicity, clinical signs, lesions, diagnostics, and ways to temper the toxic effects of each mycotoxin.

This project was also possible thanks to the help of all those who contributed to the creation of LAMIC, institution that foments and supports the development of this science. We dedicate this book to our graduate students, researchers, and collaborators. We would like to thank Special Nutrients for making this book available in English and thus available to a global audience. Finally, we would like to thank our families for their unconditional support and dedication to this cause.

1

MYCOTOXINS

MYCOTOXINS

Mycotoxins are toxic substances that are the result of secondary metabolism of diverse strains of filamentous fungi. They are organic compounds of low molecular weight and low immunogenic capacity. They are present everywhere, nevertheless they are predominate in tropical and subtropical climates where fungal development is favored by the environmental conditions. The main fungal species that produce mycotoxins belong to the genera *Aspergillus, Penicillium, Fusarium, Claviceps, Alternaria, Pithomyces, Mirothecium, Stachibotrys,* and *Phoma*.

In tropical and subtropical climates, like that of Brazil, fungal development is favored by factors such as optimal humidity and temperature conditions. More than five hundred mycotoxins produced by approximately one-hundred fungi are known. The most important can be divided into three groups: aflatoxins produced by fungi of the genus *Aspergillus*, such as *A. flavus* and *parasiticus*, ochratoxin produced by fungi of the genera *Aspergillus* and *Penicillum,* and fusariotoxins which are represented by trichothecenes, zearalenone, and fumonisins, produced by different species of the genus *Fusarium* (Table 1).

Table 1 – Main mycotoxins, primary fungi producers, food supply prone to contamination, and favorable conditions for occurrence.

Mycotoxin	Main fungi producers	Food supply prone to contamination	Main factors that lead to the production of mycotoxins
Aflatoxins	*Aspergillus flavus* and *A. parasiticus*	Almonds, chestnuts, acorn, corn and grains in general.	Inadequate storage conditions.
Zearalenone	*Fusarium*	Corn and winter grains.	Low temperature associated with high levels of humidity.
Fumonisins	*Fusarium*	Corn and winter grains.	Dry season followed by high humidity and moderate temperature.
Trichothecenes	*Fusarium*	Corn and winter grains.	Low temperature, high humidity, and storage problems.
Ochratoxin A	*Aspergillus Penicillium*	Corn and stored grains.	Storage deficiencies.

When mycotoxins are ingested, by humans or animals, they can produce different deleterious health effects, resulting in negative economical, sanitary and commercial consequences. Besides other less toxic effects, ingesting mycotoxins can also negatively affect reproductive health because of their anabolic, estrogenic, carcinogenic, mutagenic, and teratogenic properties. The danger of mycotoxins is not just the possibility of lethal effects, but rather, the harm they present to different organs and systems, decreasing optimal productive performance.

The first incidents of mycotoxicosis are present in the Old Testament, as the tenth plagues of Egypt, which is narrated in the books of Exodus and Job in the passage where Moses tries to free the Hebrews from Pharaoh's control. There is evidence of the presence of mycotoxins in the plagues that demised flocks and herds, and caused tumors and ulcers in Egyptian animal and human populations.

In the historical evolution of mycotoxins, the episode termed "Saint Anthony's fire" which occurred in the Middle Ages, specifically during the XI and XVI century, is noteworthy because it affected the population of various European countries, particularly France. The disease was characterized by outbreaks of gangrene in the population which had consumed grains contaminated by sclerotia (rye ear) of the fungus *Claviceps purpurea.* The disease is a result of the vasoconstrictor properties of ergotamine, which obstructs peripheral circulation. Furthermore, the disease also promotes oxytocia and stimulates the central nervous system, followed by a noticeable depression. During the period between 1930 and 1940, stachybotryotoxicosis caused the deaths of tens of thousands of horses in the former USSR. This mycotoxicosis is caused by a toxin produced by fungi of the *Stachybotrys* genus, such as *S. chartarum* and *S. alternans*, which grow well in humid hay and straw. The clinical manifestations of this mycotoxicosis include neurological alterations caused by tremors, diminished visual capability, often occurring epidermal necrosis, leukopenia and ulcerations in the gastrointestinal tract. In the beginning of the XX century, specifically in the years from 1941 to 1945, Alimentary Toxic Aleukia (ATA *disease*s) was responsible for making a large number of Europeans sick, killing more than 100 thousand Russians. This was a result of ingesting trichothecenes produced by cryophilic fungi of the *Fusarium* genus*,* specially *F. esporotrichioides, graminearum,* and *moniliforme*. The disease has four stages of evolution, in this order: lesions in the upper digestive tract, hematopoietic damage, damage to the

nervous system, and damage to the endocrine system. In the XIX and XX century, an epidemic called yellow rice disease struck Japan and caused a great number of deaths due to consumption of moldy rice. The disease was attributed to citreoviridin, a cardiotoxic toxin (cardiac beriberi) caused by fungi of the *Penicillium* genus. Balkan nephropathy, a disease which affected eastern European countries from 1957 to 1958, was a result of consuming food sources contaminated by ochratoxin A, produced by fungi such as *Aspergillus ochraceus* and *Penicillium viridicatum*.

Mycotoxins began receiving mayor scientific attention in the 1960s, when aflatoxins were found responsible for the deaths of more than one hundred thousand poultry in Europe. Advances in research demonstrated that mycotoxins contain extremely toxic properties which affect all mammals; therefore, mycotoxins have gradually gain scientific importance around the world. So much so that in 1988 fumonisins were attributed with different illnesses in domestic animals and were related to a large quantity of esophageal cancer outbreaks in humans. Today, more than five hundred chemical compounds are categorized as mycotoxins.

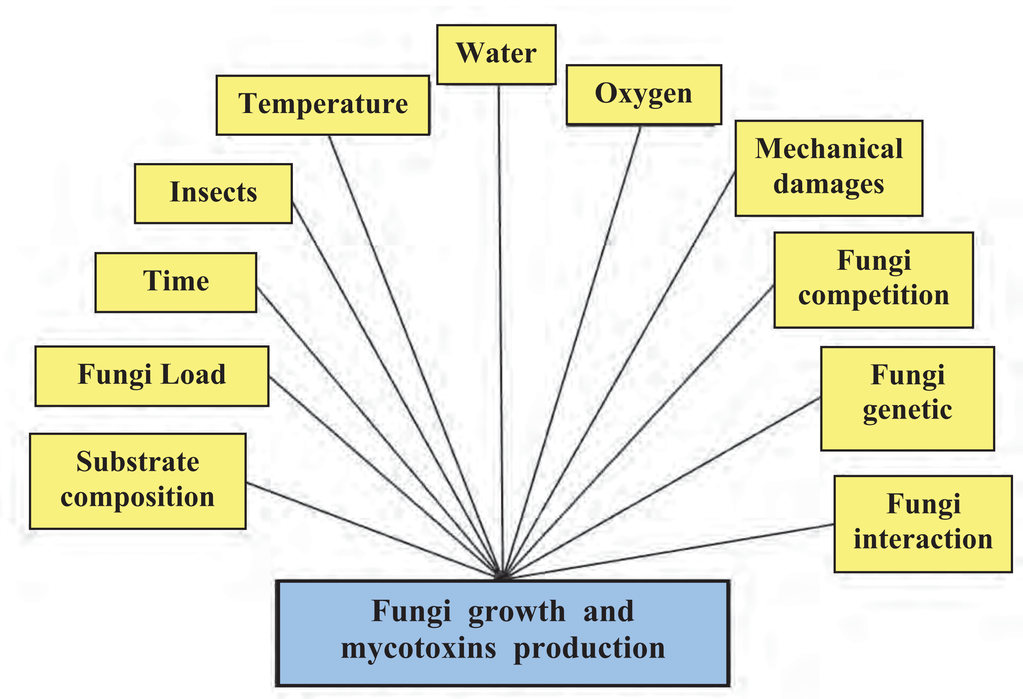
Currently, knowledge concerning the physiology, production of toxins and development of fungi producers of mycotoxins are still limited. Nevertheless, it is known that mold grows in grains and cereals, especially in peanuts, corn, wheat, barley, sorghum, and rice because they contain nutritious substrates for fungal development. Cereals and grains can lose important portions of their nutritional value when contaminated with mycotoxins which can remain present for various years, even when fungi are no longer present.

Fungal growth and mycotoxin production in cereals can occur in different phases of development: maturation, harvest, transportation, processing, or storage of grains. The difficulty of harvesting cereals in the correct stage of maturation and humidity could be another critical point that concerns the formation of mycotoxins. It is normal that cereals harvested with a high level of humidity facilitate fungi development before these cereals are dried, and even in storage, especially when these grains go through a drying process. Cereals harvested after the physiological maturation of the grain are subjected to high levels of humidity and to the onset of plagues that create favorable conditions for mold development. The transportation of cereals with high levels of humidity for long distances or prolonged periods of time, also favors the development of fungi, due to the formation of an adequate microclimate for such growth. Therefore, reducing cereal humidity through a drying process

is critical. The drying process, when done improperly, can also contribute to the proliferation of fungi in grains. In Brazil, the long lines and waiting time of trucks that transport cereal to the entrance of the drying facility frequently contribute to a hasty drying process resulting in deficiencies such as increased drier temperatures, producing mechanical and thermal damages to the grain, and consequently decreasing their immunity.

Other factors that encourage fungal development and production of mycotoxins include a series of storage deficiencies, such as high levels of humidity, uneven distribution of cereal inside silos, variations in ventilation, and incidence of pests. Understanding the various factors that can increase the formation of these toxic metabolites is fundamental in order to diminishing the production of mycotoxins (Figure 1).

Figure 1 – Intrinsic and extrinsic factors related to the production of mycotoxins.



The different effects of mycotoxins are due to their specific chemical structures, varying reactions to their ingestion by different animals influenced by species, race, sex and age, environmental factors, nutritional status, and the presence of other chemical substances. (Table 2)

Table 2 – Major mycotoxins, species affected, and main clinical signs and lesions.

Mycotoxins	Species most affected	Main clinical signs and lesions
Aflatoxins	All	Diminished weight gain, digestive disorders, hepatopathies, anorexia, ataxia, tremors and death.
Zearalenone	Swine	Hyperestrogenism syndrome (vulvovaginitis).
Fumonisins	Horses and Swine	Horse leukoencephalomalacia. Swine pulmonary edema.
Trichothecenes	Monogastrics	Feed reduction or refusal, digestive disorders with ulcerations and vomiting, and visceral hemorrhages.
Ochratoxin A	Swine and humans	Nephropathy.

In spite of a series of variations, a susceptibility to mycotoxicosis can be estimated. In order to do this, it is necessary to quantify the level of feed contamination and feed consumption, which indicates the type and severity of each disease. Mycotoxicosis can be divided into acute and chronic disorders.

The acute manifestations occur when individuals consume moderate to high doses of mycotoxins. Clinical syndromes and specific pathological signs can become present, depending on the mycotoxin ingested, the susceptibility of the species, the individual conditions of the organism, and the interaction or lack of interaction with other factors. Lesions depend on each mycotoxin (Table 3); however, the most commonly found are signs of hepatitis, hemorrhaging, nephritis, necrosis of the digestive mucosa or death.

Chronic mycotoxicosis occurs when moderate to low doses are consumed. In these cases, the acute manifestations of intoxication are not present; nevertheless, characteristic clinical symptoms such as decrease in reproductive efficiency, feed conversion, growth rate, and weight gain are present. These characteristics are only detected by detailed observation and training, or by monitoring mycotoxins levels in feed. The clinical signs could be confused with other illnesses proceeding from mycotoxicosis or from nutritional deficiencies. There are few statistics available related to the incidence of mycotoxicosis, nevertheless there is a

general awareness that the “hidden danger” (chronic intoxication) is responsible for a large portion of the losses experienced in productive animals.

The results of laboratory evaluations conducted in the last 17 years indicate that aflatoxins are the most prevalent mycotoxins in Brazil. As shown in Table 4, ochratoxin A and T-2 toxin contamination is not significant, in spite of the fact that the latter does not have a history of being diagnosed with adequate methodologies.

One important effect caused by chronic mycotoxicosis is immunosuppression, leaving the individual predisposed to other illnesses whose pathogens easily multiply with the decrease of the animal’s resistance to illness. In addition, ingesting feed contaminated with mycotoxins causes inadequate responses to vaccinations.

Table 3 - Effects of main mycotoxins in swine.

Mycotoxin	Swine stage	Level in feed (µg/kg)	Principal clinical signs
Aflatoxins	Growing / finishing	10-100	Loss of productivity, without visible clinical signs.
		200-400	Poor growth and feed efficiency.
		400-800	Hepatopathies (friable and yellow-bronze liver); immunosuppression.
		800-1,200	Significant decrease in feed intake and growth, icterus and hypoproteinemia.
		1,200-2,000	Icterus, coagulopathy, anorexia, and deaths.
	Sow/piglet	500-750	Reproductive disorders, weak piglets due to contamination through milk.
Zearalenone (F-2)	Pre-pubescent gilts	1,000-3,000	Edematose vulva, reddened and prolapsed rectum.
	Gestating Lactating sows	3,000-10,000	Edematose vulva, retention of corpus luteum and anestrus.
		25,000	Repeated heat.
		25,000-50,000	Small litter, weak piglets, edematose and reddened vulvas in neonatals.
	Replacing gilts	>25,000	Pseudogestation, nymphomania, and persistent infertility.
Fumonisins	All swine	1,000-20,000	Hepatopathies, tumors, and decrease of productivity. Enlarged heart.
		>20,000	Sharp pulmonary edema, hepatopathies, and decrease in feed intake.
Diacetoxys-cirpenol (DAS)	Growing/ finishing	2,000-8,000	Decrease in feed intake and ADG, epidermic and oral irritation, and intestinal epithelium hypertrophy.
		8,000-10,000	Complete refusal of feed.
T-2 Toxin	Growing/ finishing	< 2,000	Hemorrhaging and enteritis.
		8,000	Decrease in feed intake.
		16,000	Complete refusal of feed.
Deoxynivalenol (DON or vomitoxin)	Growing/ finishing	2,000	Decrease in feed intake and growth.
		5,000-10,000	Decrease in feed intake, and weight loss.
		12,000	Complete refusal of feed.
		20,000	Vomiting.
Ochratoxin A	Growing/ finishing	200	Renal lesions seen at processing.
		1,000	Polyuria, uremia, decrease of ADG.
		4,000	Severe renal failure.
	Females/ piglets	3,000-9,000	No alterations in estrous cycle or conception rates.

Table 4 – Results from analysis of mycotoxins in different feeds, taken during the period of 1994 to 2010 by the Laboratory of Mycotoxicological Analysis, LAMIC/UFSM.

Toxin	# Samples Analyzed	Positive %	Average (µg/kg)	Maximum (µg/kg)
Aflatoxins[1]	138,875	35.8	8.4	16,861.5
Zearalenone	105,509	29.8	74.1	17,000.0
Fumonisins[2]	45,558	64.0	1591.0	289,283.0
DON	39,451	40.1	313.1	23,740.2
Ochratoxin A	30,973	4.0	0.6	404.3
T-2 Toxin	16,939	1.0	1.2	2,133.0
Total	**377,305**	**28.4**		

[1] Results from the sum of aflatoxins $B_1+B_2+G_1+G_2$

[2] Results from the sum of fumonisins B_1+B_2

With advances in research, mycotoxins have become scientifically significant at a global level. Among the hundreds of known mycotoxins, aflatoxins are considered the most important. They are produced by the fungus genus *Aspergillus* and are classified as B_1, B_2, G_1 and G_2. These fungi develop in grains, above all in peanuts and corn. When its toxins are ingested the immediate reaction is a decrease in productivity, as well as the carcinogenic, mutagenic, teratogenic, heptatoxicity and immunosuppression effects. Aflatoxin B_1 is the most toxic of the group, mainly affecting swine and poultry. Aflatoxins were categorized as Class 1 human carcinogens by the *International Agency for Research on Cancer* (IARC) (IARC, 1987). The Brazilian Ministry of Agriculture established a maximum limit of 20 µg/kg, comprising B_1, B_2, G_1 and G_2 in food sources destined for human consumption (Brazil, 1996). The maximum limit recommended for aflatoxins in feed by the Brazilian Ministry of Agriculture is 50 µg/kg (Brazil, 1988).

The ingestion of aflatoxins decreases meat, milk, and egg production, and they can be carried by some of these food sources. Therefore, aflatoxins and products of their biotransformation can be isolated in these food sources and their derivatives, such as processed meats, cheeses, and yogurts.

Zearalenone is a toxin produced by various species of the *Fusarium* genus, especially by *Fusarium graminearum.* It causes alteration in female swine reproductive system, resulting in reproductive complications.

Ochratoxin A is produced by fungi such as *Aspergillus ochraceus* and *alutaceus,* and fungi of the *Penicillium* genus. The main effect of Ochratoxin A for swine is nephrotoxicity.

Trichothecenes is a group comprised of chemically similar metabolic toxins, produced by various species of fungi such as: *Fusarium*, *Cefalosporium, Myrothecium, Stachybotrys*, and *Trichoderma*. Approximately 200 mycotoxins make up this group, T-2 Toxin, Diacetoxyscirpenol (DAS) and Deoxynivalenol (Vomitoxin or DON) being the most well known. These toxins inhibit protein synthesis and interfere with DNA and RNA synthesis. Besides lesions in the digestive tract, especially on the mouth, pharynx, and esophagus, which are important for clinical diagnosis; animals show a decrease of productivity, as well as a deficiency in their immune system.

The chemical structures of fumonisins began to be discovered in 1988. Fumonisins are produced by fungi of the *Fusarium* and *Alternaria* genus. These fungi are present throughout the world. The most prevalent fumonisin is fumonisin B_1, responsible for pathological effects such as leukoencephalomalacia in horses, which is characterized by liquifactive necrosis of the white substance of the central nervous system, pulmonary edema in swine, hepatopathy in various species, and esophageal cancer in humans. The incidence of leukoencephalomalacia in southern Brazil is seasonal, with the majority of cases being observed during the coldest months when animal feed is supplemented with grain based rations. The corn used as a dietary supplement is frequently responsible for the presence of said pathology. It is known that *Fusarium moniliforme* develops in corn containing high humidity in favorable temperatures. The growth of the fungus is accompanied by the development of fumonisins in feed ingredients, which produces pathologies in animals. Preventive measures consist of appropriate conservation of grains accompanied by laboratory tests to detect fumonisins.

Presumable diagnosis of mycotoxins is based on clinical signs observed in intoxicated animals, and analysis of environmental data concerning harvesting and storing conditions of grains used in feed for swine. Normally the introduction of a new batch of feed,

sometimes containing altered physical characteristics is associated with intoxication. A more reliable diagnosis is done by analyzing the presence of mycotoxins in the feed of the affected animals. The most commonly used techniques are ELISA kits analyses, Thin Layer Chromatography (TLC) and High Performance Liquid Chromatography (HPLC). Mass spectrometry (GC/MS, LC/MS and LC/MS/MS) as a method of diagnosis is a recent development. It has been proven that these are the most efficient methods, and they will surely substitute all others in the near future.

Figure 2 – Equipment used to detect and quantify mycotoxins. (A) High Performance Liquid Chromatography. (B) High Performance Liquid Chromatography coupled with Mass/Mass spectrometry.

The treatment of mycotoxins represents one of the greatest challenges to clinical veterinary. The removal of contaminated feed is the first measure that should be adopted, and seems to improve prognosis. Even though there is a lack of scientific evidence, the inclusion of additional levels of sulfur amino acid to the feed has been used to decrease mycotoxicosis.

The main prophylactic measures consist of adopting harvesting and handling techniques that make fungal growth difficult, such as the harvesting of grains immediately after reaching their physiological maturity, making them less exposed to weather, and drying and stowage in adequate granary for each type of grain or grain product. Knowledge regarding fungi physiology and development and production of toxins are still insufficient, precisely because research regarding production care and storage for the majority of grains produced is limited. The choice of a given corn strain that is resistant to fungal development can diminish the probability of the presence of mycotoxins. For example, varieties of corn

that possess high concentrations of linoleic acid can be more resistant to fungal development of the *Aspergillus* genus. Hence, monitoring grains and their byproducts through adequate sampling techniques and mycotoxicological analysis before grains are used are essential practices, especially when given grains have been exposed to environmental conditions that are favorable for fungi growth. The use of organic acids can help conserve feed in risky situations. The use of additives, whether natural or modified by the addition of enzyme compounds or biological compounds to the feed, deserves scientific study. These additives have shown promising results for swine in some field cases, which are still not entirely clear.

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AFLATOXINS

AFLATOXINS

Summary

Aflatoxins are secondary metabolites of the toxigenic fungi of the *Aspergillus* genus, specifically *A. flavus* and *A. parasiticus*. Among the hundreds of known mycotoxins, aflatoxins are considered the most important in Brazil. Various compounds have been described as aflatoxins, nevertheless, only aflatoxins B_1, B_2, G_1 and G_2 have been identified as natural contaminants of grains and feed. Generally, aflatoxin B_1 is the most prevalent in various agricultural products, and it is also the most toxic amongst these aflatoxins. Aflatoxins were initially known to cause "Turkey X Disease", whose toxicosis cause high death rates for these animals in England in 1960. After this intoxication, aflatoxins became recognized as highly hepatocarcinogenic, mutagenic, and teratogenic substances for animals and humans. They can cause various toxic effects in animals, predominantly affecting the liver, thus compromising the health and productivity of intoxicated animals. Swine are particularly susceptible to intoxication due to aflatoxins. Acute intoxications frequently lead to the death of animals, nevertheless, the greatest loss results from subacute and chronic intoxications. In these circumstances, animals ingest less feed and there is a decline in productivity, a decrease of immunological capabilities, and various pathological disorders in various organs. These symptoms result in the general sickness of the affected animals.

History

Mycotoxins received significant scientific importance due to the high number of mortalities caused by aflatoxin intoxication of animals. In England, in 1960 an apparently new illness known as Turkey X Disease was recorded; more than one hundred thousand turkeys, twenty thousand ducks, and hundreds of poultry died after ingesting peanut meal allegedly imported from Brazil, and contaminated with aflatoxins. Aflatoxicosis outbreaks were immediately observed in other animal species, such as bovine, swine, sheep, and chicken that had ingested the peanut meal of the same origin. At the same time, peanut meal imported from other countries produced the same toxicological symptoms when it was ingested by animals.

The principal clinical signs present in the intoxicated animals were a loss of appetite, lethargy, weakness, and death. Histopathological examinations revealed necrotic lesions in the liver, as well as disseminated hemorrhages. After a series of investigations, the intoxications were correlated to a toxin produced by the fungi of the *Aspergillus* genus, especially *A. flavus*. Researchers had a difficult time determining that the toxin was produced by that fungal species, because the fungi were absent in most of the samples tested. This absence is due to the fact that even after the death and decomposition of fungi, the toxins they produced persist in feed and ingredients.

Aflatoxin B_1 is considered the most potent natural carcinogen known.

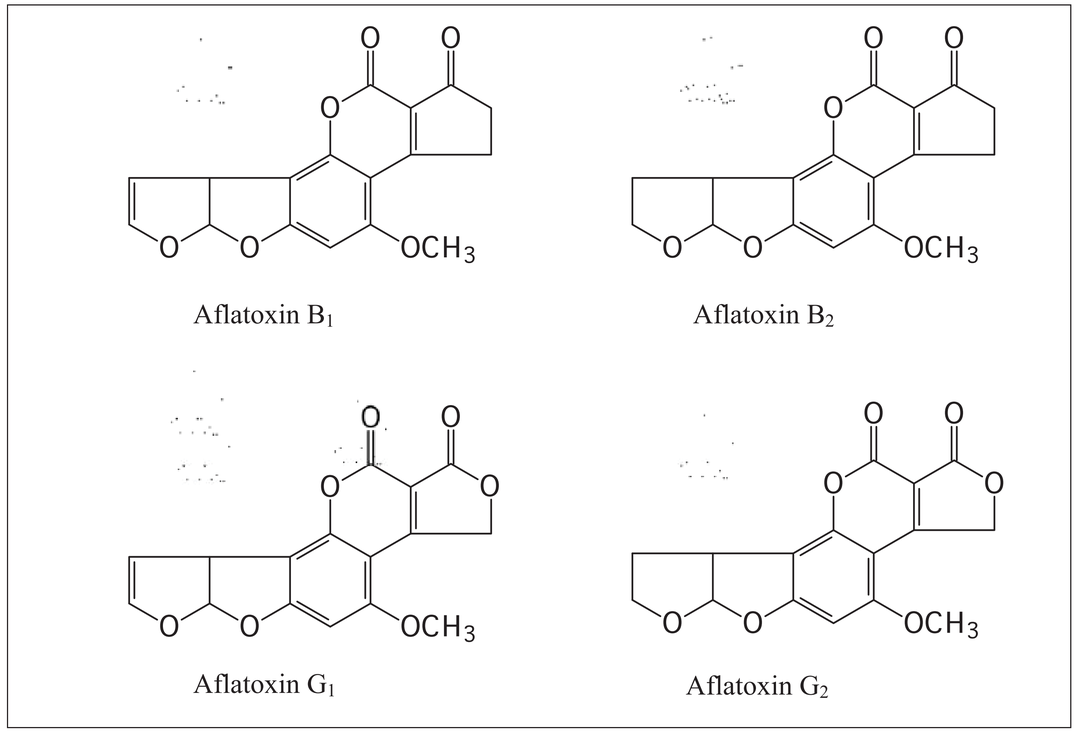
One of the first great discoveries related to toxins in peanut meal was the existence of a toxic substance that could be isolated by using high polarity organic solvents. This isolated extract emits fluorescence under the incidence of ultraviolet light; and its toxicity was proven by reproducing toxicological clinical symptoms by administrating the purified extract to healthy animals. Proof of fluorescence emission by the toxins, when submitted to the incidence of ultraviolet light, was a major scientific discovery because this characteristic was of vital importance for the development of analytical methodologies, such as Thin Layer Chromatography, to identify and quantify the newly discovered toxins. In 1962 these toxins were named aflatoxins because they were produced by fungi of the species *A. flavus*. Initially, only two toxins were identified through Thin Layer Chromatography. They were named aflatoxin B and aflatoxin G, consistent with the emission of blue or green light under the incidence of ultraviolet light. Soon afterwards, two other toxins emitting blue light were discovered. These were named aflatoxins B_1 and B_2. Two other substances emitting green light were also discovered; and named aflatoxins G_1 and G_2.

Research following the discovery, identification, and characterization of aflatoxins indicate that there are great differences in susceptibility, especially related to the species, age and sex of the animal. In the last three decades, aflatoxins and their toxicosis were widely studied in different animal species, as well as in humans. Aflatoxins constitute one of the most potent natural hepatocarcinogens known, responsible for various outbreaks of hepatocellular carcinoma in a variety of animal species.

Etiology

Aflatoxins are secondary fungal metabolites, and form part of the bifuranocumarin group. They are formed by heterocyclic molecules, with oxygen atoms and furano rings that differ amongst each other by small variations in their basic molecular structures. They are produced by *Aspergillus flavus* and *A. parasiticus*. Strains of *A. nomius* and *A. pseudotamarii* produce these mycotoxins in smaller quantities. More than twenty substances of aflatoxins are known, nevertheless, the most common in feed are aflatoxins B_1, B_2, G_1, and G_2 (Figure 3). In the majority of isolated fungi, aflatoxin B_1 is the most frequently produced, along with B_2, which is produced in large quantities by *A. flavus*; whereas *A. parasiticus* produces all four in similar quantities.

Figure 3 – Chemical structure of the four naturally occurring aflatoxins.



Other aflatoxins have already been isolated from animals' milk, meat, and urine, as is the case of aflatoxins M_1 and M_2, which are metabolites of aflatoxins B_1 and B_2, respectively. These mycotoxins are produced in the liver few hours after consuming contaminated feed.

Aflatoxins are starkly fluorescent to ultraviolet light (365 nm). Toxins B_1 and B_2 emit a fluorescent blue, hence the letter B. Aflatoxins G_1 and G_2, under the incidence of the same light wave, emit a green light, therefore deriving its letter from the word green. They are fairly soluble in moderately polar solvents such as chloroform, methanol, and acetonitrile. They have a low solubility in water and a melting point close to 269°C (Table 5). They are particularly sensitive to ultraviolet light, especially when they are dissolved in polar solutions. They are also destroyed by autoclave in the presence of ammonia and when treated with hypochlorite.

Table 5 – Physical-chemical characteristics of aflatoxins.

Aflatoxin	Chemical formula	Molecular mass	Melting point (°C)	Fluorescence emission (nm) and color
B_1	$C_{17}H_{12}O_6$	312	269	425 – blue
B_2	$C_{17}H_{14}O_6$	314	286-289	425 – blue
G_1	$C_{17}H_{12}O_7$	328	224-246	450 – green
G_2	$C_{17}H_{14}O_7$	330	237-240	450 – green
M_1	$C_{17}H_{12}O_7$	328	299	425 – violet-blue
M_2	$C_{17}H_{14}O_7$	330	293	violet
Aflatoxicol	$C_{17}H_{14}O_6$	314	230-234	425

Presence of aflatoxins in feed

The presence and magnitude of feed contamination with aflatoxins varies due to geographical and seasonal factors, as well as conditions in which agricultural products are cultivated, harvested, and stored. Crops produced in tropical and subtropical regions are more prone to contamination than are those in temperate regions, precisely because the optimal conditions for the production of toxins are predominant in regions of high humidity. Toxigenic fungi can invade and develop in a wide variety of substrates such as

Aflatoxin are principally produced with A_w >0.7, high air humidity, and temperatures between 24 and 35ºC.

cereals, seeds, and food sources during periods of growth, cultivation, harvest, transport, processing, and storage.

The presence of fungi, including those species which produce aflatoxins, does not necessarily signify a presence of toxins. Similarly, the presence of these toxins is not necessarily related to the presence of a producing fungus, since the toxins present great stability in grains, even after the deterioration of the producing fungus. The most important factors for the growth of toxigenic fungi of the *Aspergillus* genus and the production of aflatoxins in stored cereals are high relative air and substrate humidity, and the temperature at which they are stored. Relative air humidity of 80 to 85% with water activity (A_w) superior to 0.7 in cereals and temperatures between 24 and 35°C represent favorable condition for the production of aflatoxins. According to OMS (1983), the minimum, optimal, and maximum temperatures for the production of aflatoxins are 12, 27 and 40-42°C, respectively. Therefore, the contamination of agricultural products and feed by aflatoxins has a global scope. Its levels of occurrence are influenced above all by temperature, humidity, and substrate type. The interaction between these and other different factors will determine the amount of toxins produced.

The concentrations detected during various research and monitoring procedures indicate that levels showed significant variations in different regions. Although significant variations between different years in a determined region can be observed, these are probably influenced by climatic conditions, as was the case in the evaluations done in Brazil by LAMIC/UFSM over the last 17 years (Table 6).

According to studies done by the *Council for Agricultural Science and Technology* in 1989, approximately 25% of cereals harvested around the world are contaminated by mycotoxins. Corn, prominently used in feed, has an important role in contamination, for humans as well as for animals. In Brazil, various studies were done in order to detect the presence of aflatoxins in feed. The toxin was widely detected and concentrations found vary significantly.

Research carried out in São Paulo, Brazil in 1988 detected the presence of aflatoxins in 54% of peanut samples and 3.6% of corn samples. In other observations, in which hundreds of samples of corn from different regions of Brazil were analyzed, 12.3% presented different concentrations of aflatoxins.

Table 6 – Number of samples, percentage of positivity and average contamination by total aflatoxins in the last 17 years of routine analysis by LAMIC/UFSM.

Year	Number of samples	Average of positive samples (%)	Contamination average (µg/kg)	Percentage of contamination >10 (µg/kg)
1994	763	57.3	50.9	27.9
1995	785	40.9	20.8	18.5
1996	1713	35.0	33.9	18.7
1997	3051	33.2	13.3	11.0
1998	3536	21.4	28.8	9.4
1999	4376	40.6	8.8	11.2
2000	5535	31.3	9.5	11.3
2001	6199	45.3	14.6	11.1
2002	7769	62.2	11.1	15.5
2003	8341	48.3	11.2	13.6
2004	8682	31.4	4.8	4.8
2005	10792	36.0	3.9	5.4
2006	14798	41.5	12.2	12.8
2007	16848	37.2	5.4	7.4
2008	16507	27.5	3.1	5.4
2009	15304	31.0	7.0	7.7
2010	13867	23.2	8.3	3.3
Total	**138,875**	**37.84**	**14.56**	**11.47**

Three hundred eighty-two samples of corn used in animal feed were studied in 1992 in Southern Brazil. Aflatoxins were detected in 28.5% of these samples, with an average concentration of 1.9 µg/kg. After one year, 1,131 samples of grains and feed were analyzed, of which 44.3% were positive for mycotoxins, 89.9% of these were aflatoxins. In 1997 the prevalence of mycotoxins was studied in 5,335 samples of cereals and byproducts from the Southern Brazil. The authors found that 42.6% of the analyzed samples were positive for mycotoxins, with an average level of 34.5 µg/kg. The main feed ingredient analyzed was corn, 2,460 samples, which presented a positivity of 51.8%. Evaluations carried out by LAMIC/UFSM, during the last 17 years demonstrate that the occurrence of aflatoxins in corn presents a frequency of positivity of 47.2% in 63,907 samples analyzed routinely in said laboratory; with a contamination average of 10.0 µg/kg. The general positivity of 138,875

samples of all feed ingredients which were analyzed was 35.9%, with a contamination average of 8.3 µg/kg; slightly inferior than in corn because of lower averages of aflatoxin concentrations in other feed ingredients.

The simultaneous occurrence of aflatoxins with other mycotoxins was found in various agricultural products and feed ingredients. This is of significant importance because the toxic effects of these mycotoxins can be synergistic with those of aflatoxins. Although a large portion of the research reveals that the co-occurrence of aflatoxins with fumonisins, other mycotoxins such as trichothecenes, zearalenone and ochratoxin A, have been isolated in feed containing aflatoxins. In other evaluations conducted by LAMIC/UFSM, the co-occurrence of mycotoxins was confirmed. Table 7 illustrates the co-occurrence of aflatoxins with other important mycotoxins.

The co-occurrence of aflatoxin with other mycotoxins must always be considered.

Table 7 – Percentage of co-occurrence and concentrations of aflatoxins, zearalenone, fumonisins and deoxynivalenol in the last 17 years (1994 to 2010) in samples of cereals and feed routinely analyzed by LAMIC/UFSM.

Mycotoxins	Number of samples	Co-occurrence (%)	Average 1* (µg/kg)	Average 2* (µg/kg)	Average 3* (µg/kg)
Afla[1] + Zea[2]	93,358	9.2	7.4	59.2	---
Afla + FBs[3]	40,802	24.3	7.3	2084.8	---
Afla + Don[4]	23,869	8.3	3.0	109.0	---
Afla + Zea + FBs	30,427	10.4	7.3	111.0	2686.0
Afla+ Zea + Don	21,262	4.0	3.7	160.3	129.1

[1] Aflatoxins $B_1 + B_2 + G_1 + G_2$.

[2] Zearalenone.

[3] Fumonisins $B_1 + B_2$.

[4] Deoxynivalenol.

* Averages 1, 2, and 3 refer to the concentrations of mycotoxins that present both toxins, and is in accordance with the order cited in the column designated Mycotoxins.

The Brazilian Ministry of Health and The Ministry of Agriculture, Husbandry, and Supply established the maximum limit permitted of aflatoxin in food destined for human consumption at 20 µg/kg, comprising aflatoxins B_1, B_2, G_1 and G_2. The maximum limit of aflatoxins in feed accepted by the Brazilian Ministry of Agriculture, Husbandry, and Supply is presently set at 50 µg/kg. Animals intoxicated with aflatoxins normally store and eliminate the products of their biotransformation. Therefore, aflatoxins and products of their biotransformation can be isolated in these foods and derivatives such as meat, milk, cold meats, cheeses, and yogurts.

Toxicity of aflatoxins

Of the four main mycotoxins of the group, aflatoxin B_1 is the most toxic; the liver being its principal target organ. When compared to the magnitude of the toxicity of aflatoxin B_1, that of aflatoxins G_2, B_2, and G_1 are of 10, 20, and 50% respectively. Besides being hepatotoxic, aflatoxins are also highly mutagenic, carcinogenic, and possibly teratogenic in animals. Due to their high toxicity, aflatoxins are considered Class 1 human carcinogens by the *International Agency for Research on Cancer* (IARC). They present DL_{50} between 1 and 50 mg/kg for the majority of productive animal species, nevertheless, they are especially toxic for swine, presenting a DL_{50} of 0.62 mg/kg (Table 8). Aflatoxins M_1 and M_2 are products of aflatoxins' B_1 and B_2 biotransformation, respectively. These products are especially important in suckling pigs because they are eliminated through the milk of intoxicated animals.

The clinical manifestations of aflatoxicosis can be acute and chronic. Chronic illness occurs when moderate to high doses of mycotoxins are consumed, and clinical signs become evident within hours to a week after the initial consumption of the contaminated feed. In those cases, clinical signs and specific clinical pathological symptoms can appear, depending on the susceptibility of the animal species. The most frequent lesions are related to hepatitis, hemorrhage, nephritis and enteritis, followed by death. Chronic mycotoxicosis occurs when there is a consumption of moderate and low doses. In these circumstances, when animals present clinical toxicological symptoms, the signs are discrete and difficult to diagnose, and therefore can extend for weeks and months. Animals present clinical symptoms characterized by a reduction of reproductive efficiency, and deterioration of feed conversion, growth rate, and weight gain. These symptoms are detected through careful observations by experienced growers or by a permanent program of monitoring and analyzing mycotoxins in feed.

Clinical signs of chronic aflatoxicosis can be confused with a nutritional deficiency and/or other illnesses. Affected animals can also exhibit other illnesses as a result of decreased immunity.

In most cases, young animals are the most sensitive to aflatoxicosis, and a difference in susceptibility between the sexes can be observed. Generally, females are more resistant than males. This has already been scientifically proven in rodents. The difference in susceptibility in animals of the same sex in a given lot is easily detected by the uneven growth of the animals, which tends to increase with the increase in doses or intoxication period. The lack of uniformity of the lots is also severely affected by the large variety in individual susceptibility in animals of the same species.

Aflatoxicosis causes lack of uniformity in intoxicated animals of the same lots.

Table 8 – Values of lethal dose 50 (DL_{50}) of aflatoxin B_1 in different species.

Species	DL_{50} (mg/kg body weight)
Rabbit	0.3-0.5
Duckling	0.34-0.56
Cat	0.55
Swine	0.62
Rainbow trout	0.81 (intraperitoneal)
Canine	1.0
Guinea Pig	1.4-2.0
Sheep	2.0
Monkey	2.2
Chicken	6.5-16.5
Mouse	9.0
Hamster	10.2
Rat (male)	7.2
Rat (female)	17.9

Patterson, 1973 and Christensen *et al*. 1976.

Toxicokinetics of aflatoxin B_1.

Differences in susceptibility to the toxic effects of Aflatoxin B_1 in diverse species stem from variations in absorption, distribution, biotransformation, and elimination of this toxin. The presence of specific enzymes responsible for the biotransformation is considered of great importance in the susceptibility of various species to the acute or chronic effects of aflatoxins, because toxicity is exercised by the products of its biotransformation.

Absorption

Aflatoxins are absorbed by passing through a membrane barrier from an external medium to the interior of the organism, reaching the blood stream. As a result of the lipid composition of the cells that make up membranes, the liposolubility of the xenobiotic absorbed is of fundamental importance because it facilitates the passage through this membrane barrier. Therefore, aflatoxins, because of their high liposolubility, can easily be absorbed by the skin, lungs, and gastrointestinal tract.

According to the results of various researchers, the absorption of aflatoxins through cutaneous and respiratory channels is of great importance for professionals working in cereal mills. However, the easiest channel of absorption of aflatoxins occurs through the gastrointestinal tract, by ingesting contaminated feed. The absorption of aflatoxins is immediate after exposure, and seems to be completely absorbed when administered orally in combination with feed, especially when it is only moderately contaminated.

Distribution

The blood and lymph nodes distribute aflatoxins to different organs and tissues immediately after absorption. Most of the toxin is distributed to fat and soft tissue. Concentrations of aflatoxins occur in different organs, but the largest quantities can be detected in those organs responsible for biotransformation. Therefore, the liver and kidneys present the highest concentrations of aflatoxins and products of their biotransformation. Concentrations are usually inferior in other tissues and organs such as muscles, connective tissue, skin, pancreas, and lungs.

Biotransformation

The biotransformation of aflatoxins occurs in the liver. The principal hepatic enzymes are located in the soft endoplasmatic reticulum (microsomes), and in the soluble material (cytoplasma). Reactions are promoted by catalyst enzymes whose reactions are divided into two phases. The first phase consists of oxidation, reduction, and hydrolysis, making molecules more hydrophilic. During the second phase, the compounds initially produced are combined with endogenous substances (sulfates, glutathione, amino acids, and methyl and acyl groups), with the objective of facilitating removal (Figures 4 and 5).

Figure 4 – Diagram of the biotransformation of aflatoxin B_1 and its hydroxilated metabolites (AFM_1, AFQ_1 AFP_1 and AFB_{2a}), according to OMS 1983.

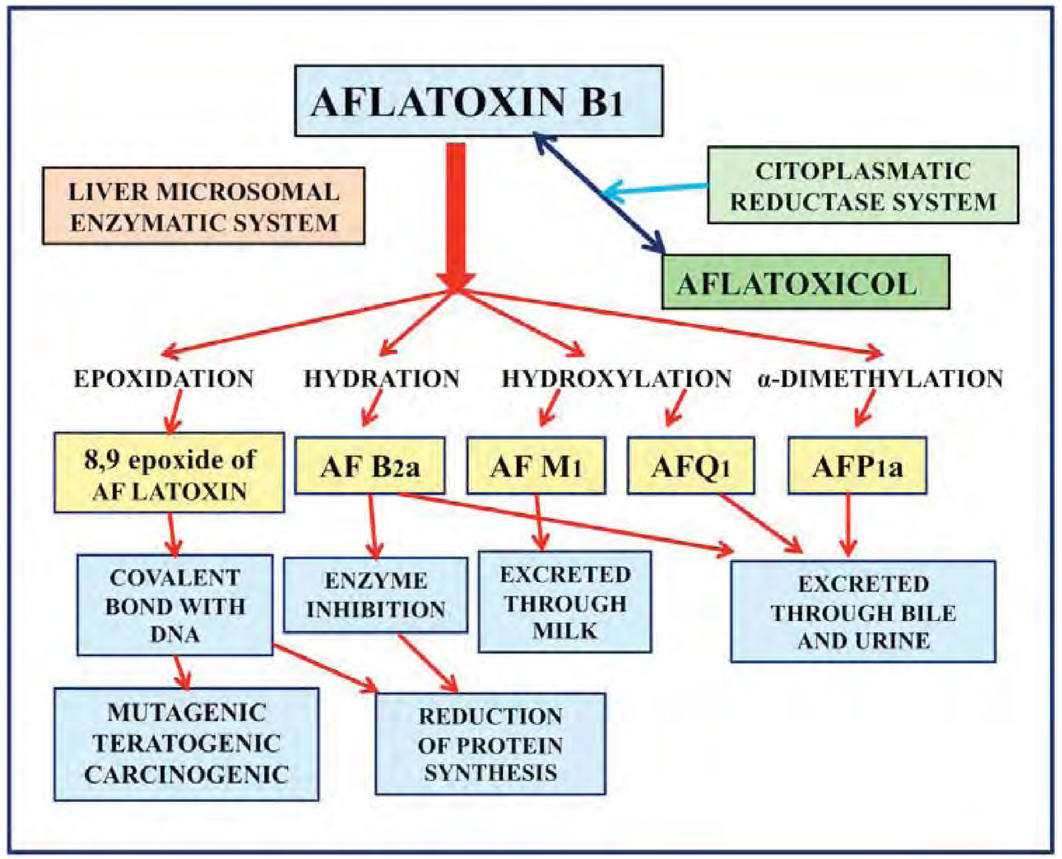
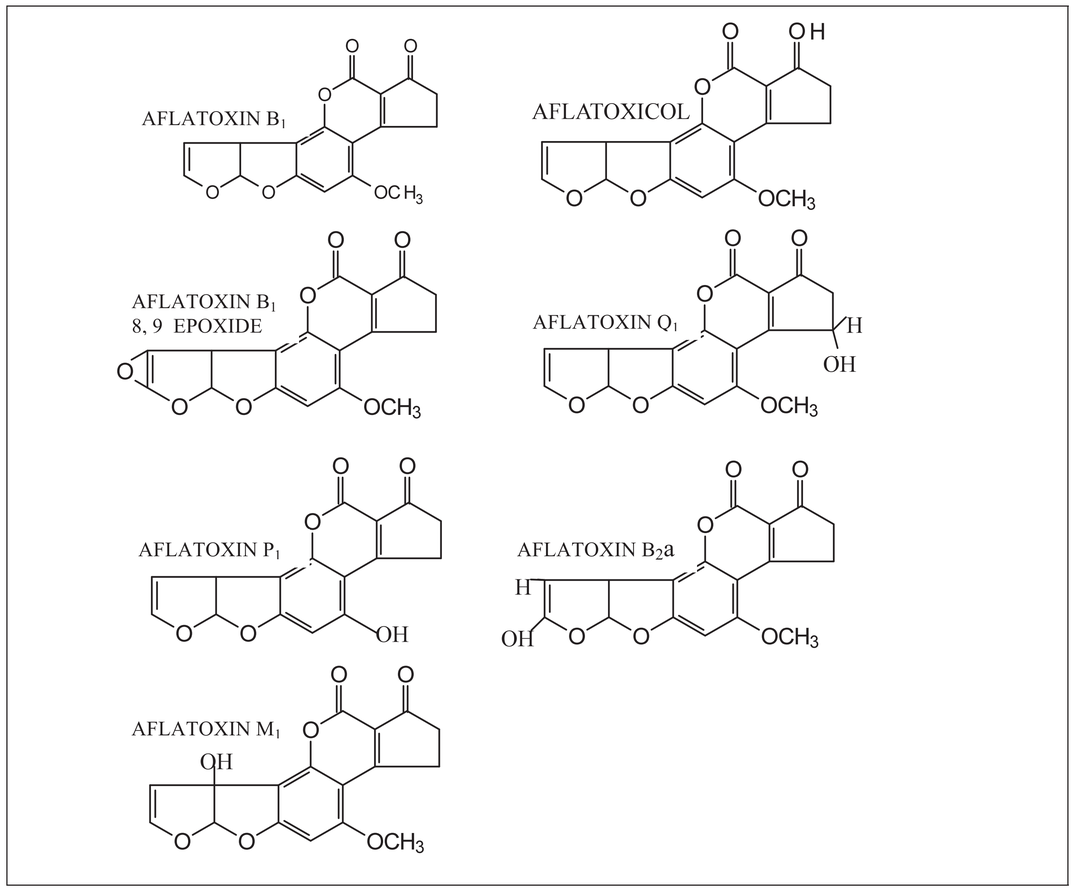


Figure 5 – Aflatoxin B_1 and its biotransformation products, according to Leeson *et al.* (1995).



The process consists of reversible and irreversible molecular alterations. Detoxification occurs by irreversible reactions whose biotransformation products are hydroxylated and hydrosoluble metabolites such as aflatoxins M_1, Q_1, P_1 and B_{2a}, which are generally less toxic that their precursors. Reversible detoxification consists in the formation of aflatoxicol, which will return to its original aflatoxin B_1 form by means of oxidation reactions that occur in the microsomal dehydrogenase.

The main activation reaction of aflatoxin B_1 is characterized by the epoxidation of the molecule, forming 8,9-epoxide of aflatoxin which has covalent bonding properties with nucleic acids, diminishing protein production, as well as causing mutagenic, teratogenic, and carcinogenic reactions. These reactions occur in the enzymatic cytochromic system P450

which is formed by the enzymes responsible for the principal oxidation reactions, especially epoxidation of covalent bonds, important in the activation of xenobiotics.

During the first phase of biotransformation, the enzymes of cytochrom P450 convert aflatoxin B_1 into different hydrosoluble products. The capacity of detoxification determines, to a large extent, the susceptibility of each species to become intoxicated by aflatoxins, and this is a result of the presence, in larger or smaller concentrations of enzyme components of cytochrome P450, which can produce more or less toxic derivatives.

Elimination

The capacity to detoxify aflatoxins by different mammals varies greatly between species. Generally, it is significantly influenced by factors such as sex, age, health state, and nutritional status. The initiation of detoxification generally occurs in the liver, coinciding with the biotransformation of aflatoxin B_1 into pharmacologically active byproducts. It is believed that this activation occurs in the nasal mucosa of swine, where higher quantities of products of biotransformation are usually found, instead of the liver. Thus, the concentration of aflatoxin B_1 derivatives in hepatic tissue of swine and the occurrence of hepatocellular carcinoma is less than the incidence of upper respiratory carcinoma. The initiation, advancement, and aggravation of respiratory illnesses in swine could be significantly elevated because of the high concentration of bioactive forms of aflatoxin in the respiratory mucosa cells. The detoxification of aflatoxin B_1-8,9-epoxide and aflatoxin M_1 in mammal tissue is achieved by the combination of glutathione catalyzed by glutathione-s-transferase enzyme. However, it is estimated that about 0.5% of aflatoxin B_1 that is ingested, will be eliminated through milk in the form of aflatoxin M_1. The elimination of aflatoxins through eggs was found in various studies done with laying hens. Aflatoxin B_1, or the products of its biotransformation (aflatoxin B_{2a}, M_1 and aflatoxicol) were distributed amongst the different parts of the eggs, reaching maximum concentrations 4-5 days following the initial administration of a constant dose of the toxin. Decline of the concentrations occurred during a similar period. Aflatoxins and the products of their biotransformation are principally eliminated through bile and feces, corresponding to approximately 60% of the ingested dose. The rest was eliminated, in similar proportions, through respiration, in the form of CO_2, and urine.

Aflatoxins can have a significant influence on respiratory diseases.

Residues

Aflatoxins can be quantified in the organs and tissues of different species such as bovines, swine, and poultry that have consumed feed contaminated with aflatoxins. The liver usually presents the highest concentrations. Aflatoxins B_1, B_2 and M_1 are also frequently isolated in other organs and muscular tissues, such as meat consumed by humans. However, the levels normally found in those tissues are low, as can be seen in tables 9 and 10.

Table 9 – Aflatoxicol and aflatoxin B_1 and M_1 residues in tissues of piglets intoxicated with a single dose (1 mg/kg live weight) contained in rice culture (B_1/G_1 = 4.3/1)

Hours after intoxication	**Tissue**	**Aflatoxicol** (µg/kg)	**Aflatoxin B_1** (µg/kg)	**Aflatoxin M_1** (µg/kg)
22	Kidney	4.47	23.6	11.2
22	Liver	1.68	36.5	6.0
24	Kidney	0.26	4.7	5.8
24	Liver	0.17	3.0	3.9
24	Muscle	0.06	2.9	1.3
72	Kidney	0.02	1.2	0.8
72	Liver	0.05	0.6	0.4
72	Muscle	0.01	0.3	0.1

Truckses *et al.* 1982

Table 10 – Quantified aflatoxin concentrations in organs of swine intoxicated for 35 days with different levels of aflatoxins in their diets.

		Experimental Groups	
Tissue	**Toxin**	**9 µg/kg of feed (90% B_1 and 10% B_2)**	**524 µg/kg of feed (90% B_1 and 10% B_2)**
Liver	M_1 µg/kg	0.071	1.479
	B_1 µg/kg	0.002	0.484
	B_2 µg/kg	0.000	0.053
Kidney	M_1 µg/kg	0.166	3.132
	B_1 µg/kg	0.027	0.681
	B_2 µg/kg	0.000	0.138
Muscle	M_1 µg/kg	0.000	0.206
	B_1 µg/kg	0.010	0.210
	B_2 µg/kg	0.001	0.027
Adipose Tissue	M_1 µg/kg	0.000	0.010
	B_1 µg/kg	0.000	0.030
	B_2 µg/kg	0.000	0.000

Beaver *et al*.1990.

Pathogenesis

Aflatoxins B_1, B_2, G_1 and G_2 have slightly different chemical structures which interact with different organic molecules of the intoxicated animal. Aflatoxin B_1 is the most prevalent and the most toxic of the group, therefore, more emphasis has been put on its study, and the majority of the noted biochemical effects refer specifically to this toxin.

The aflatoxin molecule is activated metabolically before producing its acute and chronic effects. The activation occurs in the microsomal system of the liver, mediated to a large extent by cytochrome P450. The activated metabolites are capable of interacting with macromolecules and organelles. The enzymatic hepatocyte system facilitates hydroxylation, dimethylation, reduction, and epoxidation that occur during the process of biotransformation. The reciprocal action between these types of activated molecules and the hepatic cells

apparently occurs in different sites. When it occurs in the nucleus, it inhibits RNA polymerase, DNA dependent, and the toxin bonds covalently to DNA, and in the exterior of the nuclear membrane, inhibits RNA synthesis by inhibiting RNA enzyme polymerization.

Aflatoxin increases the permeability of mitochondria and interrupts the transport of electrons, diminishing or completely inhibiting cellular respiration. Simultaneously, the lysosomal membrane increases its permeability, interfering in the transportation and exchange of nutrients and metabolites. The activation of the lysosomal enzymes and their intracellular extravasation is one of the highly toxic elements of aflatoxins, given their deleterious effects on cellular structures constitute a very harmful component in the pathological process. Yet, significant alterations occur in the endoplasmic reticulum which culminate with the degranulation of the ribosomes, inhibiting diverse metabolic functions such as protein and enzyme synthesis, and the synthesis of coagulation factor II and VII. The main metabolites formed by aflatoxins are aflatoxin M_1, aflatoxin Q_1, aflatoxin B_{2a}, aflatoxin B_1 2,3 epoxide, aflatoxicol, aflatoxicol M_1 and aflatoxicol H_1. Aflatoxin B_1-8,9 epoxide is the most toxic metabolite, and it is responsible for the alkylation of nucleic acids, and subsequently the activation of carcinogenic capacities.

The effects of aflatoxins on the different enzymatic systems, membranes, and organs compromise the lipids, carbohydrates, proteins, and nucleic acids metabolism. The interference in the metabolic process of lipids starts at the intestinal absorption level, which decreases by the interference in the excretion of lipases and bile. Furthermore, aflatoxin induces the formation of peroxides, starting from lipids, such as hydroxyl free radicals (OH^-), superoxide O_2^- and nitrogen peroxide in the liver, which have significant toxic effects on proteins, enzymes, and nucleic acids.

Aflatoxins decrease the absorption of nutrients.

Two types of interactions between aflatoxins and nucleic acids are known. One of these is the result of a weak, non-covalent and therefore reversible bond. The other, is a covalent, irreversible bond forming aflatoxin-DNA adducts.

The main carcinogenic effects are the result of the biotransformation of aflatoxin B_1, which is biologically inactive in its natural state. Aflatoxin B_1-8,9-epoxide in mammals is the active carcinogenic form. It possesses highly nucleophilic properties, with a high affinity to

establish bonds with nucleophilic sites of macromolecular components, establishing very strong (covalent) chemical bonds with diverse molecules such as proteins, RNA, and DNA. In this way, adducts which represent the initial toxic effects of aflatoxins, which can inclusively induce neoplasias, are formed.

Adducts are formed by the bonding of activated molecules (epoxides) with the basis of the DNA molecule, specifically in the N_7 position of codon 249 of the tumor suppressor gene. The points of DNA mutation can be formed by removing the bonding points formed by the adducts, or by the transvertion of the basis, which induce and promote the formation of cancer. Hydroxylated derivatives of aflatoxin B_1 have a significantly lower toxicity because they do not have the properties to establish covalent bonds and adducts with nucleic acids. The toxicity of aflatoxins depends on the formation of aflatoxin epoxides, for which it is necessary that carbons 8 and 9 of the aflatoxin molecule be unsaturated. Since aflatoxins B_2 and G_2 do not posses these unsaturated carbons, they are considered practically non-toxic compared to aflatoxins B_1 and G_1.

A large part of the toxic effects of aflatoxins occur in the hepatocytes, probably because they are the cells with the highest metabolic activity, as well as possessing high concentrations of cytochrome P450 enzymes, which are responsible for the production of the active compounds derived from aflatoxins. Knowledge of the carcinogenic effects of aflatoxins in experimental animals, and chronic exposure in humans has lead to the general consensus that aflatoxin is associated to the development of hepatocellular carcinoma, one of the most frequent forms of cancer in the world. Even though the liver is known as the main organ affected by aflatoxins, respiratory exposure to aflatoxins by means of dust inhalation, significantly increases the incidence of cancer in the respiratory tract, especially in the lungs of animals and humans.

Clinical signs

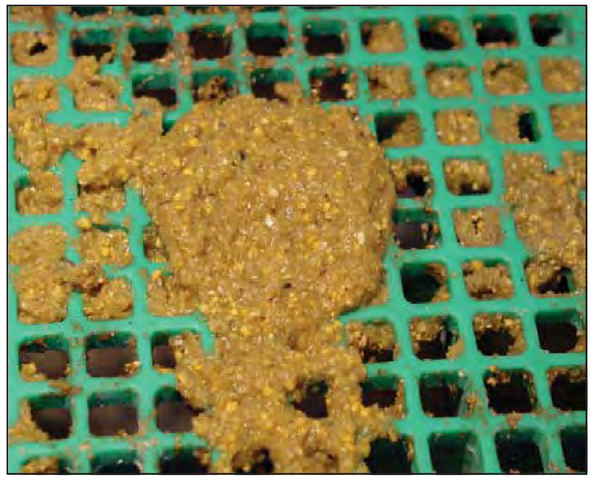
The clinical signs and the severity of aflatoxicosis can vary according to the age of the animals (fetuses and younger animals are most sensitive- Figure 6), type of aflatoxin (B_1 is the most toxic), diet composition (diets with high protein levels partially protect animals from the toxic effects), duration of exposure to the toxin, nutritional status, and, above all, on the aflatoxin concentration in feed. Thus, aflatoxicosis can occur in two clinical forms: acute

and chronic. Both are dependent on the dose and duration of exposure. Acute toxicosis is quickly recognized by hepatic lesions, clinically characterized by depression, anorexia, icterus, and hemorrhages. In super acute cases, the clinical signs are seen in approximately 6 hours and quickly result in death. In less serious cases, clinical signs begin to appear between 6 and 12 hours after ingestion, a rise in corporal temperature (40.0 to 41.1°C) can be detected, accompanied by muscular tremors and problems in motor coordination caused by hemorrhages that occur in ham musculature. Diarrhea is often present, demonstrating poorly digested feed, dark green in color and generally liquid (Figure 7).

The recuperation rate for swine intoxicated by aflatoxins is low.

Figure 6 – Abortion induced by aflatoxin intoxication. (Migliavaca & Prof. Driemeier)

Figure 7 – Paste-like feces with poorly digested feed particles resulting from aflatoxin intoxication



It is common to observe rapid involution of the mammary gland and an overall poor condition of lactating sows. After 24 hours, swine can present hemorrhagic and diphtheric diarrhea. Swine that surpass the clinical symptoms of acute aflatoxicosis, generally no longer recuperate lost weight or overall health. Rather, they remain thin with poor feed conversion, even after a long period following intoxication.

The importance of aflatoxins in swine production is not only due to the damages caused by the clinical symptoms of acute intoxication. The main economic losses are due to the ingestion of low concentrations of aflatoxins, which causes chronic intoxications and represent more than 90% of all cases. Hence, the main concern for producers should be subclinical intoxications because the level of aflatoxins found in feed is generally not enough to produce the perceivable clinical symptoms, but it is enough to significantly decrease productivity. Chronic aflatoxicosis occurs when toxins are ingested during prolonged periods in small doses and concentrations smaller than 1 mg/kg of feed. In these cases, it is common to see loss of appetite, lethargy, decrease in weight gain and general sick appearance. It is sometimes followed by diarrhea several weeks after the initial consumption of contaminated feed (Table 11). Yet, clinical signs are milder and present slow evolution. Scaly skin, curly and opaque hair, areas of red and purple coloring of the skin, lethargy, and depression can be observed. A decrease in feed consumption, decrease in weight gain, worsening of feed conversion, as well as a decrease in animal productivity can also be observed.

More than 90% of aflatoxin intoxications are chronic.

At the final stages of toxicosis, there are frequent signs of ataxia, icterus, and sometimes convulsions. Because symptoms are often not perceived until later signs appear, and because of the difficulty of clinical diagnosis, the economic consequences can be particularly severe. Furthermore, chronic intoxication is often inadequately diagnosed; often confused with problems in management, nutritional deficiencies, genetic quality of the animals, or confused with opportunistic infections which plague the animals as a result of the chronic aflatoxicosis itself. Because of the heightened difference in sensibility to aflatoxins that exists in different animals, even when the lot is made up of animals of the same descendancy, lack of uniformity in development is inevitable, forming the so called “uneven herd” in terms of weight and size of the animals (Figure 8 and 9).

Figure 8 – Comparison of the development of two pigs. 0 ppm of aflatoxin (control) and 1 ppm of aflatoxin in feed for 21 days.

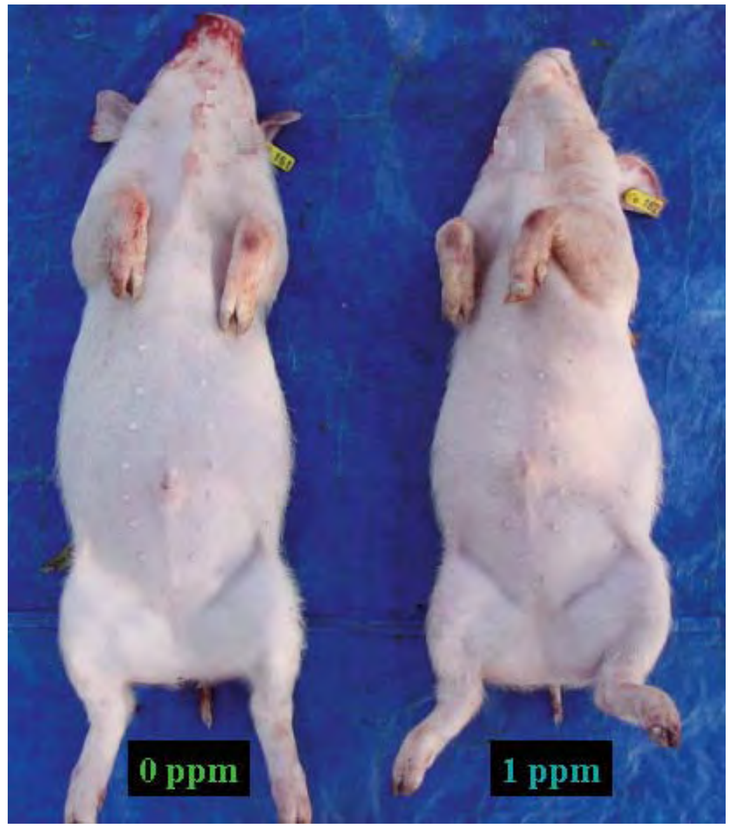


Figure 9 – Lack of uniformity in the development of pigs after an aflatoxicosis outbreak.

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Besides provoking hepatotoxicosis with the proliferation of bile ducts, hepatic steatosis, and hemorrhages, aflatoxicosis produces immunosuppression, is highly mutagenic, teratogenic, and carcinogenic. In the most severe cases, when the illness reaches the terminal stages, there are pathological alterations characterized by icterus in the mucosa membranes and in the subcutaneous tissues, as well as diffused hemorrhages, especially in the kidneys and gastrointestinal tract. Intra-abdominal hemorrhaging and/or hemorrhaging in the ribcage can also occur.

Table 11 – Clinical symptoms produced by different levels of aflatoxins in adult pig feed.

Levels (µg/kg)	Clinical symptoms
20-150	Decrease in productivity and lack of uniformity.
150-300	Decrease in weight gain and increase in susceptibility to diseases.
200	Poorer feed efficiency.
400-500	Organ lesions and variations in levels of serum proteins.
810	Increased mortality.
450-1,500	Reproductive failures.
2,000	Mortality occurs within 3 days.

Although the literature suggests that chronic intoxications in swine occur with concentrations of toxins less than 1 mg/kg, there is a consensus amongst scientists, which is confirmed by recent findings, that ingesting levels as low as 10 µg of total aflatoxins per kg of feed can cause significant decrease in swine productivity. These effects can be clearly seen in decreased feed consumption and consequently a decrease in weight gain, as well as a decrease in the average number of pigs born per sow per year.

Clinical Pathology

Hepatic lesions, besides producing a decrease in protein synthesis, increase the activity of aspartate aminotransferase, gamma-glutamyl transferase, and alkaline phosphatase enzymes. The increase in activity of hepatic enzymes such as arginase, sorbitol

dehydrogenase, and alanine aminotransferase is an indication of hepatic lesion in different species, therefore being valuable in diagnosing aflatoxicosis. Sorbitol dehydrogenase, an enzyme of the cellular cytoplasm, is indicative of hepatic lesion. However it is not commonly used in diagnostic exams because it is only present for a short period in the blood stream. Alanine aminotransferase is the preferred diagnostic tool, but is an enzyme that is found in low quantities in swine, thus being difficult to detect. Therefore, some researchers did not find significant alterations of this enzyme in swine intoxicated by aflatoxins. The serum values of aspartate aminotransferase are more sensitive to intoxication by aflatoxins, showing a significant increase of activity approximately 14 days after the initial intoxication.

Alkaline phosphatase is an enzyme that is present in various types of tissues, but it is only important in diagnosing alterations of the hepatic and bone tissues. With the exception of growing animals or those that have bone lesions, the elevation of this enzyme has a hepatobiliary origin. Cholestasis (blocked biliar flux) brings on elevated serum levels of alkaline phosphatase. Various researchers found significant increases in enzymatic activity after pigs were intoxicated with aflatoxins at rates of at least 450 µg/kg in feed.

Gamma-glutamyl transferase is an enzyme associated with the membrane of various tissues, therefore, it is an important indicator in the process leading to cholestasis. It can be highly concentrated on the edges of epithelial cells of the kidney and bile ducts because increased serum concentrations always originate in hepatobiliary tissues. Elevations of the enzyme were not observed in pigs intoxicated with 1.2 mg of aflatoxin/kg of feed for a period of 72 hours. This can be explained by the fact that gamma-glutamyl transferase only increases significantly following ciliary necrosis, severe cholestasis, and/or hyperplasic nodules. Researchers found an increase in gamma-glutamyl transferase in pigs intoxicated with 4 mg of aflatoxins/kg of feed given *ad libitum* for a length of 14 days. Concentration of 3 mg of aflatoxins/kg of feed only increased serum activity to a significant level after 28 days.

Pyruvic glutamic transaminase enzyme always presents significant increases, doubling or tripling its activity after animals are intoxicated by aflatoxins. Oxaloacetic glutamic transaminase enzymes and ornithine carbonyl transaminase are not good indicators in diagnosing aflatoxicosis outbreaks because they present a rapid increase in activity, generally between 12 and 24 hours, and their decrease is equally fast.

It is normally recommended that a combination of enzymes be used to diagnose aflatoxicosis in swine. Therefore, alkaline phosphatase (high sensitivity), aspartate aminotrasferase (moderate sensitivity), and alanine aminotrasferase (low sensitivity) are recommended.

The prothrombin time, approximately 20 seconds in swine, increases to approximately 60 seconds between 12 and 24 hours after intoxication. A decrease in plasmatic proteins always occurs after a period of intoxication due to hepatic damage.

The prothrombin time could increase when there is intoxication by aflatoxins.

Macroscopic Lesions

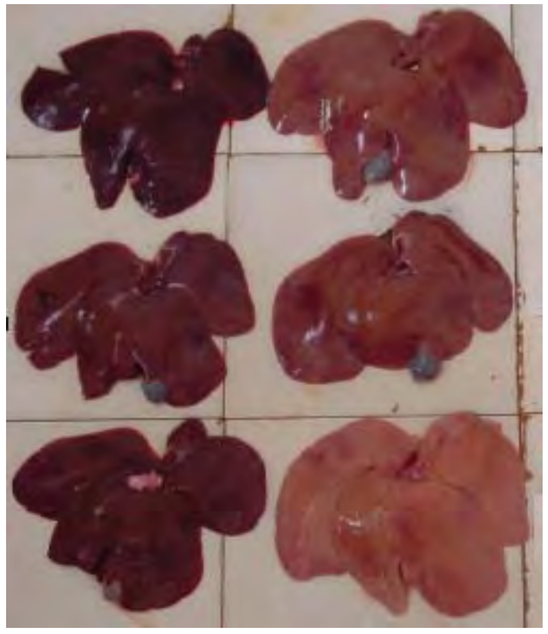
Death generally occurs in a period of 3 days when there is acute aflatoxicosis. Immediate changes evident in clinical exams are pale mucosa, and a bulging abdomen caused by the decrease of feed intake, dehydration, and gastric deflation caused by diarrhea. The presence of liquid feces can normally be found in the perineal area because poorly digested feed are eliminated in abundance, even while the animal is lying down dying.

The liver is always the most affected of all the organs because alterations start occurring from the moment of ingesting contaminated feed. After 6 hours of acute intoxication, the liver presents a pale-bronze color and appears as though it had been cooked. Twelve hours after intoxication, the liver presents hemorrhaging sites on the surface with diameters of approximately 1mm. Soon after, the liver becomes pale, edema become present, and the liver becomes friable. The gall bladder displays thickening of the walls, induced by edema with the formation of fluid in the submucosa, muscle, and serosa. The heart can exhibit areas of hemorrhaging, principally affecting the subepicardial and endocardial regions. Extensive enterocolitis can occur in the intestine, but it is most frequently present in the jejunum and the ileum, where it is possible to see disseminated hemorrhaging with free blood in the lumen. Generally, the cecum and the colon become hyperemic, with different quantities of blood on the outside.

A change in liver color is one of the first signs that an animal is intoxicated with aflatoxins.

Acute intoxications can lead to the formation of fluid accumulation in different cavities of various organs. These pools of fluids are most frequently found in the heart and the peritoneum. Jaundice in various organs and tissues can be present, especially when the intoxication becomes subacute or chronic. Jaundice of the carcass, associated with a swollen and yellow liver, are very strong indicators of intoxication (Figure 10).

Figure 10 – Hepatic changes in growing swine after 21 days of intoxication with aflatoxins. (Colum A) Normal livers (Colum B) Livers after intoxication with 1.0 mg of aflatoxins per kilogram of feed.



A **B**

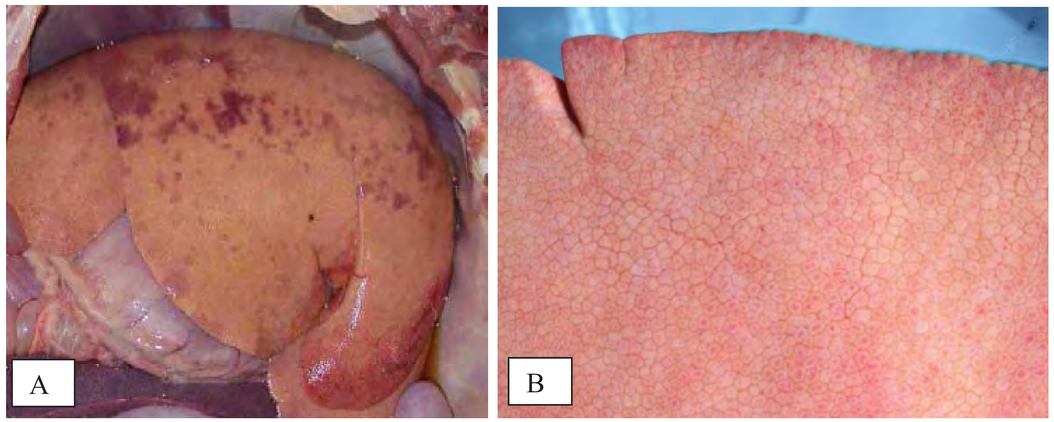
Chronic exposure to low doses of aflatoxin results in jaundice in the whole carcass. The liver can display a pale-yellowish appearance with very well defined lobes, hemorrhaging sites, especially in the parietal surface (Figure 11), and has different levels of fibrosis and cirrhosis. In the case of cirrhosis, a pale liquid (straw yellow) can be found in the abdominal and thoracic cavities, and accumulation of the liquid in the lungs leads to pulmonary edema.

Aflatoxicosis = jaundice + distorted liver.

In these conditions, the gall bladder generally appears edematous, an increase in size, with thicker content than usually found in healthy animals. Jaundice tends to be generalized, accompanied by yellowish coloration of body fluids, which can be located in various areas, even reaching the thoracic and abdominal cavities where the fluids concentrate in the spiral colon. There is a tendency of yellow liquid to accumulate in the pericardial sac of the thoracic cavity. There is also a decrease in coagulation time, therefore, it is possible to see an accumulation of blood in cavities and mucosa, other than hemorrhages in muscular tissue.

Aflatoxins levels lower than 10 ppb in feed are considered safe.

Figure 11 – Hepatic lesions caused by aflatoxicosis in pigs. (A) Pale-yellowish quality with very well defined lobes and hemorrhaging sites. (B) Red and yellow areas, giving a nutmeg appearance (Migliavaca and Prof. Driemeier).



The kidneys generally become swollen or hypertrophied and pale in color, depending on the degree of intoxication. Some researchers indicate the possibility of finding formation of areas of consolidation in the pulmonary parenchyma. DL_{50} of aflatoxins is very low (0.62 mg/kg) in pigs, and 50 μg/kg (ppb) of feed is considered a safe dose. However, because of the difficulty of sampling feed correctly, this level of toxin is regarded as being very high. Lower levels continue to be important in inducing immunosuppression, which can sometimes lead to clinical symptoms which are difficult to correlate to aflatoxin intoxication.

Microscopic Lesions

The principal toxic effects of aflatoxicosis are observed in the liver. Acute intoxications produce evident alterations, such as disruption of cell cords and granular cytoplasm with vacuoles, few hours after intoxication. Then, there is hypertrophy of cells leading to stenosis of the sinusoids and the appearance of necrotic foci randomly distributed in the parenchyma. During this evolutionary stage of lesions, compromised nuclei are not very evident. Notwithstanding, after 12 hours, necrotic foci with diameters greater than 100 μm can abound when there is a moderate to severe intoxication. After 24 hours, changes are even more evident: there is evident hepatocyte necrosis, hemorrhaging in the perisinusoidal space (Disse's space), and, above all, centrilobular congestion. Other changes, such as cellular infiltration in the centrilobular region, as well as edema, and biliary stasis associated to hyperplasia and the proliferation of bile ducts, mainly observed in subacute and chronic toxicosis, can also be present. With the chronicity of aflatoxicosis, fatty infiltration, accumulation of glycogen in hepatocytes and fibrosis, and centrilobular necrosis occurs.

Intestines are almost always affected. Enteritis can be acute and there can be hemorrhaging with necrosis of the villi. In the most severe cases, necrotic debris from the intestinal mucosa can be found in the intestinal lumen. The lamina propia and submucosa normally present high concentrations of lymphocytes, granulocytes, pasmocytes, and macrophages.

The presence of carcinomatous lesions are frequently observed in adult animals that consume feed contaminated with low concentrations of aflatoxins, starting at 10 μg/kg of feed for periods of approximately 24 months. Epidermoid and liver carcinomas exhibit metastases in surrounding tissues such as omentum and hepatic lymph nodes. However, the most frequently observed microscopic changes in the liver are diffused centrilobular necrosis, fat degeneration, and different levels of hemorrhages. In subacute cases, cellular necrosis of the hepatic parenchyma is not as prominent, but there is marked proliferation of bile ducts, fibroses, and conjunctive tissue in the interior and between hepatic lobes, which creates something like a post-necrotic cirrhosis.

Aflatoxin effects on the immune system

The immunosuppressant effects of aflatoxins have been demonstrated in laboratory and domestic animals, especially poultry. Although there is a consensus concerning immunotoxicity, its mechanism is still not completely clear. The effects that mycotoxicosis has on the complement, interferon and concentration of serum proteins are the result of hepatic damages and decrease of protein production. Other than compromising the formation of interferon and complement, it is known that aflatoxins decrease phagocytic capacity of macrophages and the migration of leukocytes and lymphocytes. They also cause aplasia of the thymus and a decrease in weight of the bursa of Fabricius in poultry, thus, principally affecting immunity. Aflatoxin B_1 principally affects T lymphocytes, including T auxiliary cells, and T suppressant cells.

Aflatoxins affect the swine immune system.

The effects of aflatoxins on immunoglobulin are also not entirely clear. Various studies indicate a decrease in IgA and IgG levels of animals intoxicated with feed containing more than 500 µg/kg of aflatoxins. However, other studies concluded that an increase in immunoglobulin concentration such as IgG can occur, and they attribute this to the inability of the damaged liver to remove the antibodies produced in the gastrointestinal tract.

The clinical effects of aflatoxins in swine can also be detected by the decrease of immunity and increase of secondary diseases in swine severely affected by aflatoxicosis. Various researchers found the co-occurrence of clinical outbreaks of aflatoxicosis with an immediate increase in septicemia infections caused by different bacteria, such as: *Salmonella*, *Pasteurella* and *Erysipelothrix*. Failure or decrease in the effectiveness of vaccines in different species has been frequently related to the presence of aflatoxins in feed, including clinical manifestations of aflatoxicosis in the animals.

Failures of vaccines can be attributed to aflatoxicosis.

The severity of aflatoxin effects on the immune system does not only depend on the levels of aflatoxins present in the feed consumed by pigs, but also on the nutritional status and general health of the animals. The presence of other illnesses or the ingestion of other toxins can exert an interactive influence on the immunological response of the intoxicated animals. Several researchers have demonstrated that a healthy diet decreases the quantity of toxic

residue in the organs and it significantly improves the function of different body organs. An increase of protein and vitamins in diets, particularly vitamins B_{12} and K, improve the animals' resistance against the effects of intoxication. Vitamin K, particularly, decreased protombine time and prevented hemorrhages. Lipotropic agents such as methionine have the important function of improving metabolism of fatty acids in the liver, reducing the severity of fatty degeneration.

Diagnosing Aflatoxicosis

The clinical symptoms of acute aflatoxicosis are easy to diagnose. The introduction of a new batch of feed, often with altered macroscopic characteristics, has been historically associated with the appearance of symptoms. Clinical symptoms as well as anatomical and pathological findings after changing feed, suggest intoxication by aflatoxins. The greatest difficulty in diagnosing aflatoxicosis is presented by the period of time in which clinical symptoms appear which can take two or more weeks, considering the concentrations usually present in commercial feed.

In hematological evaluations, there appears to be an increase in the activities of alkaline phosphatase, aspartate aminotransferase and oxaloacetic transaminase.

Chronic intoxications tend to go unnoticed in clinical evaluations of animals. In these cases, there is a loss of productivity that becomes significant when assessing the productivity of the farm.

The safest assessment used to diagnose aflatoxicosis consists in identifying aflatoxin present in feed using chromatographic methods such as Thin Layer Chromatography (TLC) and High Performance Liquid Chromatography (HPLC), and mass spectrometer such as GC/MS, HPLC/MS e HPLC/MS/MS, which are more efficient. Immunological methods, such as ELISA kits, have shortcomings; therefore, they are only used as semi-quantitative methods. The sample being evaluated should be representative of the batch, and must follow the technical procedures regarding the method of sampling collection.

Prophylaxis and Treatment

The treatment of mycotoxicosis represents one of the greatest challenges for clinical veterinary. It is therefore very important that this problem is solved by intervening in multiple processes. Considering the fact that mycotoxins are natural occurring substances in practically all food sources; they do not present immune response, and their toxic and economic effects emerge only after a determined period of ingestion (which could be a few days or hours in acute cases or weeks in chronic cases) many alternative treatments should be considered. Measures related to inhibiting and decreasing fungal development and subsequent formation of mycotoxins during farming, as well as in storage, deserve special attention. Preventing the formation of mycotoxins should always be one of the principal measures adopted. Storing feed where there is excess humidity, in silos that are poorly sealed and therefore prone to leakage and infiltration, should be avoided. The use of organic acids can help to preserve feeds in conditions favorable for mold development.

Constant and continual monitoring of mycotoxins in feed production, including appropriate sampling techniques, is the most efficient technical option, and from an economic standpoint, most viable, especially for medium to large sized swine producers.

Knowing the concentrations and frequency of aflatoxins present in diets, allows producers to make a better decisions to efficiently use absorbents to prevent and control aflatoxicosis. Due to the high sensitivity of swine to the majority of mycotoxins, especially aflatoxins, absorbents should be used when more than 50% of samples are contaminated and there is a concentration higher than 10 μg/kg. This recommendation should be considered in relation to factors such as environmental conditions, age, or diet, given each particular case. Some of the absorbents evaluated in LAMIC/UFSM display the capacity of absorbing aflatoxins *in vitro* more than 90%, which should be the minimum criterion for the employment of a product in order to avoid or ameliorate the toxic effects of aflatoxins in pigs.

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3

ZEARALENONE

ZEARALENONE

Summary

Zearalenone is a non steroid estrogenic fungal metabolite, chemically describe as a phenolic resocyclic acid lactone. It has good thermal stability and low solubility in water, but it is highly soluble in organic solvents. It is produce by various fungal species of the *Fusarium* genus, including *F. culmorum, F. graminearum* and *F. crookwellense*. These species colonize cereals and tend to become particularly important during periods of high humidity, accompanied by mild temperatures. Thermal oscillation within low temperatures is favorable for the production of large quantities of this mycotoxin. Temperatures around 25°C are favorable for fungal growth. A decrease in temperature to approximately 10°C with humidity higher than 14% triggers a secondary metabolism responsible for zearalenone production. For this reason zearalenone is a natural contaminate of cereals such as wheat, barley, rice and particularly corn, in different countries around the world. When contaminated cereals or their byproducts are ingested by different animal species, the toxin can produce estrogenic effects. Among domestic animals, swine are the most sensitive, showing clinical symptoms of intoxication starting at 0.1 mg of toxin/kg of feed consumed. Bovines can present problems related to infertility starting at 14 mg of zearalenone/kg of ingested feed. When swine consume diets contaminated with zearalenone, clinical symptoms characterized by vulvovaginitis, birth of weak piglets and stillborns, and outbreaks of splayleg often occur. There is also an obvious decrease in conception rate accompanied by repeated heat.

Zearalenone intoxication is generally manifested when concentrations surpass 100 ppb.

History

The first reported case of hyperestrogenism syndrome in swine occurred in the USA in 1928. However, a correlation between cause and effect of the outbreak was never established. Starting in 1952, the syndrome was associated with the incidence of fungi of the *Fusarium* genus in the feed consumed by the affected animals.

After more than 10 years of research, it was established that a toxin produced by the fungi of the *Fusarium* genus caused the disease, and it was then named zearalenone or F-2

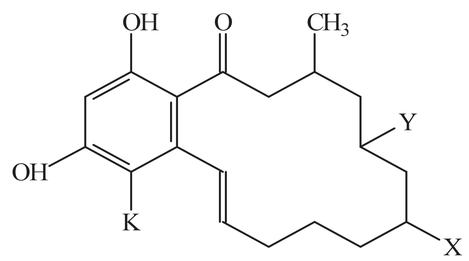
toxin. After this initial report, the syndrome was identified in various European and Asian countries, Australia, Canada, and Brazil in 1985, where the syndrome was diagnosed in various swine operations in the state of Rio Grande do Sul.

Etiology

Zearalenone, also known as F-2 toxin, gets its name from the producing fungus in its perfect state (sexual) *Giberela zeae* (*Fusarium graminearum* = *Fusarium roseum*). However, many other fungi such as *F.avenaceum, F.culmorum, F.cerealis, F.equiseti, F.moniliforme, F. semitectum, F.sporotrichiodes* and *F.oxysporum* can produce the toxin. Zearalenone is a phenolic resocyclic acid lactone (Figure 12), classified, according to its biosynthetic origin, as a nonoketide within the poliketides group. The majority of zearalenone can be produced by *Fusarium*, but only trans-α-zearalenol [6-(10-hydroxy-6-R-hydroxy-trans-1-undecenyl)-β-resocyclic-acid-lactone] occurs naturally in cereal grains, as a white crystal compound with a relative molecular weight of 318, a melting point of 164-165°C, and maximum absorption (and coefficient of absorption) in 236 nm (29,700), in 274 nm (13,909), and in 316 nm (6,020). Zearalenone is blue-greenish fluorescent when excited with long UV wavelength (360 nm), and a more intense green when excited with short UV wavelength (260 nm).

Like most mycotoxins, zearalenone also displays good thermal stability, thus undergoing little damage during the process, preparation and/or harvesting of feed ingredients. The level of toxin does not decrease when purified aliquot is submitted to 120°C for a period of 4 hours. The toxin displays stability when present in crushed corn at 150°C for a period of 44 hours, proving that zearalenone is only partially decomposed by heat during the processing of contaminated cereal. Crushing and extrusion processes carried out at 140ºC also entail significant degradation of zearalenone levels in cereals. The processing of contaminated rice can significantly decrease the presence of zearalenone, up to a 37% reduction in cooked rice. Approximately 60% of zearalenone remains during the manufacturing of breads made with contaminated ingredients, around 40-50% persists in the preparation of pasta, and 80% endure during the fabrication of cookies. The presence of zearalenone has been detected in beer produced in different countries, despite the fact that the fermentation process reduces levels of contamination by approximately 50%.

Figure 12 – Chemical structure of zearalenone and some derivatives.



X	Y	K	Name
=O	H	H	Zearalenone
=O	OH	H	8`-hydroxyzearalenone
=O	H	CHO	5-formilzearalenone
OH	OH	H	6`,8-dihydroxyzearalenone
OH	H	H	Zearalenol

Occurrence of zearalenone in feed and food

The presence of zearalenone in feed and feed ingredients is mostly limited to regions that have favorable climatic conditions, especially low temperatures (8 to 14^{o}C). There is a consensus that temperature oscillation, from low to moderate (8 to 24^{o}C), is essential for the formation of significant quantities of zearalenone. Other factors that have an important role in the production of zearalenone are high humidity of the substrate, for it is well known that fungi of the *Fusarium* genus require high humidity levels to develop. The highest quantity of toxins is produced when the percentage of water in cereal is greater than 22%, or when water activity is at approximately 0.85% or higher. The pH of the substrate is of little importance in the quantity of toxin produced, fluctuating between 3.5 and 7.5, without exhibiting significant differences in productive crops. It is common that cereals are transported over great distances, and that grains are imported from other regions and countries, therefore, clinical symptoms related to zearalenone have been observed in tropical climates.

Zearalenone occurs in a large part of agricultural products, grains, and feed, including corn and its byproducts, breakfast cereals, beer, wheat flour, breads, nuts, and a variety of other products consumed by animals and humans. Although natural contamination occurs in different grains, it is most prevalent in rice, oats, barley, corn, sorghum, wheat, and animal feed produced with these grains. Evaluation of zearalenone contamination indicated 20 to 37% positivity in Germany, 41.7% in England, 1% in Holland, 30% in Egypt, and 12.5% in South Africa. In Colombia 55.6% of sorghum, 6% of corn, 31% of rice bran, and 37.5% of swine feed samples were positive for zearalenone contamination.

In some countries, zearalenone was found in corn flour and breakfast cereals destined for human consumption, at levels of up to 70 µg/kg, correspondent to concentration levels about 400 times lower than those that cause acute effects in monkeys or rats under experimental conditions. In certain parts of Africa, substantially higher levels were occasionally found in beer and in macerated liquids prepared with contaminated corn and sorghum. In Zambia, the toxin was found in different raw materials and foods, such as cornflakes, beer, and corn at concentration levels which varied from 100 to 4600 µg/kg.

Concentrations in foods and feed vary greatly depending on climatic conditions. Zearalenone was found in 11 to 80% of wheat samples, and in 7 to 68% of barley samples collected in Southeast Germany in 1987, and from 1989 to 1993, respectively, with average annual indices of 3 to 180 µg/kg in wheat (highest concentrations of 8,000 µg/kg) and from 3 to 36 µg/kg in barley (highest concentrations of 310 µg/kg). After the 1995 harvest, year marked by heavy rains, 140 samples of wheat for human consumption were taken from all regions of Bulgaria and there was a 69% frequency of zearalenone contamination, with an average contamination of 17 µg/kg to up to 120 µg/kg in positive samples. The occurrence of zearalenone has been amply studied in France, presenting high indices of the toxin, but in low concentrations. In studies done in the USA, zearalenone was found in 6 of the 576 samples analyzed, with levels which varied from 450 to 800 µg/kg. In 1972, when climatic conditions were very favorable for *Fusarium* development, zearalenone was found in 17% of the 223 samples of corn, with concentrations that oscillated between 100 and 5,000 µg/kg.

In studies done in Buenos Aires and in the province of Santa Fe (Argentina) from 1983 to 1994, zearalenone was found in 30% of the 2,271 samples of corn collected, with an average concentration of 165 µg/kg (annual variation between 46 to 300 µg/kg) and a

maximum of 2,000 µg/kg. Concentrations in ecologically grown wheat and rye were higher than those grown with normal intensive cropping. The toxin was found in 40 of the 201 grain samples, with average concentrations of 24 µg/kg in wheat and 51 µg/kg in rye in crops that were planted and cultivated with alternative methods, and 6 µg/kg in wheat and 4 µg/kg in rye in conventional crops. The highest concentration of zearalenone was of 199 µg/kg, which was found in rye cultivated with alternative methods.

The occurrence of zearalenone in byproducts of grains and fermented grains has also been widely studied in different countries. The toxin is present in approximately 10% of fermented beverage samples, including beer, with concentrations between 8 and 53 µg/L.

Zearalenone is also frequently detected in silage made from grains and pasture, occurring principally in silage made from raw-materials after the process of maturation. Concentrations generally reach levels of up to 300 µg of toxin/kg of silage. The concurrent occurrence of zearalenone and its derivatives and other mycotoxins is very frequent because they are produced by the same molds that produce deoxynivalenol and nivalenol.

In Argentina, various studies showed contamination of zearalenone, varying from 1 to 30% of the total samples. In Brazil, 20% of 105,509 samples analyzed by LAMIC/UFSM, collected to monitor the feed industry, were contaminated. Although the average positivity is low, in certain periods of the year, the rate of positivity surpasses 45% of analyzed samples. In this laboratory, the average concentration of zearalenone found was 74.1 µg/kg, and the maximum level detected was 18.6 mg/kg.

Around 20% of feed samples analyzed in Brazil are contaminated with zearalenone.

Toxicokinetics of zearalenone

The rate of absorption of zearalenone in swine gastrointestinal tract has not been well established. It is believed, however, that around 80 to 85% of the toxin and/or products of its biotransformation are readily absorbed. Zearalenone can be biotransformed immediately after ingestion by intestinal microflora or by mucosa cells. Quickly following this, compounds are bond to endogenous substances and distributed through the bloodstream. It is also thought that there is enterohepatic circulation of the toxin because the toxin can be detected in feces for the

exact amount of time as it can be detected in the blood. The biotransformation of zearalenone occurs mainly in the liver. In mammals, it creates different steroisometric compounds, especially α and β-zearalenol, by the reduction of the ketone grouping in C_6. These compounds immediately bind to glucuronic acid. Another similarly structured compound is zeranol, which is synthetically produced from zearalenone and used to stimulate weight gain in animals. It differs from zearalenone because of the absence of a double bond between C_1 and C_2. The proportions between the products of biotransformation and zearalenone are different amongst different intoxicated animal species. It is thought that this is fundamental in explaining the difference in susceptibility of species to zearalenone intoxication.

Elimination

When a moderate dose of zearalenone is administered orally to swine, approximately 45% of the toxin and/or products of its biotransformation can be detected in urine after 48 hours. Approximately 22% of the toxin is retrieved from the feces, combining for a total recuperation of approximately 70% in 48 hours. However, a consistent pattern does not exist because other researchers have concluded that zearalenone or its biotransformation products can be detected up to 5 days after ingestion, estimating a half-life of 87 hours, when a moderate dose of zearalenone is administered orally.

Zearalenone can be secreted through the milk of milking cows after being fed with contaminated feed. The maximum quantified concentrations in cow milk, with an oral dose of 6,000 mg (equivalent to 12 mg/kg of live weight), were 6.1 µg of zearalenone/L, 4 µg of α-zearalenol/L, and 6.6 µg of β-zearalenol/L. Neither zearalenone, nor its biotransformation products were detected in milk of cows fed with 50 or 165 mg/kg of zearalenone (equivalent to 0.1 to 0.33 mg/kg of live weight) for 21 days.

Zearalenone residue in animal tissue

The amount of detectable zearalenone in tissue of intoxicated animals varies greatly, depending on the concentration of toxin in the ingested feed, time of exposure, and animal species. Few studies have been developed to determine the quantity of zearalenone and its biotransformation products in organs and tissues of intoxicated animals. However, some

results are available: in swine liver, 78 and 128 µg/kg were detected when the animals consumed feed contaminated with 40 mg of toxin/kg of feed for a period of four weeks. Chicken feed with 100 mg of zearalenone/kg of feed exhibited concentrations of up to 103 and 681 µg/kg in muscular tissue and liver, respectively. According to the recommendations by the International Committee of Risk Evaluation to Mycotoxin Exposure, the maximum levels of zearalenone residue in foods destined for human consumption are 10 µg/kg in the liver and 2 µg/kg in muscular tissue.

Because zearalenone can be transferred to beer by contaminated grains in various stages of the fermentation process, incidence of up to 58% and high concentrations of zearalenone were found in these products, including up to 2 mg/L in Nigeria, up to 53 mg/L in Switzerland, and up to 4.6 mg/L in Zambia. In similar studies, zearalenone and α or β-zearalenol were not found in Canadian, Korean, or European beers, with the exception of French beer that contained 100 µg/L. Zearalenone detection in products, such as beer, that undergo a fermentation process should be done by identifying the metabolites, especially α and β-zearalenol.

Pathological mechanisms of action of zearalenone

The capacity of zearalenone to unleash hyperestrogenism syndrome in swine has been known for many years. Contrary to other species, such as poultry and bovine, α-zearalenol is produced in greater quantities in swine. The reactions of zearalenone biotransformation to zearalenol are catalyzed by 3-α-hydroxysteroid-dehydrogenase enzyme (3-α-HSD) (Figure 13). This enzyme is also known for degrading 5-androstan-3,17-dione, which is a product of steroid metabolism. Swine appear to be the species most sensitive to zearalenone. Thus, uterotrophic activity is attributed to different zearalenone biotransformation products, especially α-zearalenol, which is produced in large quantities in this species.

Zearalenone and/or its metabolites interact with estrogenic receptors, and have significant effect on endometrial secretions, uterine protein synthesis, and uterine weight gain. Furthermore, it maintains the corpus luteum in the absence of gestation. Alterations caused by zearalenone in the reproductive tract occur as a consequence of increase in 17-β-estradiol, as well as the decrease in progesterone levels. For this reason, there are alterations in the uterine lumen, caused by changes in endometric secretion activity. Another important effect of

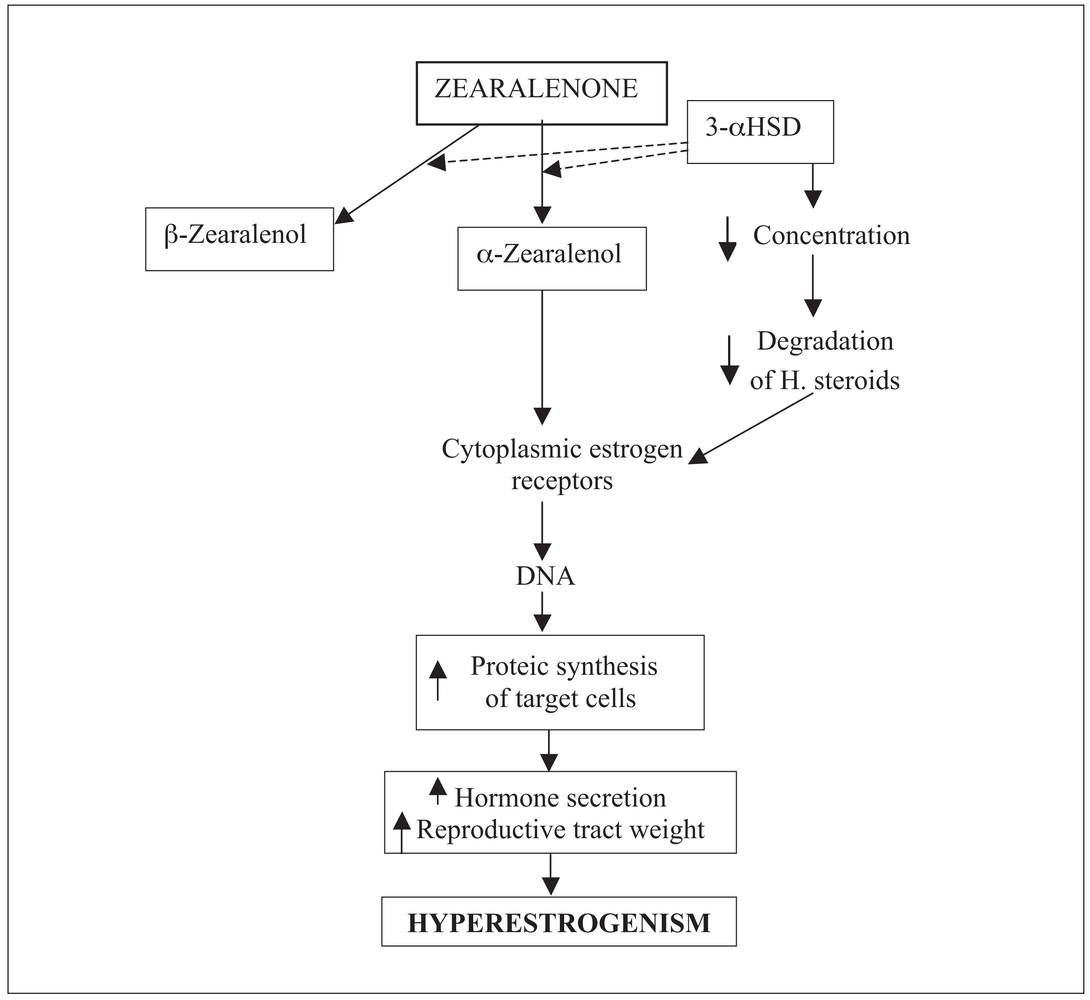
zearalenone occurs in the ovaries of young gilts, exhibiting a larger number of first stage atretic follicles. Consequently, there will be less ovulation, fewer number of embryos, and fewer number of piglets born.

Toxic effects are not observed in levels below 0.04 mg of toxin/kg of live weight/day, or below 1.0 mg of zearalenone/kg of contaminated feed. However, levels of 250 μg/kg of feed can reduce the number and weight of piglets born because sows are particularly sensitive to zearalenone during ovulation. There can eventually be abortions or mummified fetuses. International organizations, such as the Joint Expert Committee on Food Additives (JECFA) have established that tolerable levels of zearalenone should obey limits that do not have hormonal effects on swine, which is the most sensitive specie to zearalenone. According to the Committee, the maximum tolerable daily dose for human should be 0.02 μg/kg of live weight/day. However, more advanced studies are necessary in order to better evaluate the hormonal and possible toxic effects, including zearalenone's carcinogenicity. Nine countries belonging to the European Union have established zearalenone tolerance limits, mainly in grains, varying from zero to 1 mg of toxin/kg of feed. However, lower concentrations of zearalenone in naturally contaminated foods have already been responsible for causing hyperestrogenic syndrome in gilts, which displayed characteristic signs of the syndrome such as redness, hyperemia and edema of the vulva labia and mammary glands, and vesicular follicles and cysts in the ovaries.

Immunosuppressive effects of zearalenone

Various studies have been done with the intentions of evaluating the effects of zearalenone on the immune system of different animals. A large part of these studies were done on laboratory animals. Rodents intoxicated by zearalenone did not exhibit a significant increase in susceptibility to infections by *Listeria monocytogenes*. The animals did have alterations in leukocytes, lymphocytes, neutrophils, monocytes, or eosinophils.

Figure 13 – Mechanism of action of zearalenone.



Laboratory studies done on cell cultures indicate that zearalenone and its biotransformation products can cause a series of alterations in metabolic processes. Thus, exposure to 14 μg/mL inhibits DNA synthesis of lymphocytes by 50%. Other studies suggest that zearalenone and its biotransformation products can inhibit mitogenesis that causes a proliferation of lymphocytes B and T. The effects of zearalenone on swine immune system have yet to be fully studied. However, it is thought that its effects are irrelevant compared to the reproductive problems caused by this mycotoxin.

Apparently, zearalenone does not affect the immune system.

Clinical symptoms

Zearalenone and/or its biotransformation products are responsible for hyperestrogenism syndrome in swine, observed clinically as vulvovaginitis (Figure 14). The clinical symptoms associated with intoxication vary depending on the dose of toxin ingested and the age of the animal (Table 12). Symptoms tend to be more acute the younger the animal, as well as the higher the doses of toxin ingested; thus prepubertal gilts, approximately 4 months old, are generally the most severely affected.

Clinical symptoms similar to estrus are observed.

The initial onset of clinical symptoms varied greatly, appearing between 1 to 4 weeks after the ingestion of contaminated feed. It has been found that young animals are highly sensitive to diverse natural intoxications, in which they exhibit characteristic clinical symptoms. Adult animals given feed containing the same concentrations of zearalenone are not affected by that level of toxin.

Vulvovaginitis is the principal clinical symptom of zearalenone.

Sick animals display symptoms that mimic estrus, such as reddening and enlargement of the vulva labia, and edema of the mammary glands with a significant increase in volume. At times, animals can display unrest, resulting in fights or cannibalism.

Clinical manifestations normally persist for long periods, even after the contaminated feed has been substituted. Often, females' normal estrous cycle does not return. Vaginal or rectal prolapse (Figure 15), which occurs due to the relaxation of the sphincters, is not always present in contaminated herd. However, it can also be the only evident clinical symptom of zearalenone intoxication. In these cases, secondary infections generally occur, following lesions, and contaminations which occur on the mucosa surface of these organs.

Figure 14 – Zearalenone intoxication. (A) Control gilts (not intoxicated). (B) Gilts with vulvovaginitis after intoxicated with 2 mg of zearalenone/kg of feed for 24 days.

Figure 15 – Rectum prolapse in swine after being intoxicated by zearalenone.

Table 12 – Toxic effects of zearalenone in swine, according to age or stage, associated with the level of toxin in feed.

Stage	Zearalenone levels mg/kg	Clinical symptoms observed
Prepubertal gilts until first gestation	3-5	Increase in mammary gland size, edema of the vulva, increase of uterine and ovarian size. (1)
Sows	3-5	Increase of uterine size, uterine edema, corpus luteum retention, anestrous for more than 50 days. (1)
Sows	2.2	No clinical manifestations. (4)
Sows 15 days before farrowing	5	Weak newborn piglets, edema of the vulva of new born females, and splayleg. (2)
Swine 15 days before farrowing	3-4	57% of females are born with hyperestrogenism. (3)
Gilts, 20 to 30 kg	3.5-11.5	Vulvovaginitis and hypertrophia of the reproductive tract. (5)
Gilts, 64 kg	3	No change in feed consumption. (6)
Gilts, 64 kg	6-9	Decrease in daily feed intake and worsening feed conversion. Characteristic signs of hyperestrogenism. (6)
Boar	3-9	Did not affect libido. (7)
Boar	2-200	Did not affect libido or reproductive potential. (8)

(1) Etienne & Jemmali (1982); (2) Miller *et al*. (1973); (3) Lancorevic *et al*. (1977); (4) Shreeve *et al*. (1978); (5) Farnworth & Trenholn (1983); (6) Young *et al*. (1986); (7) Young e King (1986) e (8) Ruhr *et al*. (1983).

Edema of the mammary glands in sows often indicates gestation or labor. Intoxicated animals display an increase of vulva and mammary gland size the last two weeks of gestation, as well as milk in glands at least two weeks prior to labor.

Common symptoms in swine intoxicated by zearalenone are: infertility, pseudogestation, manifestation of permanent estrus, a decrease in the number of piglets born probably due to the decrease of follicle development, embryonic reabsorption, malformations and juvenile hyperestrogenism, which generally occurs in females born from intoxicated sows, a week after being born (Figure 16).

Figure 16 – New-born female piglet displaying clinical symptoms of vulvovaginitis.

Hyperestrogenism is characterized by reddening and increase in vulva size, and early development of mammary glands. According to studies done with intoxicated sows, the most critical period of embryonic survival seems to be between the seventh and tenth day of gestation. The probability of abortion in the last two months of gestation is unlikely in swine because estrogens are luteotropic in this species.

Infertility, pseudogestation, and decrease in number of piglets born are associated with zearalenone intoxication.

The refusal or decrease in feed intake is often associated with zearalenone intoxication. This is a result of the bad taste that develops in the feed which is produced by the development and intense fungal contamination, and because of the hepatic damage that this mycotoxin causes. Furthermore, the nutritional value of the grains diminishes, especially energy which is consumed by the fungal population.

Decrease in feed intake can be associated with intoxication.

One of the greatest consequences of zearalenone intoxication in swine is caused by abnormalities in the estrus cycle. Frequently, females exhibit all the typical signs of estrus, but they do not demonstrate sexual receptive behavior, refusing mount. The conception rate of the herd can decrease by up to 70% and pregnant sows generally have small litters. This

significantly influence the number of piglets born per sow per year, and the productivity of the farm. The vitality of piglets in the first 24 hours after birth is also severely affected. Consequently, suckling is done with greater difficulty, significantly increasing the number of deaths resulting from crushing and starvation, besides increasing the number of stillborns. The number of viable piglets in the first week of life is also affected. Many piglets display splayleg syndrome (Figure 17), as well as lack of coordination of the limbs.

Figure 17 – Piglet born from a zearalenone intoxicated sow, presenting splayleg syndrome.

Intoxicated piglets and females frequently present swollen and reddened vulva labia, and males and females present edema of the mammary glands various days after birth. These clinical symptoms can occur from contact of piglets with zearalenone and/or derivatives in the urine of the contaminated sows, even though in the majority of cases, the contamination occurs in gestation, through the sow's feed, or after birth, via contaminated milk.

Young boars are generally more affected than adults. The main toxicological clinical symptoms of these animals include growth of the mammary glands, edema of the foreskin that can hamper urination and cause testicular atrophy with impaired sperm quality and volume. A reduction in libido is frequently observed, even though, this aspect apparently does not interfere with the rate of conception when adult males naturally mount females.

The main effects are mostly related to reproductive animals, although growing and finishing pigs are also affected, reducing feed consumption by 100 to 150 g/day, causing significant losses in productivity.

Other mycotoxins are produced under the same climatic condition ideal for the production of zearalenone. Therefore, feed refusal and vomiting are often attributed to the simultaneous occurrence of zearalenone and deoxynivalenol.

Macroscopic Lesions

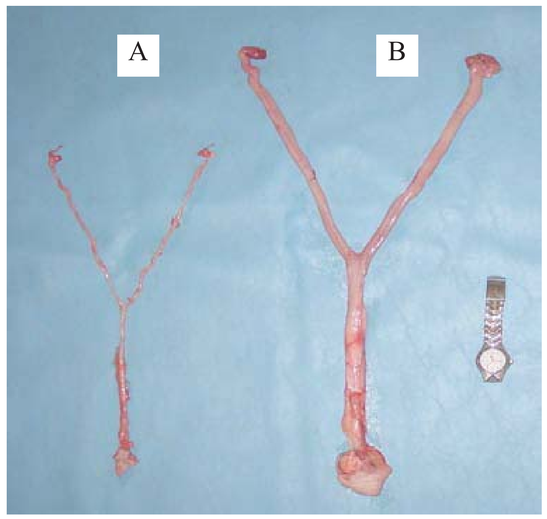
The main lesions caused by zearalenone intoxication in swine are physiological alterations in the reproductive tract which are characterized by the accumulation of liquids, forming interstitial edema, proliferation of cells, and metaplasia of squamous epithelial cells of the vagina and cervix. The vulva, vagina, cervix, and uterus appear swollen because of the combination of edema with cell hypertrophia, and hyperplasia of the components of its structures (Figure 18).

The uterotrophic effects of zearalenone bring about a significant enlargement of the uterus, particularly of the uterine horns.

The ovaries of prepubertal females present and increase in volume with a large number of small follicles; however, there is no evidence of formation of the corpus luteum.

Older females have large secondary follicle development and other follicles presented atresia. There is no evidence of ovulation because the ovaries did not present corpus luteum in the ovarian tissues of intoxicated female. Mammary glands and teats were edematous and exhibited a significant increase in size.

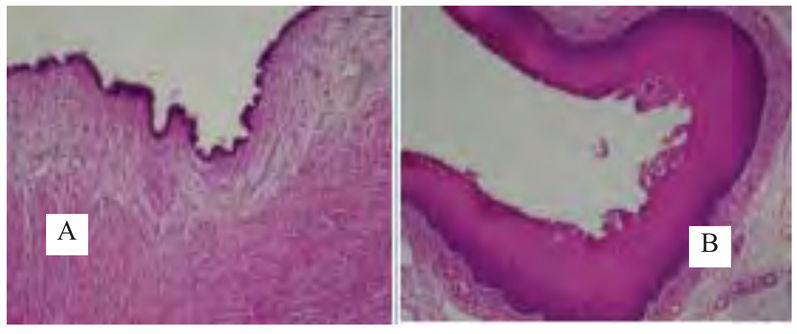
Figure 18 – Reproductive system of gilts showing alterations due to zearalenone intoxication. (A) Normal prepubertal gilt reproductive system. (B) Enlarged prepubertal gilt reproductive system after zearalenone intoxication 2 mg/kg of feed for 24 days.



Microscopic Lesions

Histological alterations in the reproductive tract of swine, caused by zearalenone, include edema of the uterine wall, and metaplasia of the epithelium of the cervix and vagina. Histologically, hypoplasic ovaries and follicular atresia were observed, with a proliferation of connective tissue. The uterine walls, cervix, vagina, and vulva were all thickened (Figure 19), and edema and proliferation in all layers of these organs due to a combination of hyperplasia and hypertrophy was evident.

Figure 19 – Microscopic alterations caused by zearalenone in the mucosa of the reproductive system of gilts. (A) Normal mucosa (control). (B) Thickened mucosa after intoxication with 2 mg of zearalenone/kg of feed for 24 days (Prof. Driemeier).



Diagnosis

The manifestation of characteristic clinical symptoms of this intoxication occurs in approximately two weeks. Therefore, preventive measures, especially diagnosing contaminated feed before ingestion, are of fundamental importance. Frequently changing feed is an indicative factor in the occurrence of the illness because the initial clinical symptoms appear after 1 to 3 weeks of using a new batch of feed.

Only macroscopically evaluating the raw material that makes up the animal's diet does not guarantee that the feed will have been correctly analyzed and free of zearalenone. Sampling procedures, analysis and management of zearalenone are the same as those described in this book for other mycotoxins.

Other than determining the presence of zearalenone in feed, plasma and feces can be analyzed. The extraction of zearalenone from feed is normally done with solvent combinations such as acetonitrile, ethyl acetate and methanol, which may be combined with water, depending on the type of matrix analyzed and the methodology used. The use of immunoaffinity columns for clarification of the samples and quantification using gas chromatography is an efficient method. Thin layer chromatography has been used often, and although some still value its use, it has been substituted by more efficient methods such as High Performance Liquid Chromatography (HPLC) combined with detecting ultraviolet or fluorescence. There are currently more sophisticated methods such as GC/ECD, GC/MS e HPLC/MS. ELISA methods are also used, but are not very sensitive, only quantifying one toxin at a time and are seriously deficient in repeating results. They are often used as semi-quantitative methods.

Treatment

Clinical symptoms, such as edema of the vulva and perinatal mortality, disappear within 3 to 4 weeks when contaminated feed is substituted with feed free of zearalenone.

Measurements to prevent the presence of this toxin in the feed should start with good agricultural and storage practices to avoid mold development. An efficient quality control system to evaluate contamination of feed ingredients can prevent the problem with relative

assurance. Redirecting zearalenone contaminated ingredients to more resistant species such as bovines and poultry, should be considered.

The use of natural or modified adsorbents deserves further scientific study, since some have presented promising results in field situations. The use of dietary hepatic protectors such as methionine and choline has had good results, especially in restoring the appetite of intoxicated animals.

Levels of this mycotoxin should not surpass 10 μg/kg of feed, which in practice means that diets destined for swine should be free of zearalenone.

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4

FUMONISINS

FUMONISINS

Summary

Fumonisin are produced by the secondary metabolism of various toxigenic fungi of the *Fusarium* and *Alternaria* genera, with *Fusarium moniliforme* recognized as the largest producer of this mycotoxin. Approximately two dozen fumonisins are known. However, only fumonisins B_1 (FB_1), B_2 (FB_2) and B_3 (FB_3) are toxicologically significant. They occur in a variety of grains, especially corn, where they display concentrations that generally induce subclinical intoxications in different species. These toxins inhibit the acetyl transferase enzyme, which is responsible for the transformation of sphingolipids. Consequently, there is a buildup of sphinganine and sphingosine in the blood stream. Equine are the most sensitive of domestic species to FB_1, manifesting clinical symptoms that include injury to the nervous system and leukocephalomalacia (ELEM). Swine are also highly sensitive to fumonisins, which can cause pulmonary edemas, among other clinical symptoms. The characteristic clinical symptoms of fumonisin toxicosis in swine include difficulty breathing and cyanosis due to pulmonary edema. Chronic intoxications are the most frequent and it is characterized by a decrease in productivity. Other than producing lesions, characteristic in this species, the liver is affected by fumonisin toxicosis outbreaks. The clinical incidence of pulmonary edema caused by fumonisins has not been greatly studied in Brazil.

Swine fumonisin toxicosis: difficulty breathing, cyanosis, and pulmonary edema.

History

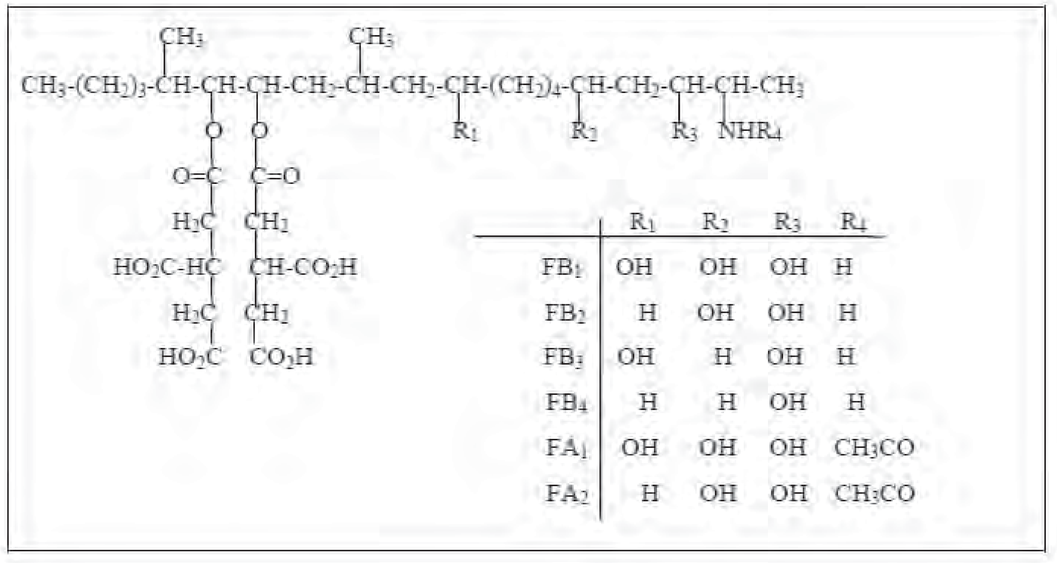
Fumonisin was first identified in 1988, therefore, its toxic effect on animals has been known for many years. Different outbreaks of equine leukocephalomalacia have been attributed to the ingestion of contaminated feed. In 1902 the illness was observed in North American equine fed with corn which was highly contaminated with *F. moniliforme*. The intoxicated animals presented characteristic clinical symptoms including neurological dysfunction and corticocerebellar lesions. From 1934 to 1935 close to five thousand equine deaths were recorded in Illinois and Iowa, USA. Clinical symptoms and lesions were identical to those attributed to leukoencephalomalacia in previous outbreaks. Different organisms were found in the feed consumed by the affected animals, among those found were

fungi from the *Fusarium* genus. Cultures of *Fusarium moniliforme* in sterile food were used to reproduce the disease, which confirmed the toxigenic potential of the fungus and its role in the production of certain toxins. In Brazil, the illness was first described and accounted for in the state of São Paulo in 1950. Beginning in 1982, hundreds of outbreaks have been reported in Brazil. Scientists believe that the disease appears to be more important than indicated by the few reports available. Mistakes in diagnosing the illness in cases that did not result in death and where feed was not monitored could have distorted the perceived prevalence of the disease.

Etiology

In 1988 the structural formula of fumonisin B_1 (FB_1) molecule was discovered, but the natural occurring mycotoxin was first isolated in 1990. Dozens of fumonisin molecular structures have been isolated, among them: FB_1, B_2, B_3, B_4, B_5, A_1, A_2, A_3, AK_1, BK_1, C_1, C_3, C_4, P_1, P_2, P_3, PH_{1a} and PH_{1b}. The predominant mycotoxin produced by strains of *Fusarium moniliforme* is FB_1, a diester of propane 1, 2, 3-trycarboxylic acid and 2-amino-12, 16 dimetil-3, 5, 10, 14, 15-pentahydroxycosan, where carbons 14 and 15 of the hydroxyl groups are esterified with carboxyterminal propane 1, 2, 3-trycarboxylic acid group (Figure 20)

Figure 20 – Molecular structures of some fumonisins (Norred, 1993).



Fumonisins are highly polar molecules, soluble in water. They are more soluble in acetonitrile/water or methanol and insoluble in organic solvents. FB_1 is very resistant to high temperatures, exhibiting few losses in the drying and processing of grains. Half (50%) of FB_1 is recuperated when it is submitted to 150°C, 125°C, 100°C and 75°C for 10, 38, 75 minutes, and 8 hours, respectively. Other researchers have tested the stability of FB_1 and FB_2 for 60 minutes at 150°C in media containing different potential of hydrogen. There was a 80 to 90% loss of toxins at pH levels of 4.0; 18 to 30% at pH levels of 7.0, and 40 to 52% at pH levels of 10.0.

Thermal processing of foods does not affect fumonisins.

Occurrence of fumonisins in feed and food

Fumonisins are mycotoxins, secondary metabolites of fungi, especially of the *Fusarium* genus. Fumonisins are produced mainly by *F. moniliforme*, but other species such as *F. proliferatum*, *F. nygamai*, *F. anthophilum, F. dlamini*, and *F. napiforme* are also producers. It has been recently shown that fungi of the *Alternaria spp* genus also produce fumonisin, but in smaller quantities. Fungi of the *Fusarium* genus develop and produce the largest quantities of fumonisins in climatic conditions with temperatures between 15 and 25°C and high contents of substrate humidity, above 20% or water activity higher than 0.9. Fungi of the *Fusarium* genus are often called "field fungi" because the largest concentrations of toxins are seen in grains that are exposed during tillage to these basic conditions for fungal development. The predominant flora of different grains which are freshly harvested, generally contain fungi of the *Fusarium* genus. However, it is known that the incidence of fumonisins is significantly higher in cereals that are exposed to bad weather during and after the maturation of the grains. This generally occurs when grains are not harvested or stored immediately after physiological maturation.

High humidity + mild temperatures = production of fumonisins.

Fumonisins are found globally, especially FB_1, which is responsible for approximately 70% of all quantified fumonisins in cereals and their byproducts. FB_2 and FB_3 occur jointly in smaller proportions. Normally, only FB_1, FB_2 e FB_3 are detected when fumonisins are produced under natural conditions. Numerous researchers have proposed that fumonisins C_1, FB_4, FA_1, and FA_2 can only be produced in laboratory conditions. However, apart from FB_1, FB_2, and FB_3 some researchers have found other fumonisins such as FB_4, C_1, C_3 and C_4 (which had only been isolated in laboratory conditions) in corn samples which were sufficiently damaged. Agricultural products frequently contaminated with fumonisins include: oatmeal, barley, bran, rice, corn, and their derivatives.

The presence of FB_1 at concentrations between 44 and 83 mg/kg is very frequent in corn coming from the Republic of Transkei in Southern Africa, a region with a high incidence of human esophageal cancer, but even higher concentrations of up to 117 mg of FB_1/kg have been found in corn produced in this region. The incidence of high concentrations of fumonisins in human food in Transkei, Italy, and China was epidemiologically compared to the incidence of esophageal cancer in the population. This comparison showed that there is a highly significant index of correlation between the two.

Concentrations of up to 7.2 and 8.85 mg of FB_1/kg of corn and its derivatives were found in the USA and South Africa, respectively. FB_1 was detected at levels between 0.2 mg/kg and 330 mg/kg in feed involved in episodes of intoxication in different animal species (such as horses and swine).

In Europe, analyses to detect naturally occurring FB_1 and FB_2 performed on corn samples from various countries, demonstrated that batches of cereals frequently present 100% positivity. Although samples have demonstrated a high rate of positivity, concentrations normally found are low, varying from 0.055 and 5.0 mg/kg of feed.

50 to 90% of corn from any given region can be contaminated with fumonisins.

In South America molds of the *Fusarium* genus also find favorable conditions for development and production of fumonisins. In surveys conducted in Uruguay on 64 samples of feed, 32 samples (50%) were positive for FB_1, with contamination varying from 0.005 up to 6.34 mg/kg. In the province of Cordoba, Argentina, 50 hybrids of corn analyzed presented 100% positivity, with concentrations ranging from 0.185 up to 27.05 mg/kg of FB_1 and 0.04 up to 9.95 mg/kg of FB_2.

In Brazil, the presence of fumonisins in cereals and feed was found by various researchers. The mycotoxin has greater occurrence in the Southern states of the country, but it is also present in the Southeast, Goias, and Mato Grosso do Sul. The most frequently monitored products are corn, cornmeal, and feed for poultry and swine. The incidence of fumonisins has been found in most of the samples that have been analyzed, presenting 50 to

90% positivity. Concentrations of fumonisins are generally low, between 0.005 and 15 mg/kg of samples, with an average of less than 1 mg/kg of feed. Nonetheless, samples containing concentrations higher than 50 mg of FB_1/kg of feed were frequently found.

In the state of Rio Grande do Sul, 407 samples (267 samples of corn, 92 samples of feed, 8 samples of oatmeal, 5 samples of rice bran, 8 samples of soybean meal, 14 samples of barley, and 13 sample of wheat) obtained from different storage facilities and feed mills from the Southern region of Brazil were analyzed during the period between 1996 and June 1998. Of the samples analyzed, 32.2% were positive for FB_1, with levels of contamination varying from 86 µg/kg to 78,9 mg/kg. The highest concentration found and the percentage of positive samples were 14.2 mg/kg and 75%, respectively in rice bran; 68.3 mg/kg and 30.4% in feed; 78.9 mg/kg and 35.2% in corn; 2.4 mg/kg and 7.7% in wheat; 175.5 µg/kg and 25% in oatmeal; 2.4 mg/kg and 14.3% in barley. Soybean meal did not present contamination levels above the quantification limit of the method used (50 µg/kg).

In evaluations done in the last 17 years on samples from the main grain producing states of Brazil, LAMIC/UFSM analyzed fumonisins in 45,558 samples, finding an average concentration of FB_1 + FB_2 of 1591.1 µg/kg. Corn represented 22,746 of these samples, with an average contamination of 1,989.7 µg/kg, found in 75% of this grain. Feed submitted for fumonisin analysis presented 72% contamination of all samples with an average level of 1,592 µg/kg..

Toxicokinetics of fumonisins

Absorption

The bioavailability of FB_1, when administered orally, is relatively low in various species of domestic animals. The absorption of the mycotoxin ingested by swine was around 3 and 6%. Due to the low absorption of FB_1 in the gastrointestinal tract, high concentrations are eliminated in feces. Elimination through urine is low, despite the fact that parenteral administration could have invert quantities eliminated through these two ways. Approximately 30 to 45 minutes after intragastric administration in pigs, the toxin could be detected in plasma, with maximum concentrations appearing between 60 and 90 minutes, reaching no detectable levels in a few hours.

Distribution

The distribution of radioactivity in the tissue of pigs fed with a diet containing FB_1 radioactive labeled by ^{14}C was tested using 3 mg of toxin/kg for 12 days and 2 mg of FB_1/kg until completing 24 days of intoxication. At day 25 these animals were fed uncontaminated rations for 9 days. Samples from different organs were collected on days 3, 6, 12, 24, 27, and 33 of the experiment. Radioactivity was detected from the first sampling, with levels of concentrations in the tissues increasing until day 24. The highest concentrations in tissue were found in the liver and kidneys (160 and 65 µg/kg, respectively). After 3 days of consuming uncontaminated feed, radioactive labeled residues in the organs decreased by close to 35%. After the 6th day of ingesting a fumonisin free diet, only traces of radioactivity were detected. At the end of the experiment, no residue was detected in the plasma, spleen, muscle, brain, adrenal glands, fat, or skin.

Elimination

The elimination of fumonisin from the plasma is fast in all species. Swine, particularly, present a relatively long terminal elimination phase (γ) of t½ + 182.6 minutes, and the plasmic concentration of toxin is less than the detectable limit (18 µg/kg) 4 hours after administration, when the purified toxin is administered orally in moderate doses. It is also known that the elimination of fumonisins occurs for a longer period when naturally contaminated feed is ingested. Recovery from the toxin is higher than 90%, mostly eliminated through feces and only 0.6% through urine. Based on the evaluation of concentrations, enterohepatic circulation, and elimination, it has been concluded that the bioavailability of FB_1 is around 4%.

Residues

Fumonisins are quickly eliminated from the organism of the intoxicated animals, especially when contaminated feed is substituted by feed not containing the toxin. Poultry and bovines are very resistant to fumonisins, eliminating the toxin through eggs and milk when consuming feed with high concentrations of these mycotoxins. During the period of intoxication, animal hair incorporates the toxin in the area of growth. This finding demostrates the possibility of stipulating the periods when the animal was intoxicated by quantifying the toxin from different sections of animal hair strands.

Pathogenesis

The molecular structures of fumonisins are similar to those of sphinganine and sphingosine, which are responsible for the formation of sphingolipids (sphingomieline and glycosphingolipids - Figure 21) in animals. More than three hundred sphingolipids are synthesized in the endoplasmatic reticulum of eukaryotic cells from a combination of serine with palmitoyl-CoA. They are very important in maintaining the integrity of the cellular membrane, regulating surface cellular receptors, ion pumps, and other systems that are vital to the functioning and survival of the cell. The glycosphingolipid galactosylceramide lipid is the main constituent of myelin, which is a component of oligodendrocytes membrane and Schwann cells in central and peripheral nervous system, respectively.

Studies done on rodent hepatocytes have demonstrated that fumonisins block the formation of sphingolipids by inhibiting the enzyme N-acyltransferase. Although the mechanism of action is not completely understood, an outline representing the mechanism has been proposed based on the similarity between fumonisins and sphingolipid molecules (Figure 22).

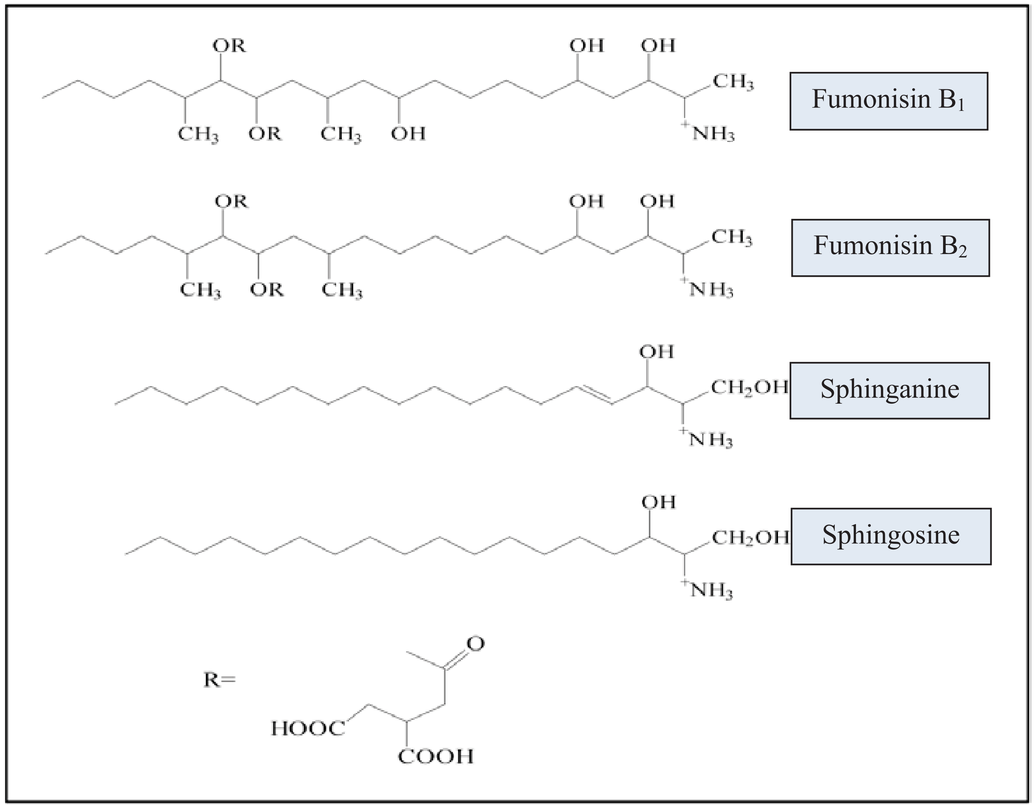
The accumulation of sphinganine and, sometimes sphingosine, has been demonstrated *in vitro* using cells from the intestinal epithelium of swine, and *in vivo* with the intoxication of ponies, pigs, and chickens. There was partial or total inhibition of sphingosine and of enzyme N-acyltransferase was due to hepatotoxicosis in pigs. Some researchers have proposed that there could also be an activation of intravascular macrophages in the lungs of pigs. The presence of those phagocytic cells adhere to the endothelium of pulmonary capillaries, with subsequent release of vasoactive substance, could be the cause of pulmonary lesion susceptibility of pigs intoxicated with fumonisins.

Accumulation of membrane material was also detected in the periphery of cellular nuclei, components of the vascular endothelium of pig lungs that consume fumonisins. This alteration was not found in endothelial cells of other organs or in lungs of other species, leading researchers to conclude that this alteration in the lungs could be a result of an increase in capillary permeability which induces pulmonary edema in intoxicated pigs.

Other researchers have found a series of alterations in cardiac dynamics and suggested that the formation of pulmonary edema in swine could be caused by contractile deficiency of the left side of the heart and not by increased capillary permeability of the lungs.

The pathogenesis of fumonisins in swine suggests cardiotoxic action.

Figure 21 – Chemical structure of fumonisins B_1, B_2, sphinganine and sphingosine.

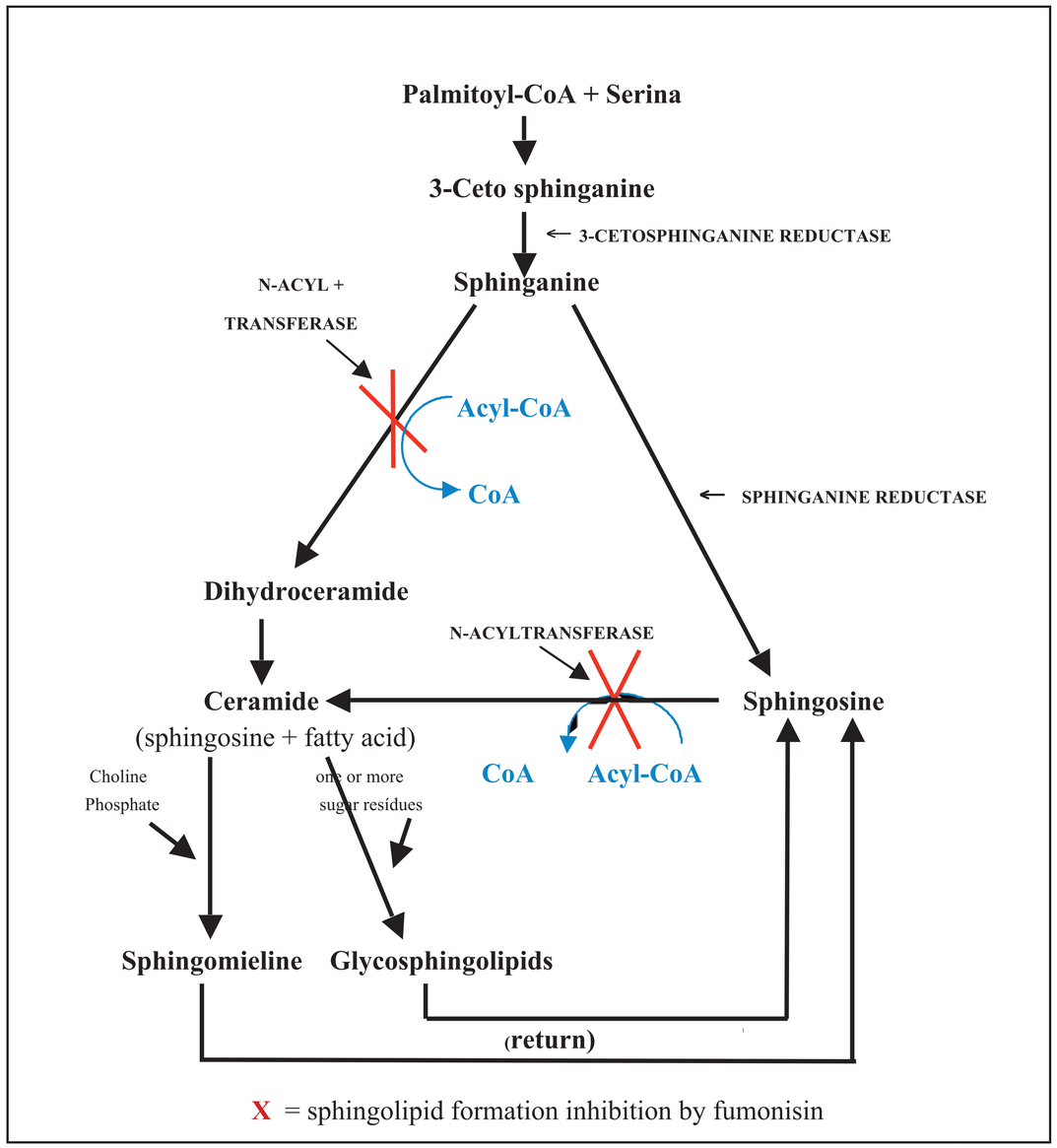


Wang *et al*., 1991

Fumonisins can exert carcinogenic activity on rat hepatocytes when exposed to high doses of the toxin. These toxins are mitogenic in fibroblasts of mice, stimulating cellular multiplication due to the increase in sphingolipid concentration, as well as products of its metabolism. They induce apoptosis in intoxicated animals and in cell cultures, through mechanisms that are still unclear.

The incidence of fumonisins in food destined for human consumption has been epidemiologically related to incidences of esophageal cancer since 1976 in Transkei, Italy, and China. Based on toxicological evidence, the *International Agency for Research on Cancer* (IARC) has declared that toxins produced by *Fusarium moniliforme* are possible human carcinogens (class 2B carcinogen).

Figure 22 – Mechanism of sphingolipid formation inhibition by fumonisin.



Wang *et al.*,1991

Immunosuppressant effects of fumonisins on swine

The immunopathogenic potential of fumonisins preoccupies the animal industry, even though few studies show the real importance of fumonisins in the decrease of intoxicated animal immunological capacities. However, statistical studies confirm the involvement of FB_1 in the occurrence of *Mystery Swine Disease* (known today as PRRS, caused by the virus–PRRSv) in the United States. Researchers have concluded that the association of fumonisin toxicity and PRRS were significant in 21 herds studied. Of the 12 that presented clinical signs of the disease, 8 consumed feed containing more than 20 mg/kg of FB_1, and of the 9 herds that were not affected by the disease, only 1 consumed feed containing more than 20 mg of FB_1/kg. Decrease in the concentration of macrophages in the lung was found beginning with 1 week of intoxication in piglets fed a diet containing 20 mg of FB_1/kg. Associated with this decrease in immunity, a greater susceptibility to infection by *Pseudomonas aeruginosa* was seen. The increase in susceptibility to *Escherichia coli* in intoxicated piglets has also been proven. Doses of 0.5 mg of FB_1/kg of live weight/day during 7 days were sufficient to significantly increase the bacterial dissemination when *Escherichia coli* was administered orally. Although animals did not develop lesions derived from the intoxication by FB_1, researchers concluded that bacterial colonization, after 24 hours, was significantly higher in the lungs, spleen, kidneys, and greater still in the digestive organs such as the ileum, cecum, colon, and mesenteric lymph nodes. It was also observed that animals intoxicated with FB_1 presented fewer number of local inflammatory cytokines.

Pneumopathies could be associated to the ingestion of fumonisins.

Clinical symptoms

Acute intoxication of swine with fumonisins induces clinical symptoms characterized by pulmonary edema, which generally occurs between 3 and 5 days after the initial consumption of contaminated feed. In these cases, death can occur within a few hours. Chronic intoxication occurs as a result of consuming feed with low concentrations of the toxin for a prolonged period of time, causing hepatic damage in affected pigs. Concentrations of FB_1 higher than 10 mg/kg in feed are considered unsafe for swine. However, natural outbreaks of pulmonary edema have been found in swine fed diets contaminated with 1 mg of FB_1/kg (Table 13). Various researchers found that swine at

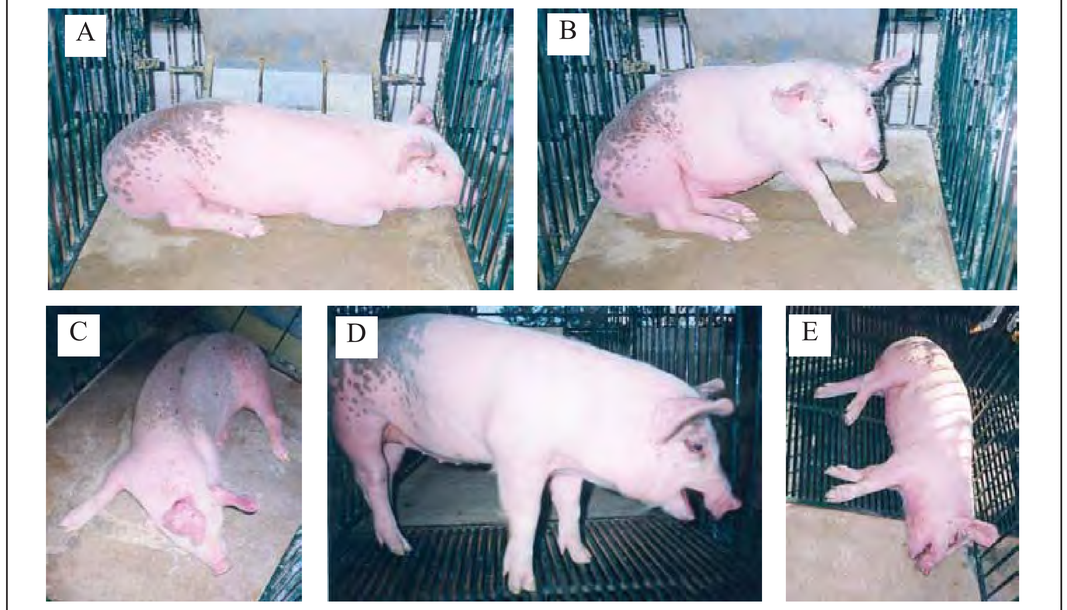
Respiratory problems could be associated with fumonisin intoxication.

reproductive age are more sensitive to intoxication by fumonisins when compared to growing piglets. However, stress factors, such as nutritional and management deficiencies could also be important factors in the onset of intoxication. Females are less sensitive than males, but gestating females that are intoxicated generally have offspring with pulmonary lesions characteristic of pulmonary edema. Levels of fumonisins were not detected in the milk of sows that were fed rations containing concentrations of 100 mg FB_1/kg of feed and nursing piglets did not present any signs of intoxication. Abortions resulting from intoxication by fumonisins generally did not cause complications in subsequent gestations.

The principal clinical symptoms in swine chronically intoxicated by fumonisins are nonspecific and occur as a consequence of hepatic lesions, leading to an overall sickly appearance of the animal, and a decrease in weight gain. These symptoms can be easily confused with malnutrition, genetic deficiency, inadequate management and/or other afflictions that also cause clinically sickly appearance of the animal. The herd generally presents a significant increase in non-uniformity. There is usually a decrease in feed intake and weight gain, which can often lead to significant worsening of food conversion. Bristles present a decrease in shine, coarseness, and animals roam more frequently through the pen without eating.

Acute intoxication of swine by fumonisins is clinically easier to detect because it is characterized by pulmonary edema. The most characteristic signs seen in affected animals include anorexia, lethargy, open mouth, increase in respiratory frequency, jaundice, and cyanosis of the skin, most evident on the ears, snout, sclera and mucosa membranes. Excessive salivation can also occur, as well as hepatic encephalopathy syndrome, a humid snoring sound when listening to the lungs, and watery diarrhea. After the decreased in intake induced by the contaminated feed, respiratory difficulties, comprised of superficial and abdominal breathing associated with an increase in frequency, normally occur between 12 and 24 hours. Characteristic symptoms of pulmonary edema appear close to 3 to 5 days after consumption of contaminated feed. If toxicosis persists, more severe symptoms such as ataxia of the posterior limbs, dog sitting position, lateral decubitus, a short period of agony, and death are rapidly observed (Figure 23).

Figure 23 – Sequence of clinical symptoms in pigs with pulmonary edema induced by intoxication with fumonisins. (A) Apathy and prostration on the onset of clinical symptoms of pulmonary edema. (B) Dog sitting position in order to relieve pulmonary pressure. (C) Cyanosis of extremities and intensification of apathy. (D) Increase in respiratory difficulties and initiation of oral respiration. (E) Death of animal due to intense pulmonary edema.



Hemoptysis and bloody feces can be present in some animals of the intoxicated herd. Generally more than 80% of the animals of the same lot are affected. When intoxication is not severe and contaminated feed is immediately replaced, the recuperation rate of the lot can reach up to 100%. However, if intoxication is very severe, all affected animals can die within a few hours.

Table 13 – Maximum recommended levels of total fumonisins ($FB_1 + FB_2 + FB_3$) in corn, corn-based feed, and total feed of various species.

Animal Species	Maximum limits of total fumonisins in corn and its byproducts (mg/kg[1])	Correlation Factor[2]	Maximum limits of total fumonisins in feed (mg/kg[1])
Equine[3]	5	0.2	1
Rodents	5	0.2	1
Unscaled fish	20	0.5	10
Swine	10	0.5	10
Ruminants[4]	60	0.5	30
Mustelids[5]	60	0.5	30
Chicken[6]	100	0.5	50
Ruminants and laying Hens[7]	30	0.5	15
All others[8]	10	0.5	5

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[1] Total fumonisins = $FB_1 + FB_2 + FB_3$.
[2] Portion of corn or corn mixed with other feed.
[3] Includes donkeys, zebras, and other wild equine.
[4] Includes cattle, sheep, goats, and other ruminants for human consumption, older than 3 months old.
[5] Wild animals bred for fur production.
[6] Turkeys, hens, ducks, and other poultry for human consumption.
[7] Includes laying poultry, roosters, milking cows, bulls, and buffalo.
[8] Includes canines and felines.

Clinical Pathology

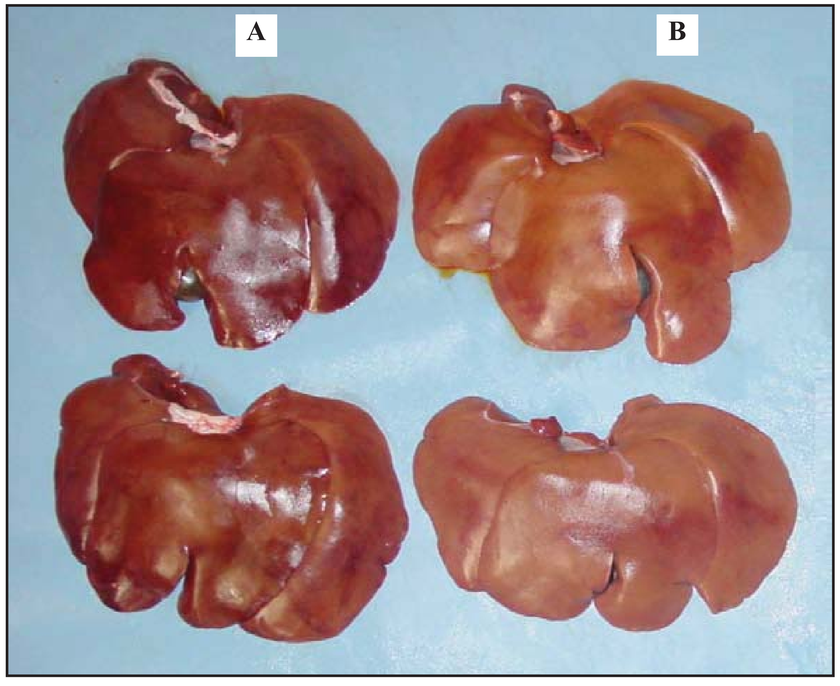
Determining the clinical biochemical parameters is of great importance in the evaluation of the extension and prognosis of the pathology. Generally alterations in biochemical concentrations are indicative of progression of the pathology of a given organ such as the liver, kidneys, or pancreas. Swine intoxicated with fumonisins present a significant increase in bilirubin, and in enzyme activity (aspartate aminotrasferase and glutamyltransferase), and above all, in alkaline phosphatase. Increased levels of serum cholesterol generally occur on the 10th day of intoxication, and enzyme alterations occur even earlier. An increase in sphinganine and/or sphingosine, or an increase in the proportional concentration of these sphingolipids, has been considered to be a biological marker of intoxication with fumonisins. It is currently known that other mycotoxins also interfere with the concentrations of these two substances. Hematological parameters such as erythrocytes/mm^3, hematocrit levels, hemoglobin concentrations, absolute hematometric indices, and leukogram are not easily affected by fumonisin-toxicosis in pigs. Total protein concentrations and albumine can, however, be affected, especially after severe hepatopathies.

Pulmonary edema ?
Remember fumonisins.

Macroscopic Lesions

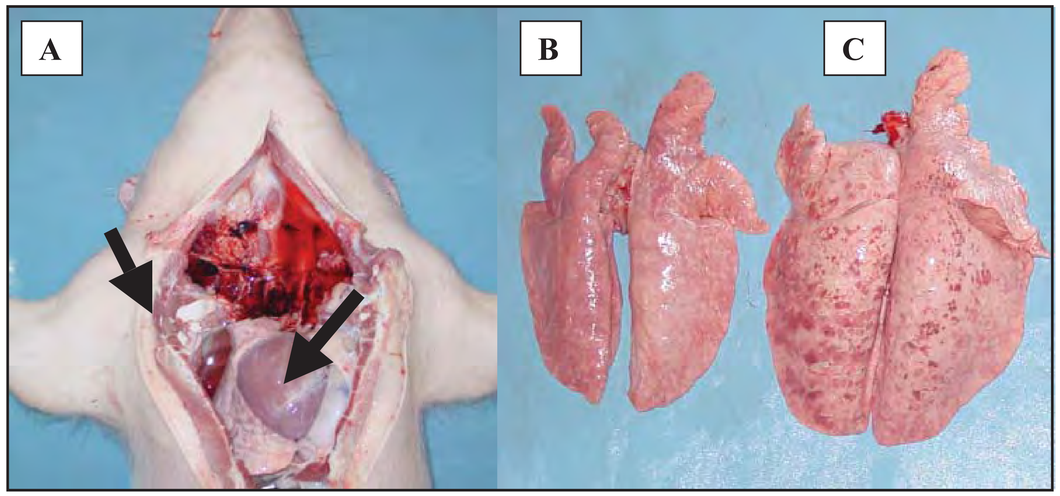
The necropsy of pigs intoxicated with high doses of fumonisins showed extensive pulmonary edema which frequently evolved into hydrothorax, jaundice, and darkening and hardening of the liver. Exposure to low doses during prolonged periods induced hepatic degradation and necrosis, interfering with protein synthesis, weight gain, and general animal performance (Figure 24).

Figure 24 – Hepatic damage in pigs intoxicated with 30 mg of fumonisins/kg of feed for 21 days. (A) Livers of pigs not intoxicated with fumonisins. (B) Livers of intoxicated pigs presenting yellow coloration.



Necrosis of the medullary region, resulting from fumonisin intoxication is normally associated with hepatosis. Pathological changes observed in pigs affected by pulmonary edema are characterized by the presence of beige-clear or yellowish-gold transudate in the thoracic cavity, which characterizes more severe cases of pulmonary edema and hydrothorax. Concentrations greater than 500 mL of this yellowish-gold liquid can accumulate, initiating the coagulation process when it comes into contact with air (Figure 25).

Figure 25 – Occurrence of pulmonary edema in pigs intoxicated for a period of 21 days with 30 mg of fumonisins/kg of feed. (A) Liquid from edema (arrows). (B) Lung of non-intoxicated swine. (C) Enlarged lung of intoxicated swine.



The lungs of pigs affected by pulmonary edema did not collapse when removed from the chest, and they presented an increase in size with rounded edges. The lungs are heavier because of the concentration of liquid in the parenchyma and air conduits. Edema predominantly occurs in the interlobular and interstitial region. When the accumulation is interlobular, it predominantly appears in the hilum, extending through the lung, separating the lobules by 3-5mm spaces. Exudate seeping through the pulmonary surface was observed, as well as distended septa and removal of the pleura, separating it from the pulmonary parenchyma. Little liquid is normally found in the bronchioles, bronchi and trachea.

Few pathological changes can be detected in different organs of pigs affected with pulmonary edema. However, some researchers assure that ventricular walls of the heart can become more flaccid. The incidence of ulcerative lesions in the esophagus and stomach, sometimes associated with the formation of hepatic and esophageal hyperplasia, can be observed in some chronically intoxicated animals. An increase of mediastinal and peribronchial lymph nodes was also observed in some animals.

Microscopic Lesions

Microscopic pulmonary lesions of pigs affected with pulmonary edema are similar, including an accumulation of acidophilic fibrin in the alveoli and interlobular lymphatic vessels. This protein rich fluid is present in abundance in the subpleural lymphatic vessels and has little cellular infiltration. The septum presents a small number of mononuclear cells and neutrophils. Some alveoli have few mononucleous cells and large amounts of capillary hyaline thrombi are found in the lungs. In cases of acute pulmonary edema, lesions in other organs are rarely found, but, hepatopathies with foci of necrosis can occur. Hepatic changes, insufficient to cause clinical pulmonary edema, are observed with greater frequency in animals that suffer chronic intoxication with low doses. Random hepatocellular necrosis, nuclear pleomorphism, an increase in the amount of mitosis, deformed hepatomegalocytosis cells and focal necrosis of hepatocytes can occur.

Diagnostic, treatment, and prophylaxis

Diagnosing acute swine fumonisin-toxicosis is easily done by identifying clinical symptoms. However, a definitive diagnosis is based on both the presence of lesions and analysis of the presence of fumonisins in feed.

Observation of clinical symptoms leads to diagnosis.

Various analytical methods such as Thin Layer Chromatography, Gas Chromatography, Gas Spectrometry, ELISA, Capillary Electrophoresis, and High Performance Liquid Chromatography (HPLC) have been developed to quantify the amount of fumonisins in feed. Animals intoxicated by fumonisins, can also have an increase in sphinganine and/or sphingosine concentration in their blood stream, which can sometimes be used as biological markers of intoxication with fumonisins.

Intoxication of pigs with fumonisins does not have a specific treatment. The clinical symptoms of acute intoxication disappear approximately 3 days after withdrawing the contaminated feed.

Prophylaxis consists of implementing measures that lead to the inhibition of fungal development in grains and ingredients used in feed, and/or a system which monitors the presence of mycotoxins in feed.

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5

TRICHOTHECENES

TRICHOTHECENES

Summary

Trichothecenes (TCT) form part of a group of chemically similar toxic metabolites. They are produced by various fungal species of the *Fusarium*, *Cefalosporium, Myrothecium, Stachybotrys*, and *Trichoderma* genera. More than a hundred mycotoxins form part of this group, which is divided into macrocyclic TCT and non-macrocyclic TCT. The toxic effects of macrocyclic TCT such as baccharinas, roridinas, satratoxinas, and verrucarinas have yet to be studied in swine. Non-macrocyclic TCT are divided into type A and type B. The main constituents of type A include toxin T-2, toxin HT-2, and diacetoxyscirpenol (DAS). The main constituents of type B are deoxynivalenol (DON or vomitoxin), fusarenon-X, and nivalenol. TCT occur in various grains and their byproducts, especially those cultivated during winter because the temperatures and high humidity of this season favors the development of the molds which produce these mycotoxins. The mechanism of toxic action of TCT is based on inhibiting the synthesis of protein and interfering with the synthesis of DNA and RNA, affecting cells of high metabolic activity and active multiplication. In addition to feed refusal, TCT induce the formation of ulcerative lesions in the gastrointestinal tract and dermatitis, which is important in clinical diagnosis. Animals present decrease in productivity concurrent with immunosuppression.

Trichothecenes production mainly occurs with cool ambient temperatures.

History

In the 1930s, a stachybotryotoxicosis caused the death of tens of thousands of equine in the former USSR. The intoxication was the result of feed contaminated with satratoxins. Ulcerative stomatitis lesions, bleeding, leukopenia, inflammation, and intestinal necrosis are characteristic of this intoxication, and clinical symptoms include blindness and lack of coordination. In the beginning of the XX century and especially between 1941 and 1947, alimentary toxic aleukia (ATA diseases) made a large number of Europeans sick, causing more than 100 thousand deaths in Russia. The illness was related to the ingestion of wheat, rye, and other grains which had been contaminated by TCT produced by cryophilic fungi of the *Fusarium* genus, especially *F. sporotrichoides, graminearum* and *moniliforme*. The four

evolutionary stages of the disease occur in the following order: caustic lesions in the upper digestive tract, bloody vomit and diarrhea which last from 3 to 9 days. The second stage occurs for 2 to 8 weeks and is characterized by hematopoietic damage such as leukopenia, agranulopenia and lymphocytosis which decrease resistance to bacterial infections. The third stage is characterized by the formation of petechiae that can reach up to a few square centimeters in size on the skin. These lesions are frequently accompanied by necrotic lesions in the oral cavity which can cause asphyxia and death in approximately 30% of intoxicated animals. The last stage is characterized by dermatitis resulting from direct contact with the toxins. Furthermore, there is a decrease in the productivity of intoxicated animals in every stage of the illness.

Etiology

TCT is made up of a chemical group of toxic secondary metabolites with the same basic structure (Figures 26 and 27). They are composed of sesquiterpenoids, which are characterized by a 12,13-epoxy-trichothecium-9-ene skeleton type.

Table 14 – Main non-macrocyclic trichothecenes.

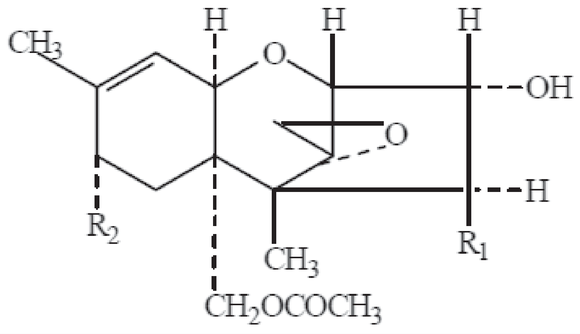
Type	Toxins	Main fungal producers
A	T-2 toxin, HT-2 toxin Diacetoxyscirpenol (DAS), Neosolaniol.	*F. sporotrichoides*, *F. poae, F. oxysporum, F. semitectum* e *F. equiseti.*
B	Nivalenol, Deoxynivalenol (DON), 4-acetyl-nivalenol (Fusarenon-X), 3-acetyl-deoxynivalenol (3-Ac-DON)	*F. graminearum, F. culmorum, F. crookwellense, F. sporotrichoides, F. poae, F. tricinctum* e *F. acuminatum.*

Adapted from Pasteiner (1994) and Pittet (1998).

TCT producing molds belong to species of the *Trichoderma, Trichothecium, Myrothecium, Stachybotrys, Cylindrocarpon, Verticimonosporum, Cephalosporium, Phomopsis* and especially *Fusarium,* such as *F. graminearum* and *F. tricinctum* genera. The presence of these fungi in food sources does not imply the presence of toxins, but it is an important indicator of potential contamination. Approximately two hundred types of TCT are

known; however, only a dozen are known to have toxicological importance. They are divided in two large groups, according to their molecular structure: simple chain, non-macrocyclics (Table 14), and macrocyclics, which have a macrocyclic ring between C_4 and C_{15}. TCT macrocyclics are easily distinguishable from non-macrocyclics through thin layer chromatography, because of their intense fluorescence when seen under ultraviolet rays. They are generally more toxic than non-macrocyclics. Frequently, the ingestion of TCT macrocyclics is related to the occurrence of stachybotryotoxicosis and immediate death of animals that have ingested contaminated feed. Non-macrocyclics TCT are divided into four groups: A, B, C, and D. The most important belong to groups A and B. Type B have a group of conjugated carbonyl in position C_8, which is absent from type A TCT. The main representative of macrocyclic TCT (type C) are: verrucarins, roridins, satratoxins and baccharins. Crotoxin, a non-macrocyclic diepoxytrichothecene belongs to type D trichothecenes.

Figure 26 – Molecular structure of some type A trichothecenes.



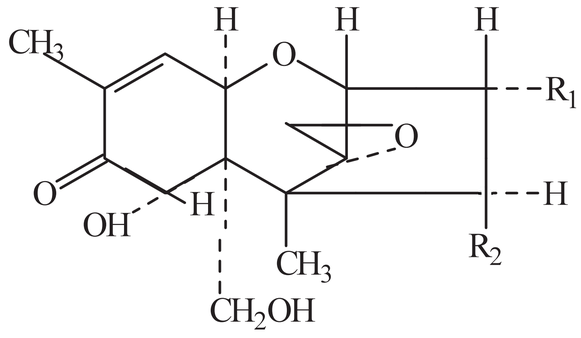
	R_1	R_2
Toxin T-2	$OCOCH_3$	$OCOCH_2CH(CH_3)_2$
Toxin HT-2	OH	$OCOCH_2CH(CH_3)_2$
Neosolaniol (NEO)	$OCOCH_3$	OH
4,15-Diacetoxyscirpenol	$OCOCH_3$	H

Type A TCT are soluble in apolar solvents such as chloroform, ethyl acetate, acetone and ether. Type B TCT are soluble in polar solvents, such as water, acetonitrile, and alcohols. Other TCT are normally soluble in solvents used for type A TCT. The resistance of some TCT was tested in grain processing. Experimental corn milling shows that around 67% of T-2

toxin was removed. DON proved to be the most stable amongst TCT during food processing given that 50% of the toxin can be lost through baking. It has been found that using sodium carbonate during the elaboration of food increases the loss of toxins. Significant losses of T-2 toxin and DON were observed when extracting oil from contaminated corn. Only traces of T-2 toxin were detected in malt after the fermentation of barley. Deoxynivalenol does not appear to be as unstable during the fermentation process in the production of beer. Out of fifty samples of Argentinean beers analyzed for the presence of deoxynivalenol in 2000, 44% had levels between 4 and 221 μg/L, with 18% of samples having levels above 20 μg/L. Generally, TCT are relatively stable at high temperatures. This stability is attributed to the protection of the epoxide ring against nucleophylic attacks. Thus, the melting point of T-2 toxin is 152°C, the melting point of Diacetoxyscirpenol is 164°C, the melting point of Nivalenol a 223°C, and that of Deoxynivalenol is 153°C.

The melting point of TCT surpasses 150ºC.

Figure 27 – Molecular structure of some type B trichothecenes.



	R_1	R_2
Nivalenol (NIV)	OH	OH
4-acetyl-nivalenol(Fusarenon-X)	OH	$OCOCH_3$
Deoxynivalenol (DON)	OH	H
3-Acetildeoxinivalenol(3-Ac-DON)	OCOC	H

TCT of most toxicological and economical importance in the swine industry include deoxynivalenol (vomitoxin; DON) and T-2 toxin, followed by diacetoxyscirpenol (DAS) and nivalenol (NIV).

Occurrence of trichothecenes in feed and food

TCT occur globally as a natural contaminant of grains and food sources. Their incidence has been recorded in Asia, Africa, North and South America, and Europe (Table 15). The occurrence of TCT depends on various factors, such as crop strain and its toxigenicity and climatic conditions, including water activity, preferably above 0.96. This occurs especially when harvest takes place under rainy, cloudy, and cool conditions, with temperatures ranging from 6 to 24°C. Trichothecenes are mostly present in Southern Brazil in grains harvested during winter, when temperatures and humidity levels are adequate for the development of toxin producing fungi. Significant concentrations are frequently detected in oats, barley, corn, wheat, and hay, while low levels of the toxin are usually found in rice, rye, and sorghum. Levels of natural contamination of DON, DAS, T-2 toxin, and nivalenol normally reach up to 10 mg/kg. Levels of up to 15-40 mg/kg are rarely detected. The incidence of DON in food sources is frequent at levels between 0.1- 42 mg/kg, with an average between 2.4 mg/kg and 4 mg/kg.

Contamination by TCT is a major problem in Southern Brazil because of climatic conditions.

Globally, DON is the most common contaminant of grains, as well as nivalenol (NIV) in certain regions. The concurrent presence of other TCT and other *Fusarium* toxins such as fumonisins, moniliformin, and zearalenone in the same batch of grain is possible.

In 1988 five hundred samples of grains (wheat, barley, rye, corn, and others) from 19 European, Asian, and South American countries were analyzed for NIV and DON by Japanese researchers. Approximately 40 to 50% of the samples were positive for NIV and DON, with an average concentration of 267 and 292 µg/kg, respectively. Barley had the highest incidence of mycotoxins, being that in 139 of the samples analyzed the average levels of NIV and DON were of 401 and 149,

Vomitoxin (DON) is the most frequent TCT and is often present with other mycotoxins.

respectively. Of the 222 samples of wheat analyzed, 50 and 39% were positive for NIV and DON, respectively, with an average of 127 µg/kg and 488 µg/kg.

In samples of wheat and barley from Japan and Korea, levels of NIV were much higher than those of DON, while DON was the principal contaminant in samples from Argentina, Canada, China, Poland, and Germany.

Table 15 – Occurrence of deoxynivalenol (DON) in grains.

Product	Country	Samples	Incidence	Levels (µg/kg)
Wheat	Bulgaria	140	67%	50-1,800
Wheat	Switzerland	205	87%	20-3,000
Wheat	Switzerland	154	62%	10-3,010
Corn	Canada	283	86%	20- 4,090
Wheat	Canada	2311	33%	10-10,500
Barley	Germany	240	81%	2- 4,764
Wheat	Germany	445	91%	3- 20,538
Wheat	Argentina	60	93%	100-9,250
Wheat	Argentina	40	80%	300- 4,500
Barley	Canada	117	100%	30-15,790

Adapted from Pittet (1998).

In Canada and the United States, the most commonly found TCT are DON, followed by NIV, T-2 toxin, and HT-2 toxin, and rarely DAS. In Argentina, DON is one of the main toxins found in primary food sources, with levels between 200 and 4,500 µg/kg in corn. In other evaluations, Argentinean researchers found DON in 93% of wheat samples analyzed, with average levels of 1,798 µg/kg. Some samples of wheat flour presented contamination between 250 and 9,000 µg/kg with an average of 1,309 µg/kg.

To evaluate the occurrence of TCT contamination in wheat, researchers analyzed samples of grain harvested in the main producing areas of Argentina, during a period of 6 years, comprising 1,056 samples. The occurrence of DON was found in 524 (49.6%) samples, with concentrations between 47.5 and 570.9 µg/kg.

Levels of TCT contamination in grains are significantly related with the quantity of plant residue in the grains. Thus, the presence of portions of cob, stalk, stunted grains, and residues can cause and increase in contamination of up to 5 folds. Therefore, there is a tendency to find higher concentrations of toxin in older grains.

Approximately 10% of the samples of analyzed grains cultivated in the state of São Paulo were found to be positive for TCT. It was also found that concentrations rarely exceed the limit of 1 mg/kg. However, the incidence of TCT in grains cultivated in Southern Brazil is higher, frequently reaching levels higher than 30%. Barley, rye and wheat have the highest incidence of contamination, with average contaminations of 1 mg/kg, reaching levels of up to 15 mg/kg.

The incidence of different TCT in feed and feed ingredients from the principal grain producing states of Brazil was analyzed during the last 15 years in LAMIC/UFSM. The incidence of T-2 toxin was analyzed in 16,939 samples. Less than 1% of samples were positive, with an average contamination of 1.2 µg/kg. The presence of DON was higher, found in 15,809 of the 39,451 samples (40%), with an average contamination of 313 µg/kg, especially in winter grains such as wheat and barley.

Toxicokinetics of trichothecenes

The dynamic of absorption, transportation, and distribution by tissues and organs was studied using markers such as ^{14}C. The study is important, especially in order to measure and prevent the risks of human exposure through consumption of contaminated meat products.

Absorption

Studies testing trichothecenes absorption were done by administering the toxin in three different ways: oral, dermic, and parenteral. The highest concentrations reached through oral exposure are found in the blood stream one hour after administration. The amount of toxin absorbed in the gastrointestinal tract is low, close to 1%, causing local caustic action. Studies done with oral doses in various species show that the maximum radioactive labeled TCT appear in the blood after one hour of being administered. Distribution can be seen in different organs, especially in the liver, kidneys, and in bile.

The first reactions to natural exposure are inflammation of the skin, mucosa, and gastrointestinal tract. Absorption of the toxin through the skin is slow and the subcutaneous adipose tissue serves as a reservoir. Among different TCT there is a difference in absorption since the highest concentrations in the tissues and organs can be detected when T-2 toxin is administered. Levels of DON are generally lower because it is largely eliminated through the feces.

Distribution

The distribution of toxins occurs in different organs and tissues, especially in the liver, kidneys, stomach, and skin. The highest levels of toxin were found in tissues after approximately 3 to 4 hours of initial oral exposure, and after close to 30 minutes when exposure was parenteral. Distribution to adipose and muscular tissue occurred 6 hours after ingestion. In the majority of cases, the toxin was no longer detected 24 hours after administering the contaminated feed.

Mechanism of action

The adverse effects of trichothecenes are the result of the interaction of the toxin or its metabolites and the target tissue. The largest toxic effects of trichothecenes are a result of interference with protein synthesis, followed by a decrease in nucleic acid production. Immediate effects are seen in the function of cell membranes, enzymatic activity, and immune function. The peptidyl transferase enzyme, integral component of ribosomes, responsible for the elongation and termination of the polypeptide chain, is the specific site of action.

TCT are the most potent interferers of protein synthesis.

TCT are also associated with hemorrhages in humans and animals. Prothrombin time significantly increases, thus the primary factor of hemorrhages is a result of a decrease in the VII factor of blood coagulation. They cause lymphoid necrosis in the thymus, spleen, and lymph nodes, and a decrease in the production of immunoglobulin and serum antibodies.

Biotransformation

Biotransformation consists of a change in molecular structure, generally becoming more polar. Its toxic activity is diminished because there is a reduction in passage through the cellular membrane. Consequently, there is an increase in the excretion of the toxin, especially through the kidney, because of a decrease in reabsoption of nephron levels. Biotransformation occurs as a result of the activation of a series of enzymes located in the endoplasmatic reticulum of various types of cells, especially hepatocytes. Two enzymatic processes take place: the first phase involves oxidation, reduction, and hydrolysis, followed by the second phase in which conjunction reactions and synthesis occur. Like many xenobiotics, TCT are biotransformed biphasically. Three main reactions occur: deacetylation (hydrolysis), hydroxilation (oxidation), and epoxidation (reduction). The hydrolysis of esters is known as the principal metabolic pathways of TCT, containing esterified chains, as occurs in T-2 toxins and diacetoxyscirpenol (DAS), such as the hydrolysis of ester C-4, being the primary sight of attack. Oxidation reactions have only been observed in T-2 toxin. The reduction of bond 12, 23-epoxide by the anaerobic microflora present in TGI is an important detoxification reaction.

Elimination

The elimination of trichothecenes occurs quickly through feces, but it mainly occurs through urine because of the conjugation of endogenous molecules to the toxin, facilitating elimination. Despite enterohepatic circulation of the toxins, elimination is quick after the removal of contaminated feed. Approximately 75% of the toxin is eliminated within 24 hours, and the toxin is nearly undetected in the bloodstream after 48 hours. The half-life of the toxin in the bloodstream is around 16 hours.

Residues

TCT are eliminated quickly from the body, hardly detected in animal byproducts, except in adipose tissue. Residues can be found in hen eggs and cow milk. The concentrations of these toxins decrease rapidly when animals resume a diet of uncontaminated feed.

Pathogenesis

TCT are potent inhibitors of protein synthesis in eukaryotic organisms. Their inhibiting action occurs effectively in different organism, including fungi, plants, and animals. The greatest toxic effect of TCT is a result of the primary inhibition of protein synthesis, by inhibiting the enzymes responsible for the elongation and termination of the polypeptide chain, such as the ribosomal enzyme of the subunit 60S, peptidyl transferase, blocked by the linkage with TCT molecules. Thus, the main mechanism of action is the inhibition of the polypeptide chain by affecting tRNA and subunits of ribosomal cross-linking. Immediate effects are observed in cellular membrane function, enzymatic activity, and immune function. Rapid proliferation cells such as intestinal, mucosa, lymphoid, and erythrocyte cells, are the most affected by inhibition of protein synthesis. The second cellular groups to be greatly affected are those of the vital organs such as the liver, kidneys, and pancreas. It is thought that the greater toxicity of TCT in young animals occurs in function of the increased metabolism of these animals.

The cytotoxicity of TCT was verified in the erythrocyte membrane but its mechanism of action is not completely understood. Therefore, it is known that the necessary quantity to create a significant effect is much greater than what can normally be detected in the bloodstream of animals affected by TCT toxicosis.

The main effect of the inhibition of nucleic acid production seems to be the induction of a decrease in protein synthesis. The inhibition of RNA seems to occur through a nonspecific secondary mechanism, in other words, as a result of the decrease in protein production whose synthesis is coordinated. The inhibition of DNA production is tied to the lack of new protein, recently synthesized, in order to initiate the S phase of DNA replication. Consequently, the DNA deficiency is also tied to the decrease in protein production and vice-versa. TCT are potent teratogenic and carcinogenic agents because of the damage they pose to DNA. These effects are mainly carried out by products of TCT biotransformation.

Induction of resistance to intoxication by TCT, found in mammalian and yeast cells, is attributed to the alteration of subunits of ribosome 60S, decreasing the affinity to bind with all TCT. Another type of resistance is attributed to a recessive gene, responsible for the codification of protein L-3 of ribosome 60S, which is the largest protein structural constituent of peptidyl transferase enzyme. The mutant protein can increase the resistance of cells to

some TCT up to a hundred times. A second mechanism of resistance was found in cells that do not have ribosomic alterations, thus being less permeable to toxins.

Immunosuppressive effects

TCT belong to a select group of mycotoxins of great immunosuppressive effect. The inhibition capacity of these toxins on protein synthesis, and interaction with cell membrane apparently affects the suppression capacity of the immune system. Acute intoxications present several deleterious effects such as degradation and necrosis of rapid division cells, such as the intestinal mucosa, spleen, thymus, bone marrow, and lymph nodes. The production of immunoglobulin and antibodies decreases. Various experimental intoxications have shown the damaging effects on immune system cells, host resistance, and immunoglobulin production. Chronic intoxication with TCT results in a significant increase of susceptibility to diverse pathogens such as: *Cryptococcus*; *Candida*; *Listeria*; *Mycobacterium*; *Salmonella* and the virus that causes type 1 Herpes. The interaction of TCT with infectious diseases seems to occur when the mechanism of resistance depends on cellular immunity. Likewise, phagocytic capacity decreases significantly when intoxicated with T-2 toxin, which can lead to the occurrence of aspergillosis in afflicted animals. The effect appears to be induced mainly by the decrease in neutrophil migration, phagocytosis of macrophages, decreased organ transplant rejection, and delayed hypersensitivity. The decrease in immunity resulting from intoxication by trichothecenes is evidenced by an increase in specific illnesses caused by opportunistic pathogens, unleashing secondary infections. Furthermore, intoxicated animals show a poor response to vaccinations.

Susceptibility to pathogens increases in chronic TCT intoxications.

The incidence of secondary infections increases when TCT are present.

Clinical signs and lesions

Swine and other monogastric animals are very sensitive to TCT. Nivalenol and DON have similar toxigenic potential; they induce refusal of feed and decrease in weight gain or even weight loss in intoxicated pigs. A combined level less than 0.4 mg/kg of feed is acceptable for swine. However, concentrations greater than 2.0 mg/kg of feed are always unacceptable. Biological responses to TCT occur few minutes after contact. This suggests a

rapid absorption in exposed areas, especially in the gastrointestinal tract and the skin, under natural circumstances of exposure. It should be considered that other mycotoxins produced by the fungi of the *Fusarium* genus such as fumonisins, moniliformin, and zearalenone could also be present in feed consumed by pigs intoxicated with trichothecenes. It is very important to consider the possibility that various trichothecenes mycotoxins could be present in the same feed.

Although the protein synthesis inhibition seems to be the greatest biochemical effect of TCT, it has still not been proven which proteins are significantly inhibited. Nevertheless, they affect cell division in the gastrointestinal tract, skin, lymphoid and erythroid organs. Hemorrhaging observed in animals intoxicated by TCT are due to ulcerations caused by the toxins, associated to a decrease in coagulation factors. Hemorrhaging lesions and clots are frequently observed in the gastrointestinal tract, and may even cause hemoptysis.

Using feed contaminated with more than 2 mg/kg is not recommended for swine.

Complete refusal of feed ingestion can be observed in swine when DON concentrations are greater than 15 mg/kg of feed, and starting at 1 mg/kg of feed a decrease in feed intake can be observed. Feed contaminated with concentrations between 0.3 mg/kg and 0.5 mg/kg merely induce a decrease in weight gain, and feed refusal is not necessarily manifested in a clinically significant form. Concentrations of 1.3 mg/kg are economically significant because of the decrease in animal performance, and levels greater than 2.5 mg/kg decrease weight gain by 50%. Starting at 11 mg/kg, animals show weight loss and complete refusal of feed. Concentrations greater than 20 mg/kg of feed induce vomiting (Figure 28).

Figure 28 – Vomiting caused by ingestion of deoxinivalenol.

The most characteristic clinical symptoms of TCT toxicosis in swine are a decrease or refusal of feed, vomiting within 15 minutes and an hour after ingesting contaminated feed, bloody diarrhea, affected feed efficiency, decrease in weight gain, loss of body weight (beginning when 12 mg/kg of feed is ingested), dermatitis, salivation, hemorrhages, abortions, and nervous disorders. Macroscopic lesions after necropsy are not always clear, although an increase in liver volume, lymphonode hemorrhaging and erosions in the stomach and intestines can sometimes be observed. Characteristic lesions in pigs are rarely observed in outbreaks of natural intoxication with trichothecenes because swine starkly decrease ingestion of feed when the toxin is present.

Only a decrease in appetite and weight gain is normally observed in TCT intoxication.

Deoxinivalenol (DON)

Among the different types of domestic animals, swine are the most susceptible to intoxication with DON, and males are more susceptible than females. Clinical signs characteristic of swine intoxication are: a decrease or complete refusal of feed, vomiting, digestive disorders, and decrease in weight gain, and even weight loss in more severe cases.

Macroscopic lesions caused by DON intoxication are rarely observed in sick animals. However, it has been reported that animals have manifested alterations one week after intoxication, with mild to moderate redness in the fundus of the stomach and small intestine, associated with increased edematous mesenteric lymph nodes.

An increase in liver size in growing pigs was reported at 3.5 mg/kg, and a decrease in plasma protein, albumin, calcium and inorganic phosphorus levels were also observed. However, other researchers have observed a tendency toward a decrease in liver weight in piglets when ingesting DON levels of 1.2 to 3.6 mg/kg, and it has been recently shown that 6 mg/kg significantly reduce the liver size of prepubertal gilts. This discrepancy in the effect of DON on liver size might be due to the presence of additional mycotoxins in the study conducted with growing pigs.

Histological lesions consist of vascular congestion of the stomach and intestinal mucosa. Mild to moderate erosion can be observed in the squamous region of the stomach, with infiltration of lymphocytes and macrophages and multifocal degeneration of epithelial

cells of the fundus. Degeneration and necrosis can occur in lymphoid organs such as Peyer's patches and intestinal, mesenteric and bronchial lymph nodes, accompanied by lymphoid depletion. These alterations can also occur in the tonsils, thymus, and spleen.

T-2 toxin

Moderate intoxications with T-2 toxin usually comprise toxin concentrations of 2 to 8 mg/kg of feed. Young swine are more resistant and can tolerate levels of up to 10 to 12 mg/kg of feed. Nevertheless, concentrations of the toxin that are considered safe for swine are below 0.5 mg of T-2 toxin per kg of feed. The complete refusal of feed normally occurs beginning with 16 mg/kg of feed, but, significant reductions in feed intake occur beginning at 3 mg of T-2/kg of feed.

Lesions caused by T-2 toxin are mainly observed in the upper digestive tract.

T-2 toxin causes lesions mainly in the upper gastrointestinal tract. However, ingesting feed with high concentrations of the toxin can induce diarrhea and perineal lesions because of the contact with residual toxins in feces (Figure 29). This suggests that the toxin has a direct action, without the need of metabolic activation.

Figure 29 – Diarrhea and irritation caused by T-2 toxin in swine.

An increase in the incidence of different types of tumors with an increase in levels of toxin ingested, leads to believe that the immunosuppressive effects of the toxin are specifically attributed to the tumor initiator process effect. T-2 toxin inhibits protein synthesis

in various systems and its toxic effects are evident in cell division, which could explain its teratogenicity. Glucose metabolism is significantly affected in pigs intoxicated by T-2 toxin. Hypoglycemia is caused by improper absorption of carbohydrates and an increase in glucose consumption. The capacity to produce glucose by cortisol using amino acids decreases. With a decrease in glucose concentration, there is also a reduction in fatty acid concentrations in intoxicated animals, which indicates a disorder in energy metabolism. An increase in enzyme serum activity such as aspartate aminotrasferase which can be seen with concentrations of 0.5 mg of toxin/kg of feed, is indicative of hepatic lesions. Pigs intoxicated with concentrations of 2 mg of T-2/kg of feed have a significant reduction of lymphocytes, erythrocytes, and hemoglobin concentrations. A decrease in lymphocyte T concentrations can be seen beginning with contaminations of 0.5 mg of toxin/kg of feed.

Macroscopic lesions on the skin can be observed after a week of consuming feed contaminated with at least 4 mg of T-2/kg. Dermatitis and scabby formations appear preferentially on the skin of the snout, the upper region of the nose, lip commissures, on the posterior region of the ears, and around the prepuce. Similar lesions are also frequent in the oral mucosa and in the dorsal region of the tongue. T-2 toxin also has cardiotoxic properties. Subendothelial hemorrhages and white multifocal myocardial petechiae can be observed at a macroscopic level. At a microscopic level, multifocal edema, mononuclear cell infiltration, hyalinization of myofibrils, vacuolization, and disorganized contractions of strips of myofibrils with pyknotic nuclei can occur. Areas of edema and fibrillar disorganization can be observed at an ultrastructural level. Clinically, intoxicated pigs present a decrease in cardiac output, blood pressure, blood circulation to various organs and systems, as well as an increased heart rate.

Problems with reproduction are rarely observed in pigs intoxicated by normal levels of trichothecenes found in grains and feed, however, experimentally, T-2 toxin causes infertility, abortions, small newborn piglets, and a decrease in the number of piglets per litter.

Nivalenol

Pigs intoxicate with up to 5 mg of nivalenol/kg of feed rarely present a significant decrease in weight gain, clinical symptoms of intoxication, or refusal of feed. Lesions resulting from nivalenol intoxication rarely occur, but, nephropathies and gastric ulcerations have been observed in pigs intoxicated with feed contaminated with 2.5 to 5 mg of nivalenol/kg. After 3 weeks of intoxication, the number of spleen cells and concentrations of IgG decreased significantly, and an increase in IgA concentrations was found.

Diacetoxyscirpenol (DAS)

DAS is one of the most toxic mycotoxins among trichothecenes. As of 2 mg/kg of feed, DAS always presents significant toxic effects in swine. There is a decrease in feed consumption, presumably resulting from lesions in the central nervous system, as well as oral and intestinal lesions caused by the toxin. Anorexia becomes apparent when swine ingest feed containing concentrations greater than 10 mg of DAS/kg. Sublethal intoxication by this toxin results in cell depletion and necrosis of lymphopoietic organs, multifocal necrosis in the intestine, and diffused necrosis in the germinal epithelium, leading to progressive tubular degeneration in the testes. Erosive lesions can be observed in the oral cavity of intoxicated pigs. After a few days of intoxication, linphopenia, neutropenia, and anemia occur.

DAS is highly toxic for swine.

Diagnosis, treatment, and prophylaxis

The presumptive diagnosis is based on observation of clinical signs of the animals intoxicated, and by analyzing environmental data regarding the collection and storage of grains used in swine diets. However, the definitive diagnosis is achieved by determining the presence of the mycotoxin in the feed consumed by affected animals, through analysis.

Species chronically afflicted by TCT have an excellent prognosis, if and when contaminated feed is removed. Specific therapies to treat animals intoxicated by trichothecenes have still not been scientifically proven. Changing contaminated feed for feed free of the toxin should be the first measure taken. Symptomatic treatment and supportive therapy could help the recovery of affected animals. It is advised to provide highly nutritious feed, avoid stress, and control secondary infections, especially when dealing with chronic toxicosis. The most adequate treatment for acute or superacute intoxications could be oral administration of a toxin absorbent, thus avoiding the absorption of the toxin by the gastrointestinal tract, and decreasing enterohepatic circulation of its metabolites. The benefits of adding vitamins and antioxidants in order to prevent intoxication or help the recovery of intoxicated animals have yet to be determined.

Monitoring and using grains free of TCT is the best preventive measure.

One of the main preventive measures is adopting cultivation and management techniques that hamper fungal growth. These include, harvesting grains immediately after physiological maturity, thus leaving them less exposed to weather, and drying and storing them in warehouses suitable for each type of grain and/or their byproducts. Monitoring grains and their byproducts through mycotoxicological analysis before using them in feed is also a good measure to take, especially when grains have been exposed to environmental conditions that favor fungal development.

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6

OCHRATOXIN A

OCHRATOXIN A

Summary

Ochratoxins are part of a group of seven toxic metabolites produces by fungi of the *Aspergillus* and *Penicillium* genera. Although a number of ochratoxins are known, only ochratoxin A is of toxicological importance. It occurs in a large number of cereals and grains, including corn, coffee, beans, and peanuts. Swine are highly sensitive to this mycotoxin, being one of the most toxic for this animal species. Records on natural intoxication of swine by ochratoxin A demonstrate its occurrence in concentrations of 200 μg of the toxin per kg of ingested feed. The kidneys are the main organ affected by this toxin and at a molecular level of this organ there is an interference of DNA and RNA metabolism due to the inhibition of enzyme tRNA synthetase. There is also an interference with mRNA, altering glycogenesis by inhibiting the phosphoenolpyruvate enzyme. The main clinical symptoms of ochratoxicosis are an increase in water intake and polyuria. A decrease in feed intake, worsening in feed conversion and weight gain are all present when feed is contaminated with moderate to high doses of this mycotoxin. During necropsy or during slaughtering of intoxicated animals, kidneys appear to have a pale yellowish or gray coloring and are enlarged. Histologically, renal disease is characterized by tubular degeneration, interstitial fibrosis, and glomerular degeneration, which reduces the reabsorption of electrolytes and proteins and increases urinary excretion by osmotic retention of glomerular filtrate that will be excreted.

Ochratoxin A increases water intake and affects production performance.

History

High incidence of characteristic lesions of swine nephropathy was first recorded in Denmark in 1928. The government determined that all enlarged kidneys should be analyzed for the presence of ochratoxin. Animal carcasses with concentrations of ochratoxin A greater than 10 μg/g in the kidneys, which corresponds to 50 μg/ml of blood, were condemned at slaughter. Significant levels of chicken nephropathy caused by ochratoxin were also found in this country.

In 1957 and 1958 an endemic nephropathy occurred in residents of areas near the Danube River in Yugoslavia, Romania, and Bulgaria (Balkan nephropathy), affecting 3 to 8% of residents, particularly those in rural zones practicing subsistence agriculture and who primarily consumed what they harvested from their farms. Women between 30 and 50 years old were the most affected. Research done in Yugoslavia revealed that 6.6% of people had concentration between 3 and 5 μg/g of ochratoxin in their blood serum. In the 1960s, researchers suggested that the sickness could be related to the ingestion of some toxin produced by plants or fungi. In 1965, ochratoxin was finally extracted and purified from a culture of *Aspergillus ochraceus,* which gave it its name. Up until that point, researchers had not been able to establish a direct relationship between its incidence in food and the occurrence of nephropathy. In 1974, researchers found very strong evidence that the illness was caused by the ingestion of ochratoxin present in food. It was soon discovered that ochratoxin occurred in food sources and feed, and from that point it was considered to be the etiologic agent of nephropathy, in both swine and humans, thus explaining what had occurred in the Balkans.

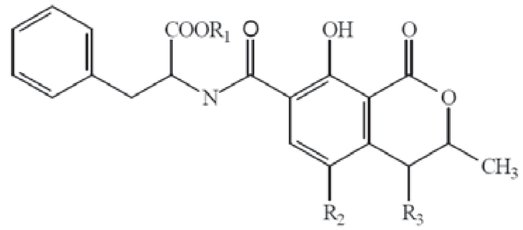
Etiology

Ochratoxins are part of a group of metabolites with an isocumarin structure linked to the amino acid L-β-phenylalanine (Figure 30). It is highly soluble in organic solvents and is slightly soluble in water. There are at least seven known ochratoxins, but only ochratoxin A seems to have significant toxicological importance. The co-occurrence of ochratoxins B and C has been found in small proportions. Food samples containing 16 mg/kg of ochratoxin A can have close to 8% and 2% of ochratoxin B and C, respectively. Once produced, ochratoxin A is highly stable during food processing. Even when subjected to temperatures of 250°C, the toxin is not completely destroyed. The half life of the toxin in low humidity wheat was determined 700 minutes at 100°C, 200 minutes at 150°C, 12 minutes at 200°C, and 6 minutes at 250°C. When wheat had high humidity levels, the half life is less: 145 minutes at 100°C, 60 minutes at 150°C, and 18 minutes at 200°C. The stability of ochratoxin during fermentation has been widely studied. It has been determined that losses can be significant, degrading up to 70% of the toxin present. Approximately 98% of ochratoxin A can be degraded by rumen microorganisms, especially by protozoa that produce ochratoxin α. Ochratoxin is also highly stable in pork muscular, kidney and liver tissue, with minimal losses when cooked.

Ochratoxin A is the etiologic agent of Balkan nephropathy.

Ochratoxin is stable at high temperatures.

Figure 30 – Chemical structure of ochratoxins.



Types of ochratoxin	Radicals		
	R_1	R_2	R_3
Ochratoxin A	H	Cl	H
Ochratoxin B	H	H	H
Ochratoxin C	C_2H_5	Cl	H
Ochratoxin A methyl ester	CH_3	Cl	H
Ochratoxin B ethyl ester	C_2H_5	H	H
Ochratoxin B methyl ester	CH_3	H	H
4-Hidroxiochratoxin A	H	Cl	OH

Occurrence of ochratoxin A in feed and food

Ochratoxin A is produced by a single species of fungus, *P. verrucosum,* belonging to the *Penicillium* genus. However, it is also produced by fungi of the *Aspergillus* genus, especially *A. ochraceus* and other related species such as *A. carbonarius, A. alliaceus, A. auricomus, A. glaucus, A. melleus,* and a small number of strains of the species *A. niger*. These groups differ in their ecological niches, products affected, and frequency of their occurrence in different geographical regions. *P. verrucosum* only grows at temperatures below 30°C with low water activity levels, close to 0.8. It is therefore only found in temperate regions with low temperatures, and it is the only source of ochratoxin A in grains and their byproducts in Canada and Europe.

Grains are extensively used in animal feed in Europe, and because ochratoxin A is relatively stable during animal metabolism, this mycotoxin can also be found in some animal products in Europe, especially in pork liver and kidneys. *P. verrucosum* does not occur in the tropical or subtropical regions. Therefore, grains of those regions rarely contain high levels of ochratoxin A. *Aspergillus ochraceus* preferentially grows in climates with moderate temperatures with water activity levels above 0.8, found sporadically in large scale food storage, including grains, but rarely is the cause of substantial concentrations of ochratoxin A in food. It can also contaminate coffee beans during solar drying process, and it is a source of ochratoxin A in coffee beans while the fruit is still immature. *A. carbonarius* grows in high temperatures, and it is widely related to fruit contamination during ripening, especially grapes. It is highly resistant to light and survives solar desiccation because of its black spores. It is the source of ochratoxin A in fresh grapes and apples, dried fruit, wine, and coffee.

Ochratoxin A is essentially restricted to countries of the northern hemisphere. Thus, prevalence rates have been recorded between 5.6% to 45.0% in grains cultivated in Germany, 14.3% in the Czech Republic, 14% in grains and 13% in feed destined for swine in Poland, in England 20% of wheat and up to 78% of inadequately stored grains were contaminated, and in Canada 5.3% of grains show positivity for ochratoxin A.

The assessment of contamination as a result of heterogeneous distribution of ochratoxin A in agricultural products becomes more feasible when evaluating the presence of the toxin in blood, especially in serum. This evaluation becomes much more representative because of the ability of ochratoxin A to remain present for a period of 30 days, linked to blood protein, which facilitates diagnosis.

In more than 30 thousand batches of feed from Brazil that were evaluated in LAMIC/UFSM, the presence of the toxin was only found in 4% of samples, with only one sample contaminated with more than 500 ppb.

Toxicity of ochratoxin A

According to findings by the International Committee of Risk Evaluation to Mycotoxin Exposure, ochratoxin A is highly toxic. Therefore, the Committee provisionally established that the maximum tolerable limit of ochratoxin A for humans would be 112

ng/kg/week. This limit was establish based on the maximum level of ochratoxin A tolerated by swine without showing deterioration in renal function, which is 0.008 mg/kg of live weight/day, which corresponds to approximately 0.2 mg of toxin per kg of feed. Lethal dose 50 (LD_{50}) values of ochratoxin A for some animal species can be seen in Table 16.

Table 16 – Lethal dose 50 (LD_{50}) values of ochratoxin A, orally administrated.

Species	LD_{50} (mg/kg of body weight)
Dog	0.2
Mouse	46-58.3
Chicken	3.3
Duckling	0.5
Rat	20-30.3
Newborn rat	3.9
Swine	1.0

Harwig *et al.* (1983).

Toxicokinetics of ochratoxin A

Absorption

Most domestic animal species rapidly absorb ochratoxin A through the stomach because of its acidophylic properties (pKa = 7.1). However, the greatest amount of toxin is absorbed through the small intestine, especially by the proximal jejunum portion. In this region, ochratoxin A can even be transferred against a concentration gradient when the toxin is present in its liposoluble non-ionized form, depending on the pH of the mucosa surface.

The toxin is principally eliminated through the bile, which is quickly reabsorbed. Its reabsorption is also highly efficient in the active ion transport system of the proximal and distal convoluted tubules of the kidneys. The total quantity of ochratoxin A observed along the digestive tract is of approximately 66% in swine, 56% in rat, and 40% in chicken. Oscillation of toxin concentrations in blood plasma is common since biliary excretion is elevated causing immediate intestinal reabsorption of the toxin, characterizing ochratoxin A enterohepatic circulation as very effective. Absorption through the lungs also seems to be very efficient since absorption in animals inoculated with pure

Ochratoxin A can be absorbed by the respiratory system.

crystallized toxin through the traquea was estimated to be 98%, with a half life of approximately 127 hours.

Distribution

Ochratoxin A reaches the bloodstream, immediately bonding with proteins, especially serum albumine and other macromolecular structures. Once ochratoxin A is absorbed, concentrations of the toxin and its biotransformation products in the tissues and residue in plasma depend on a series of important factors. This is important in order to evaluate the natural occurrence of residues in animal tissues. The main factors are related to the amount of time that the animal ingested contaminated feed, the concentration of the toxin in the feed, if the ochratoxin A contamination is natural or purified, the degree to which the toxin binds to proteins, the plasmatic half-life, and the amount of time the animal received a diet free of ochratoxin A before slaughtering.

The association constant for the binding of ochratoxin A to serum albumine varies among different species of domestic animals. The association constant in swine is 7.1 x 104/mol, 5.1 x 104/mol in chickens, and 4.0 x 104/mol in rats. The fraction of ochratoxin A bound to serum albumine and other macromolecules constitutes a mobile reserve of mycotoxin, becoming available to release into tissues after a long period of time. Studies done with animals that have low concentrations of albumin, show that they eliminate the toxin quicker. This indicates that the transference of ochratoxin A from the bloodstream to hepatic and kidney cells is facilitated when it is not bound to this protein. Therefore, in swine, the rate of detoxification of ochratoxin A from the blood is slower than from the kidneys, liver, and other tissues. The fraction of ochratoxin A that does not bind to plasma proteins is approximately 0.02% in humans and rats, 0.08% in monkeys, 0.1% in mice and swine, and 22% in fish.

After administering oral doses, maximum concentrations of ochratoxin A in the serum were found in intervals of 10-48 hours in swine and rats, 2-4 hours in bovines, 1 hour in rabbits, and 33 minutes in poultry.

The distribution and partial accumulation of ochratoxin A occurs primarily in the kidneys, followed by muscle, liver, and fat tissue, respectively. Only traces of the toxin can be found in erythrocytes. It is possible to determine ochratoxin A in the blood of fetuses of

intoxicated sows, which reached levels of around 50% of those of the pregnant female. Large differences in the plasmatic half-life of ochratoxin A were observed in different species when moderate oral doses of the toxin were administered; 510 hours in monkeys, 72-120 hours in swine, 55-120 hours in rats, 6.7 hours in quails, and 4.1 hours in chickens.

Biotransformation

Ochratoxin A is biotransformed into atoxic molecules by various organs through hydrolysis, principally producing ochratoxin α and L-phenylalanine. Cecum bacterial flora plays an important role in the hydrolysis of the molecule involving chymotrypsin and carboxypeptidase enzymes. Ochratoxin α has high levels of enterohepatic circulation. Therefore, much of it is eliminated through the urine, up to 30% of the toxin administered orally. Another product of ochratoxin A biotransformation is 4-OH (4R and 4S), which is less toxic than the original form. It is produced in the liver or in the kidneys of different species. This process of detoxification is carried out by cytochrome P450 enzymes. Ochratoxin C, a product of ochratoxin A biotransformation, which can be produced in rumen fluid, is as toxic as ochratoxin A. Ochratoxin B, a chlorinate derived from ochratoxin A, can occur with ochratoxin A in grains. It is less toxic than ochratoxin A, biotransformed into 4-OH-4-OH-ochratoxin B and ochratoxin-β.

Elimination

The elimination of ochratoxin A through bile is very efficient. However, enterohepatic circulation is constant, restoring a large quantity of the toxin excreted by the bile, which is the main path of elimination of the toxin its biotransformation products. The relative contribution of ochratoxin A excretion in all species is influenced by the degree to which the toxin binds to blood macromolecules, as well as by the level of enterohepatic circulation. Elimination seems to be proportionately quicker in animals fed with diets containing low concentrations of ochratoxin A.

Residues

Residues of ochratoxin A are rarely detected in ruminants because the toxin undergoes hydrolysis by the action of rumen microflora, producing ochratoxin-α and L-β-phenylalanine. It can be found in the tissues of swine and chicken. Chicken fed diets

containing 2 mg of ochratoxin/kg for a period of 8 weeks had concentrations of the toxin in the liver and kidneys, but not in fatty tissues. Swine had a harder time eliminating ochratoxin A, and had the highest concentrations of the toxin in their blood, followed by the kidneys, lean meat, liver, and fatty tissue. When given feed containing 1 mg of ochratoxin/kg, swine eliminate the toxin to non-detectable limits in the kidneys, liver, and plasma in 4 days. Studies show that animals need to be fed diets free of ochratoxins for a minimum of 30 days before being slaughtered in order to guarantee that the carcass will be free of residues.

Ochratoxin A free diet should be given 30 days before slaughter to guarantee the absence of the toxin in tissues.

Research done with 9,132 samples of serum taken from swine from 7 European countries show that 38% of the serum sampled has detectable levels of the toxin. Residues of ochratoxin can also be detected in human tissues. Studies done in Germany with blood serum from 306 people showed that 56% were positive for the toxin. It is believed that this high level of positivity is a result of constant exposure to contaminated food such as contaminated grains and contaminated animal products such as meat and milk. Egg consumption could also be an important source of contamination, given that layers that ingest high levels of ochratoxin also eliminate it through eggs.

Pathogenesis of ochratoxin A

The primary effect of ochratoxin A is related to the inhibition of protein production. It decreases the synthesis of RNA and the formation of DNA adducts. These effects are attributed to the phenylalanine chain of ochratoxin A molecule, which competes and inhibits phenylalanine-tRNA enzyme synthesis which is of fundamental importance in protein chain initiation process. This deleterious effect is reduced when the cell is filled with L-phenylalanine, since animals treated with L-phenylalanine before intoxication show significantly lower lethal effects. It has been concluded, because of this prophylaxis, that the inhibition of protein synthesis is the main cause of acute toxicity produced by ochratoxin A. One of the principal chronic effects of ochratoxin A is related to its carcinogenic properties, which were found in various species. In humans, this property has solid epidemiological findings associated with evidential studies performed *in vitro.* Based on this information, the International Agency on Cancer Research (IARC) classified ochratoxin A as a possible human carcinogenic (Class 2B).

Secondly, ochratoxin A also interferes in carbohydrate metabolism, particularly gluconeogenesis. It affects the codification of renal mRNA, inducing a decrease in the production of phosphoneolpiruvate carboxykinase, an important enzyme in gluconeogenesis. This enzyme acts as the energy source of decarboxylation of oxaloacetate to phosphoenolpyruvate. It also has an important function in the conversion of intermediate components of citric acid into glucose and glycogen. Gluconeogenesis is one of the main metabolisms of carbohydrates in the renal cortex. Since the enzyme phosphoenolpyruvate carboxykinase is reduced, these reactions are affected, leading to serious injuries in the nephron, and renal dysfunction, which is characteristic of ochratoxin A intoxication. Changes in glomerular filtration and impaired function of the proximal convoluted tubules lead to the loss of ability to concentrate urine, causing glucose and protein levels in the urine to increase. Ochratoxin A interference in the metabolism of cellular proteins, DNA, and RNA confer also immunosuppressive characteristics to this mycotoxin.

Lipid peroxidation induced by ochratoxin A occurs in various organs and systems, predominantly affecting the functions of cell membranes. Mitochondria membrane is also severely damaged, significantly impairing metabolic changes, cell respiration, and energy production.

Clinical symptoms and lesions of swine ochratoxicosis

Ochratoxin A intoxications generally occur in the spring and summer months. On some farms, intoxication occurs randomly but in these cases the disease is usually associated with feed or feed ingredients that have had serious storage problems at some stage of conservation. Pigs become extremely thirsty, increasing water consumption up to four folds when acute intoxication is present (more than 1 mg of ochratoxin A per kg of feed). Consequently polyuria, which is characteristic of the intoxication, occurs. Frequently, animals drink their own urine, especially when water is not readily available. These symptoms can be accompanied with a decrease in feed consumption, poor feed efficiency, diarrheal diseases, and bloody urine.

Polydipsia and polyuria are characteristic of ochratoxin A intoxication.

Doses of 200 µg/kg of feed were sufficient to cause nephropathy in animals, leading to negative effects on feed conversion and weight gain. In these cases, clinical symptoms of

ochratoxicosis are very discrete. They are followed by reduction of feed intake and weight gain. Polydipsia, polyuria, and lumbar and abdominal depletion were also observed one or two days preceding death. Death rates can reach up to 90% in affected herds. Furthermore, there is a possible interference with sperm quality in males.

Macroscopic lesions

Although different organs can be affected by swine ochratoxicosis, the kidneys are the most affected, presenting alterations such as the following: enlargement, pale-yellowish coloration or mottled, as well as cystic or fibrotic characteristics (Table 17). When kidney disease reaches its final stage, from the formation of moderate to severe fibrosis, the renal surface has undulations or roughening due to a reduction of its internal mass.

Table 17 – Classification of kidneys with nephropathy resulting from ochratoxin A according to macro and microscopic alteration in 506 swine at slaughter. Listed in chronological order of intoxication development in natural outbreak. (Stoev *et al*., 1998).

Group	Kidney Characteristics	Enlargement (%)	Number of cases	Frequency (%)
1	Mottled	Up to 20	230	45.40
2	Enlarged - mottled	20-200	210	41.50
3	Enlarged and pale	100-300	40	7.91
4	Cystic	± 100	20	3.95
5	Fibrotic	20-60	6	1.19

Group 1 kidneys were slightly enlarged and were a bit more pale than normal. They also had some clear spots on their surface measuring 1 to 2 centimeters in diameter as well as pale streaks on the renal parenchyma oriented toward the pelvis. Density was somewhat affected and was normal or slightly high when palpated.

Group 2 kidneys were mottled and their density was slightly high. They had white-grayish spots, sometimes confluent, thus spanning a large part of the surface. The spots were

usually confined to the cortex, but were able to penetrate the marrow. The accession of the renal capsule to the parenchyma was often evident.

Group 3 kidneys were pale or white-grayish in color and extremely enlarged, reaching a cylindrical shape. Density at palpation was significantly high, and there was rippling of the surface.

Group 4 kidneys were double the size of normal kidneys of pigs of the same age, and were white-grayish or mottled. Density at palpation was high, with wavy or rough surface in some cases. Cysts on the renal surface that were 2 mm to approximately 5 cm in diameter were detected. These cysts contained clear or slightly murky liquid derived from dilatation of tubules whose epithelial cells were atrophied.

Group 5 kidneys were slightly enlarged in older reproductive animals, had a white-grayish hue, and very protruding ridges. At palpation, kidneys were much more dense than normal.

Renal lymphatic nodes of groups 2 and 3 presented nodular or diffused reactive processes, while group 5 kidneys and in some cases those of group 3 and 4 had trabecular connective tissue proliferation due to a reduction of lymph tissue.

Microscopic lesions

The main microscopic lesions associated with swine ochratoxicosis occur in the kidneys. Initially two types of alterations occur: degradation that affects epithelial cells of proximal convoluted tubules, and proliferation of connective tissue infiltrating mononuclear cells in the interstice. The beginning of the degeneration of epithelial cells of proximal convoluted tubules occurs through karyolysis, plasmolysis, cariopicnose and increasing size of the nucleus or nuclear vesicles. Eventually, granular degeneration, hyaline droplets, or vesicular degeneration can be observed. In more advanced stages of the intoxication, necrosis and desquamation of the epithelium of the tubules affected is very common. Frequently, the lumen of the proximal convoluted tubules shows necrotic material accompanied by grainy or hyaline material. One of the few glomerular alterations is related to the dilatation of the capsular space containing serous fluid. The interstitium shows mild focal edema and focal limited growth of fibroblastic tissue and infiltration of mononuclear cells around the damaged tubules. At that stage, lesions characterize an acute nephrosis. The advancement of the

pathology brings on an increase in the amount of tissue fibrin, lymphocytes, monocytes, and plasma cells. The renal interstitium can contain lymphatic cysts containing serous fluid and hemorrhagic petechiae which are usually concentrated in the subcapsular region. At a still more advanced stage, the distal convoluted tubules show atrophic changes and the first changes of glomeruli are found. They are characterized by hyalinization and sclerosis. Most of the glomeruli, especially those located in the region of greatest proliferation of connective tissue, immediately become atrophied, hyaline, or sclerotic.

Although the main organ affected by ochratoxin is the liver, concentrations greater than 5 mg/kg of feed can lead to degenerative alterations in other organs. The most frequent are related to enteritis, necrosis of lymphoid tissues and degeneration of fat, all in the liver.

Clinical Pathology

Although ochratoxin A mainly affects the kidneys, causing significant increase in serum enzyme and blood component activity, such as decrease in protein concentrations, urea, cholesterol, triglycerides, and potassium, diagnosing ochratoxicosis is done by analyzing ochratoxin A concentration in serum. The evaluating method of ochratoxin A intoxication is the quantification of circulating ochratoxin associated with clinical symptoms, especially endemic regions. Alterations in volume and composition of urine are also characteristic signs of swine ochratoxicosis. Polyuria, an increase in eliminated urine quantity, is also significant. The main alterations in the composition of urine are related to an increase in protein and glucose concentration. The presence of blood can indicate serious renal lesions at nephron levels, or the presence of bloody tumors induced by chronic ochratoxicosis.

Diagnosing ochratoxicosis is easily done by detecting toxin levels in plasma.

Effects on the immune system

Ochratoxin A is eliminated from the organism 3 to 4 weeks after contaminated feed is removed. The difficulty in quickly eliminated the toxin from the organism contributes to the highly toxic property of ochratoxin A in various organs and systems. It has immunosuppressive properties in fetuses, young animals, and adults. The main effects are related to a decrease in phagocytosis and the production of lymphocytes.

Vaccine failure may be associated with ochratoxicosis.

Therefore, susceptibility to bacterial infection significantly increases. The efficacy of vaccines is also significantly affected by ochratoxin A, especially in young pigs.

Diagnosis of intoxication

Clinical diagnosis of ochratoxicosis can be done in endemic regions. Thirst and polyuria suggest the presence of the toxin in feed. Alterations of enzyme concentrations such as gamma-glutamyl transferase and phosphoenolpyruvate may also aid in the diagnosis. Ochratoxin has peculiar pharmacokinetic properties. Because of the high stability and relatively long half-life of ochratoxin A in blood, it can be detected in plasma/serum for a period of 30 days when a single dose is ingested with the diet. Thus, the quantification of this mycotoxin in plasma has a high diagnostic value because it is possible to use the blood of a single animal to determine the presence of the toxin in the whole herd, since all consume the same feed.

Ochratoxin A can be detected in feed, plasma, and tissues. The methods of diagnosis are ELISA kits analysis, Thin Layer Chromatography, and preferably High Performance Liquid Chromatography, which detects levels of up to 100 ppt (ng/kg). More precise equipment and methods such as mass spectrophotometry coupled with liquid chromatography are currently being used to detect the toxin.

Prophylaxis and treatment of ochratoxin A intoxication

Prophylactic measures are the only clearly effective means to minimize the damaging effects of all mycotoxins, including ochratoxin A, especially in endemic regions. Possible practices to minimize the effects of ochratoxicosis include analyzing samples of feed, using adequate techniques, giving feed to more resistant animals and using adsorbents. Only absorbents which have been proven to be effective in reducing toxicity through *in vitro* and *in vivo* experiments should be used. Contaminated feed could also be given to ruminants, since it is know that the species has ruminal microflora which is responsible for the degradation of large portions of the molecules of this mycotoxins.

There is no specific treatment for pigs intoxicated with ochratoxin A. However, some measures should be implemented immediately in order to reduce the toxic effects in animals that have been exposed to the mycotoxin. Changing contaminated feed for one free of

ochratoxin A should always be the first measure taken. Supportive treatments can be used to improve the organic conditions of the affected animals.

Currently, studies are being conducted with the objective of reducing the half-life of ochratoxin in the bloodstream of intoxicated animals. Trials with orally administered phenobarbital in doses of 80 mg/kg of live weight for 5 days or oral administration of 20 mg of 3 metylcolantrene/kg of live weight for 2 days make animals more resistant to ochratoxin A intoxication. They will also significantly decrease LD_{50} levels. More detailed studies are necessary in order to increase the efficiency of these products used to prevent or treat swine

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7

OTHER MYCOTOXICOSIS IN SWINES

OTHER MYCOTOXICOSIS IN SWINE

Among the hundreds of other mycotoxins that can occur in swine feed, there are some that have been cited by the literature as having importance or causing deleterious effects when administered individually or having synergistic effects when associated with other mycotoxins (Table 18).

Table 18 – Main toxin effects and fungal producers of some mycotoxins.

Mycotoxin	Main mold producer	Main effects in swine
Ergot alkaloids	*Claviceps purpurea*	Gangrenous necrosis, agalactia and reproductive disorders.
Cyclopiazonic acid	*Aspergillus flavus* *Penicillium commune*	Digestive and central nervous system disorders.
Fusaric acid	*Fusarium moniliforme*	Digestive and immunological disorders.
Citrinin	*Penicillium citrinum*	Nephrotoxic.
Stachyobotryotoxin	*Stachybotrys alternans*	Digestive tract hemorrhages and necrosis, and leucopenia.
Sterigmatocystin	*Aspergillus flavus e* *Aspergillus parasiticus*	Hepatic lesions.
Phomopsins A and B	*Phomopsis leptostromiformis*	Jaundice and hepatic lesions.
Moniliformin	*Fusarium moniliforme*	Cardiotoxic.
Oosporein	*Chaetomium trilaterale*	Nephrotoxic, inducing visceral gout.
Rubratoxin	*Penicillium rubrum*	Hepatic lesions (necrosis) and disseminated hemorrhages.

ERGOT ALKALOIDS

History and etiology

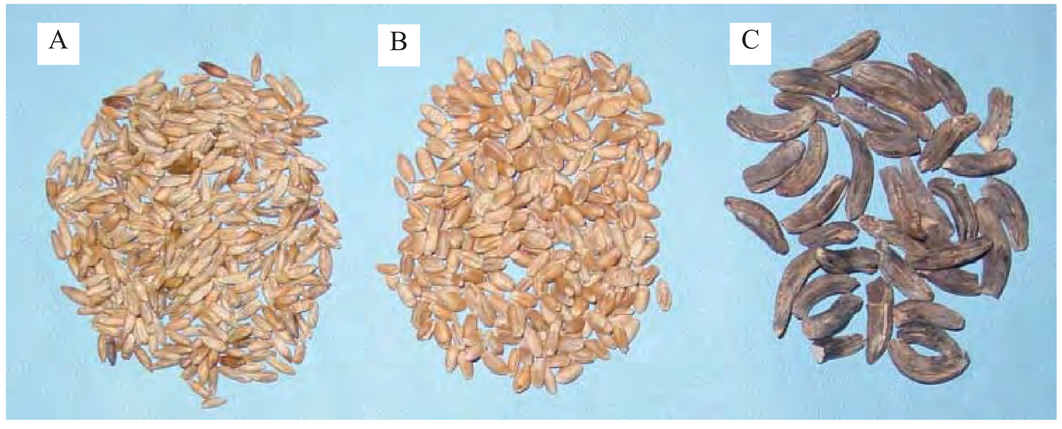
A focal point in the evolutionary history of mycotoxicology is the episode called "Saint Anthony's Fire," which occurred between the XI and XVI century, affecting various European countries, especially France. The illness was characterized by gangrene outbreaks in

populations that consumed grains contaminated by sclerotia of the fungus *Claviceps purpurea* (spurred rye) or other less frequent species. The illness is caused by the vasoconstrictive properties of ergot alkaloids which hamper peripheral circulation. It also has oxytocin properties, which increases stimulation of the central nervous system followed by depression.

The formation of sclerotia occurs when mold ascospores invade flower ovaries of different gramineae plants, where they develop, producing a yellowish-white mycelium mass. The sclerotia preserves some of the characteristics of the seed, however, it increases its size and renders it angular, similar to rooster spurs. External characteristics may vary greatly, depending on the different *Claviceps* species, reaching between 3 mm and 8 cm in length, with a black or dark brown appearance, and a hard consistency. The weight of 100 units varies between 8 and 24 grams, displaying different shapes: elongated, curved, or rounded (Figure 31).

Ergot alkaloids occur mainly in gramineae plants.

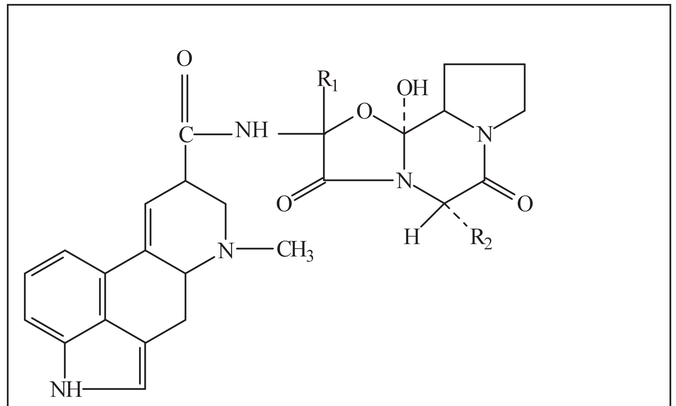
Figure 31 – (A) Rye (B) Wheat (C) Ergot sclerotia which can occur in wheat or rye.



After grain maturation, scleration fall to the ground where they remain viable. Germination and release of ascospores which invade the ovaries and stigma of the plant can occur after a few months of adequate temperatures and humidity conditions. Spore formation occurs within a few days. Mature sclerotia quickly form, with many ascospores, which replace the seed. Toxicologically, sclerotia have different chemical substances. They are called ergot alkaloids and its basic structure is composed of a tetracycle called ergoline, which is a substance derived from indole (Figure 32). According to its chemical properties, alkaloids are subdivided into three groups of toxins: those derived from lysergic acid, those derived

from clavine, and those of peptide composition. The most important are derived from lysergic acid and dimetylalgoline alkaloids. The main *Claviceps purpurea* alkaloids, and pharmacologically most important are ergocornine, ergocristine, ergocriptine, and ergotamine. Different toxins can be produced by a variety of fungi, such as: *C. fusiformes, C. paspali, C. africanus, C. sorghi* and fungi of the *Neitiphodium* and *Epichloe* genera.

Figure 32 – Molecular structure of four Ergot Alkaloids.



	R_1	R_2
Ergocornine	$CH(CH_3)_2$	$CH(CH_3)_2$
Ergocristine	$CH(CH_3)_2$	CH_2-C_6H_5
Ergocriptine	$CH(CH_3)_2$	$CH_2CH(CH_3)_2$
Ergotamine	CH_3	CH_2-C_6H_5

Occurrence

The occurrence of *Claviceps* spp and sclerotia are universal, primarily affecting countries that have temperate climates during spring, when seeds are forming. The development of *Claviceps purpurea* and production of ergot alkaloids occurs when fungal ascospores infect the inflorescence of gramineae plants such as rye, rice, wheat, barley, oats, sorghum, corn, and other crops used for grazing. Infested grains generally have proportions of sclerotia to non contaminated seeds less than 5%. The concentrations of ergot alkaloids in spurred grains can vary greatly, generally inferior to 0.9%, depending on the site and host plant species. The concentration of the different toxic subtances also varies greatly. There are

known occurrences of ergotamine exclusively in rye used for pasture, but the main toxic principle (71%) found in rye seeds has been ergocristine.

Mechanism of action, clinical signs, and lesions.

Ergotamine has considerable biological activity, being one of the most important alkaloids produced by *Claviceps* spp. Ergot alkaloids, contained in sclerotia, ingested through feed present an absorption rate of approximately 90%. The effect on feed digestion is generally insignificant. Pig metabolism is very efficient in biotransforming ergot alkaloids, which are eliminated quickly, especially through urine. Detectable concentrations are rarely found in the tissues of organs of intoxicated animals. An increase in water intake by intoxicated pigs is apparently due to the unpleasant flavor of the contaminated feed. The increase in water levels in the intestines probably increases feed solubility and absorption. In addition, it should contribute significantly to the renal elimination of the biotransformation products.

The prevalence of ergotamine use in human medicine is due to its oxytocic and vasoconstrictive properties. Clinical symptoms in different species include ischemia of the extremities, such as ears, snout, legs, and tail. Severe outbreaks include the presence of gangrene, which frequently leads to the loss of extremities and/or organs due to degenerative and necrotic processes. Heart rate decreases and blood pressure increases with the increase in quantity of alkaloids ingested.

Symptoms of human intoxication by ergot alkaloids may lead to convulsive ergotism, manifested in muscular tremors, spasms, mental incapacity, hallucinations, sweating, and fever for several weeks, suggesting a stimulating action of serotonin, since it is known that ergot alkaloids are serotonin agonists. Bovine display four syndromes derived from ergotism intoxication that are clearly defined: hyperemic syndrome, ergotism gangrenous skin, convulsion syndrome, and reproductive syndrome. The toxic effects of ergot alkaloids are less severe in swine than in bovine.

Gangrene and lesions on the extremities are characteristic symptoms of ergot alkaloids intoxication.

Sows exposed to ergot alkaloids present retardation in embryonic development, reabsorption, abortion, and increased perinatal mortality of the litter. Gangrenous ergotism has been diagnosed in piglets of intoxicated sows, even though hypogalactia or agalactia are always the first clinical symptoms of the intoxication, which can be produced with a single dose of ergocriptine administered orally 24 hours after delivery. It is believed that the primary effect is the inhibition of prolactin. A decrease in the size of the nipples occurs concurrently with a decrease in milk production. Dysthermic syndrome, which occurs because of the inability to dissipate heat, can occur in all intoxicated species.

Severe reproductive effects can be observed in ergot alkaloid intoxication.

Swine intoxicated by ergot alkaloids can present lesions in the internal organs. The stomach mucosa can have an edematous appearance, often scaly, which frequently lead to ulcerations. The small intestine can exhibit hyperemia, enteritis, and sometimes, hemorrhaging. Necrotic areas can be seen in the liver, often extending to 30% of the organ. The heart wall may have petequial hemorrhaging and there may be bloody streaks in the muscle. Mesenteric lymph nodes, lungs, and kidneys rarely have lesions that can be detected macroscopically. Hematologic alteration or changes in coagulation time are rarely observed. Animals display an increase in nitrogen elimination through the urine, resulting in a significant decrease of nitrogen in serum. Nonetheless, clinical symptoms in intoxicated animals are very dependent on the concentrations of each active principle of the sclerotia ingested. Since various concentrations of several alkaloids can be found in infested seeds, clinical symptoms can vary significantly between outbreaks.

Various effects of ergot alkaloids on the central nervous system have been scientifically proven. Recently, purified derivatives of ergotamine were used to treat headaches, Parkinson, and were used as prolactin inhibitors. However, the effects of ergot alkaloids on the central nervous system of swine are of little significance. The neurological symptoms present in intoxicated pigs are similar to those observed in other domestic species. The symptoms are caused by the vascoconstrictive properties of ergotamine and ergocristine, which cause ischemia of the central nervous system. Convulsion syndrome can also be present, generally accompanied by hypersensitivity and ataxia.

Diagnosis

The clinical signs that appear in pigs that have ingested ergot sclerotia depend on the alkaloids present, the quantity ingested, and the amount absorbed. There is a decrease in ingestion of contaminated feed, with partial or complete refusal of feed, especially when the contaminant is ingested for the first time. These effects tend to significantly diminish as pigs adapt to ingesting contaminated feed. Fluctuations in the quantity of feed ingested can also depend on the quantity of alkaloids present in the sclerotia and on the presence of other contaminating fungi, which reduce the palatability of the feed.

Effects depend on the quantity and chemical makeup of the sclerotia ingested.

The clinical signs of ergot alkaloid intoxication are not always characteristic, especially when related to reproductive disorders. External lesions observed in other intoxicated species are not characteristic in swine. It is possible to see lesions on internal organs, depending on the quantity of each alkaloid contained in the sclerotia. The existence of lesions and assessment of clinical symptoms can be important in reaching a presumptive diagnosis. The presence of sclerotia in feed is very significant as a diagnostic tool. Concentrations between 1 and 2% of sclerotia in feed cause a significant decrease in feed ingestion as well as in weight gain. Rates of 0.05 to 0.1 % of sclerotia in the diet also moderately decreases feed consumption in swine when ergocristine is the main alkaloid present.

The presence of 0.05% of spores in feed can cause a decrease in appetite.

Treatment

There is no specific treatment for swine intoxicated with ergot alkaloids; however, secondary infections must be treated. Treatment such as administering intravenous fluids can improve the prognosis of intoxicated animals. Different treatments have been used unsuccessfully in an attempt to decrease ischemia that alkaloids induce in the extremities. Treatment with intra-arterial sodium nitroprusside and hydroxycobalamin associated with increased diuresis and hydration seem to be effective in reducing the adverse effects of intoxication by ergot alkaloids. However, the most suitable option is the removal of the contaminated feed.

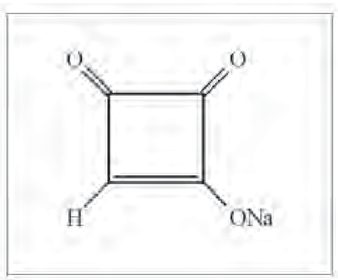
MONILIFORMIN

Moniliformin is a mycotoxin produced by a limited number of species from the *Fusarium* genus, such as *F. moniliforme, F. proliferatum, F oxysporum, F. sporotrichoides, F. acuminatum, F. chlamydosporum, F. graminearum, F. solani* and *F. equiseti*. These molds, under favorable ecological conditions, such as water activity above 0.9 and mild temperatures, can develop in vegetables and grains that are cultivated around the world.

Etiology

Moniliformin is a crystalline, hydrosoluble (hydroxycyclobutanedione) substance, with a sodium or potassium radical (Figure 33). In order to handle it in a laboratory environment and analyze the toxin, it is necessary for it to be stable, especially when it is in the form of sodium salt, which is less stable than potassium salt. Good stability is achieved by adding tetrabutylammonium dihydrogen sulfate to the solvents. The toxin is moderately stable in grains stored at room temperature. Six days after milling of naturally contaminated corn and wheat, 30% of the mycotoxin can be lost. Moniliformin is less stable under conditions of high temperatures. Losses of up to 62% were observed in corn and 85% in wheat when these were exposed to temperatures between 100 and 150°C, respectively, for two hours. Moniliformin is more stable in acid pH, so that when the toxin is exposed to pH 4.0 for 60 minutes at 150°C, there is a 5% loss, whereas when exposed to pH 10 at the same temperature for the equal amount of time, losses can be higher than 80%.

Figure 33 – Chemical structure of moniliformin



Occurrence

The natural occurrence of moniliformin in different grains has been investigated. It has been found that this mycotoxin is prevalent in practically all grains and cereals used in swine feed, especially corn, wheat, rye, triticale, oatmeal, rice, and sorghum. The incidence of the toxin in samples of corn has been found at concentrations of up to 25 mg/kg, but the most prevalent levels reach up to 2 mg/kg with a positivity of approximately 70% of samples analyzed by different researchers. Concentrations greater than 400 mg/kg have also been found in naturally contaminated corn, reaching up to 645 mg/kg in corn exposed to conditions favorable for fungal development. There is evidence to suggest that the highest levels of moniformin are found in sorghum, which naturally presents the toxin, even after proper care and handling of seeds. More than 50% of samples contaminated with moniliformin are concurrently contaminated with other mycotoxins such as zearalenone, fumonisins, and trichothecenes.

Mechanism of action

The mechanism of pathological action of moniliformin, after ingested by animals, consists of the suppression of piruvate dehydrogenase and α-ketoglutarate dehydrogenase enzyme system. Thus inhibiting the incorporation of piruvate to the Krebs cycle, causing alterations in the cell membrane, oxidative damage in the cells, a decrease in osmoregulatory functions, and inhibition of cellular respiration. The main consequences of these alterations are acute multifocal degeneration, myocardial necrosis, and heart attack.

Clinical signs and lesions

Moniliformin can induce acute toxicity, especially in young animals, even when the mycotoxin is present in feed at relatively low concentrations. The symptoms of toxicity are more evident when 5 mg of toxin per kg of feed is consumed. Animals consuming concentrations starting at 16 mg of moniliformin per kg of feed exhibit a significant increase in aminotransferase and alkaline phosphatase enzymes, as well as an increase in serologic calcium and creatinine. Moniliformin also reduces weight gain and increases heart size.

Animals intoxicated by moniliformin generally display muscular weakness, difficulty breathing, cardiac dysfunction, cyanosis, coma and death, which generally occurs suddenly.

Macroscopic or histopathological lesions are not always present in animals that die as a result of acute moniliformin intoxication. Sometimes, ascites, hydropericardium and increased weight and volume of the heart and kidneys, as well as cardiac paleness can be observed. According to findings in experimental animals and mechanism of action, moniliformin is suspected of having significant involvement in the induction of so-called sudden death syndrome in domestic animals, especially in broiler chickens.

Sudden death preceded by weakness and respiratory and cardiac problems can be associated with moniliformin.

Diagnosis

Definitive clinical diagnosis of moniliformin intoxication is very difficult to conclude because the presence of moniliformin in grains is associated with other mycotoxins produced by other molds of the *Fusarium* genus, making the diagnosis complex. However, a definite diagnosis can be established by detecting the toxin in feed consumed by the animals. The quantification of moniliformin in feed can be done through thin layer chromatography, and high performance liquid chromatography. Recent technological advancements have lead to the development of analytical methods using liquid chromatography in conjunction with mass spectrophotometer to quantify moniliformin in feed.

Little is still known about the importance of moniliformin in commercial animal production, but the sudden death of domestic animals is frequent enough to suggest that moniliformin has a significant role in its occurrence. Research on the real incidence of moniliformin in different grains used in feed intended for different species of domestic animals, especially swine and poultry, is needed in order to uncover the clinical and economic relevance of this mycotoxin.

CITRININ

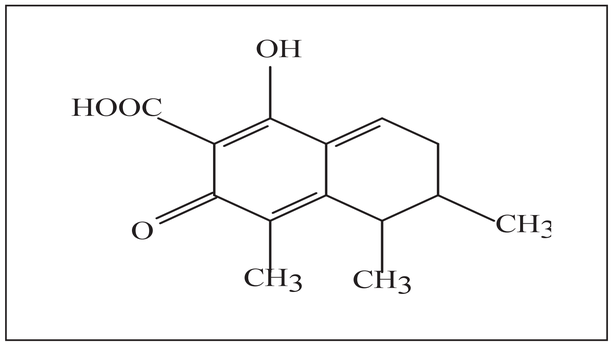
History and etiology

Citrinin (Figure 34) was isolated for the first time from the *Penicillium citrinum* fungus in the 1930s. Its incidence occurs mainly in cereal grains. It is highly nephrotoxic and affects most domestic animals.

Occurrence

It is currently known that citrinin is produced by different fungi of the *Penicillium, Aspergillus* and *Monascus* genera, and certain species of *P. camemberti* (used in cheese production), *A. orizae* (used to produce miso, soy sauce, and beverages made with rice: "sake") as well as *M. ruber* and *M. purpurea* (used for the production of red pigments).

Figure 34 – Molecular structure of citrinin



The main toxicological importance of the toxin is due to its high incidence in rice cultivated in the East, especially Japan, where it is associated with yellow rice disease. It is also prevalent in wheat, oatmeal, barley, peanut, tomato, and corn, with the highest concentrations varying between 1.0 μg/kg and 80,000 μg/kg. Various studies have proven the relatively high co-occurrence of citrinin and ochratoxin A in food sources.

Citrinin and ochratoxin very often occur simultaneously.

Mechanism of action, clinical symptoms and lesions

Citrinin initially received attention because of its bactericidal properties. However, its toxicity is also very high, impeding its use as an antibiotic. Interference with electron transport in the mitochondria seems to be the mechanism of action of the toxin. Mutagenic effects have been found in studies done with cell models.

The nephrotoxicity of citrinin is similar to that of ochratoxin A. When the two toxins were present in the same feed, the affected animal exhibited synergistic toxic effects. Citrinin seems to exert its toxic effects when concentrations reach levels above 200,000 μg/kg of feed for a period of at least 2 months. Ochratoxin A has a similar toxicity at concentrations of

approximately 200 μg/kg of feed. Glomerulonephritis is the most evident lesion, characterized by edema, degeneration, scaling, and dilation, particularly in the proximal convoluted tubule. Citrinin can also be responsible for cholinergic responses, resulting in bronchial constriction, vasodilatation, and smooth muscle contraction. Oral administration of 20 to 40 mg/kg for a period of 42 hours normally only induces nephrotoxic effects in pigs. Animals intoxicated by citrinin have enlarged kidneys, up to five times their normal size, with a gray-yellowish color, and a large number of small capsular cysts that are formed by the thickening of tubular segments. Thickening of basal membrane and formation of connective tissue in the affected kidneys can also be seen.

An increase in kidney size is the main macropathological symptom.

Levels of citrinin between 20 to 40 mg per kg of live weight are enough to decrease weight gain, causing weight loss and glycosuria. Proteinuria is usually low and the creatinine and urea may increase five folds.

Diagnosis

Citrinin intoxication of swine can be clinically diagnosed in endemic areas, based on characteristic symptoms and kidney lesions. However, since this toxin does not have the endemic features necessary for this to happen, the quantification of the toxin in feed is indispensable.

Treatment

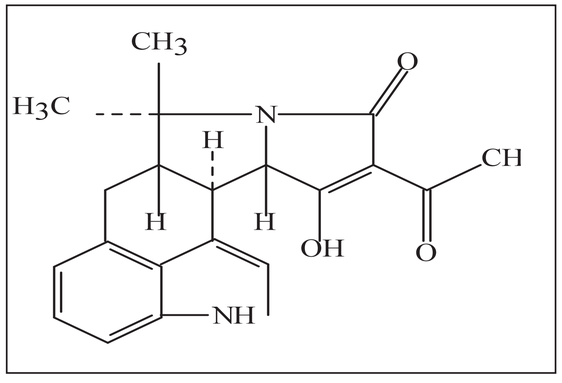
There is no known treatment for intoxicated animals. It is a good practice to immediately replace the contaminated feed.

CYCLOPIAZONIC ACID

History and Etiology

Cyclopiazonic acid (CPA) is an indole tetramic acid (Figure 35) produced by some molds of the *Penicillium* and *Aspergillus* genera. It was initially isolated from cultures of *Penicillium cyclopium* and then from *Aspergillus versicolor, A. flavus, A. orizae* and *A. tamari*. A large quantity of *Penicillium* fungi such as *P. camemberti*, *P. verrucosum, P. patulum, P. puberulum, P. viridicatum, P. oryzae* and *P. commune* also produce CPA. Swine are very sensitive to CPA, which produces oral lesions, edema, intestinal lesions and hemorrhages, and muscular necrosis.

Figure 35 – Molecular structure of cyclopiazonic acid.



Occurrence

CPA has a high incidence in corn and peanut, but also in cheese and meat products. The incidence in peanut, particularly, can be very high, reaching levels higher than 6,000 μg/kg. Most aflatoxin producing fungi also produce CPA. Research has found that *A. flavus* species simultaneously produce aflatoxins and CPA, but *A. parasiticus* species tend to produce aflatoxins. The co-occurrence of aflatoxins and CPA in seeds and food sources is very common since 50 to 75% of *A. flavus* species produce both toxins in laboratory conditions. That percentage is lower under natural conditions, and quantities produced are generally lower than 200 μg/kg.

CPA and aflatoxins can occur simultaneously in the same food source.

Mechanism of action

CPA is rapidly absorbed when ingested orally. It is distributed by organs, especially by the liver and muscles, and is eliminated through milk, urine, and feces in approximately 6 days, when moderate doses are given to swine. Pigs do not show symptoms when levels are less than 200 μg/kg of feed. Levels higher than 1,000 μg/kg of feed are unacceptable. The toxic effects of cyclopiazonic acid appear to be caused by the interference of mineral metabolism, such as calcium, iron, and magnesium.

Clinical symptoms

The most frequent clinical symptoms of swine intoxicated with cyclopiazonic acid include anorexia, fever, dehydration, decrease in weight gain, diarrhea, ataxia, immobility, rigidity, and death. Signs indicating the implication of the central nervous system are frequently observed. The presence of CPA in swine diets frequently leads to refusal of feed, probably due to lesions in the gastrointestinal tract of affected animals.

CPA leads to death preceded by anorexia and problems with the digestive system.

Lesions

Swine intoxicated by CPA initially exhibit lesions along the gastrointestinal tract. The most frequent lesions include hyperemia, edema, hemorrhages, and ulceration. Focal necrosis is most commonly seen in the liver, spleen, kidneys, pancreas, and muscles, and it can be present in other tissue. Liver fat degeneration can also be seen.

Diagnosis

Diagnosis of cyclopiazonic acid intoxication is often difficult, especially because some of the characteristic symptoms are similar to those produced by trichothecenes. The occurrence of CPA in food sources is often concomitant with the occurrence of aflatoxins. Therefore, lesions can be the result of the additive or synergistic effects of both mycotoxins.

Treatment

There is no specific treatment for animals intoxicated by CPA. Maintenance treatments can be adopted. The first step that should be taken is to change the contaminated feed for a toxin free diet.

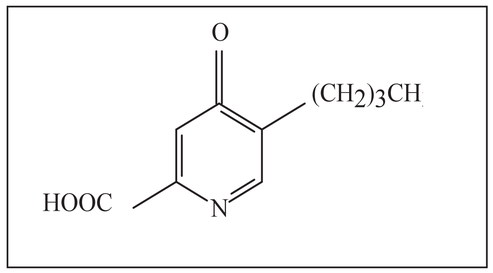
FUSARIC ACID

History and etiology

Fusaric acid occurs in different cereals and their byproducts because it is produced by a large number of molds. Its effects include digestive complications, especially vomiting. The toxin is classified as a phytotoxin (Figure 36) produced by different species of fungi of the *Fusarium* genus, especially *F. moniliforme*.

Vomiting and digestive tract disorders are the main problems caused by fusaric acid.

Figure 36 – Molecular structure of fusaric acid.



Occurrence

Canadian researchers have concluded that fusaric acid is easily detected in feed destined for swine. Quantities between 10 and 40 mg/kg of feed occur in the majority of contaminated feed, but concentrations higher than 130 mg/kg have also been found in corn. Fusaric acid seems to be a toxin of high co-occurrence with other fusariotoxins, such as fumonisins, trichothecenes, moniliformin, and others.

Mechanism of action and lesions

Fusaric acid inhibits the enzyme involved in the synthesis of norepinephrine (dopamine-beta-hydroxylase) showing potent hypotensive action in several animal species.

Clinical signs

Normally, fusaric acid concentrations in feed are insufficient to cause effects in intoxicated pigs, but when it is associated with other fusariotoxins, especially trichothecenes, the synergetic effects lead to feed refusal and induce vomiting. Intoxicated swine exhibit hypertension, increase in cardio-respiratory frequency, and decrease in weight gain. Fusaric acid is also an important immunosuppressant.

Diagnosis

Fusaric acid intoxication always suggests the co-occurrence of other mycotoxins in the same feed. In these cases, toxemia is aggravated by the synergetic action of these mycotoxins, as is the case with trichothecenes and fumonisins.

The incidence of fusaric acid is generally associated with other fusariotoxins.

Treatment

There is no known treatment for this mycotoxin, except for substituting contaminated feed for one that is toxin-free.

OOSPOREIN

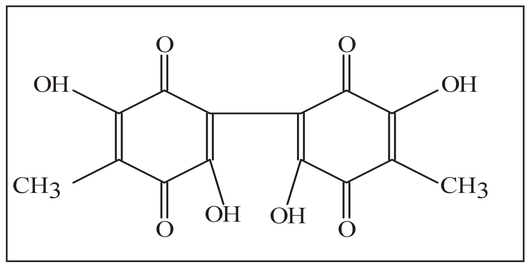
History and etiology

Oosporein (Figure 37) was first isolated from a mycelium of *Oospora colorans* in 1944. Many fungi which grow in different cereals, grains, and other food sources, produce this mycotoxin, especially *Chaetomium trilaterale*.

Occurrence

Few incidences of oosporein occurrence have been reported; however, concentrations of 300 μg/kg of the toxin have been detected in moldy corn.

Figure 37 – Molecular structure of oosporein.



Mechanism of action and lesions

Beginning in 1974, the toxic effects of oosporein have been studied in only a few species. Its potential toxicity is similar to that of aflatoxin, with LD_{50} of 0.612 mg/kg.

Oosporein affects various organs, such as the liver, the spleen, and especially the kidneys. The most important toxic effects of oosporein can be seen microscopically on the proximal convoluted tubule of the kidneys. The greatest damage is necrosis of the tubule epithelium, although other effects can also be observed: interstitial granulomas, fibrosis, dilatation of tubules, and hyperplasia of the remaining centrilobular cells of convoluted tubules. The most evident macroscopic lesions are observed in the kidneys, which normally appear enlarged and are pale. The liver can also be enlarged, but the most evident symptom is the deposit of crystal clear urate on the viscera of chronically intoxicated animals.

Kidney, liver, and spleen damages can be caused by oosporein.

Clinical symptoms

Hyperuremia is one of the characteristic symptoms of oosporein intoxication, but other symptoms such as a decrease in feed intake and a general sickly appearance are generally associated with intoxication. Ataxia can eventually develop.

Diagnosis

Clinical diagnosis can be reached in endemic regions based on clinical evidence and pathological alterations. Calcification in the abdominal viscera, forming gout generally occurs. To confirm diagnosis, the toxin should be detected in the ingested feed.

Treatment

There is no specific treatment for animals intoxicated by oosporein, but there are some measures that can be taken to treat these animals, such as administering hepatic protectors.

UNCOMMON SWINE MYCOTOSICOSIS

Rubratoxin is a *Penicillium rubrum* metabolite, which is associated with hepatic lesions (necrosis) and wide spread hemorrhages in different domestic animals. Swine that consume high concentrations of this toxin display acute illness with high mortality, without fully displaying characteristic clinical symptoms. In these circumstances, animals exhibit intense erythema, especially on the ears and ventral region, with a bright red or purple coloration, without having characteristic lesions on internal organs. The toxin is often associated with aflatoxin, resulting in clinical symptoms of both mycotoxins. Rubratoxins A and B have the highest occurrence, significantly aggravating the clinical symptoms of aflatoxicosis.

Clinical symptoms of aflatoxicosis are aggravated when associated with rubratoxin.

Sterigmatocystin is a precursor to aflatoxin B_1, but it has a lower toxicity, although it maintains some hepatotoxic and carcinogenic properties. Its toxic action is due to the covalent bonding with DNA, in proportions that are significantly less (20 to 30%) than aflatoxin B_1.

Stachyobotriotoxin is produced by *Stachybotrys alternans,* which also produces other mycotoxins that cause hemorrhages and necrosis of the digestive tract and leucopenia. Animals initially exhibit stomatitis and salivation with subsequent coagulopathy and hemorrhages in multiple organs.

A series of lesser important mycotoxins can occur in swine feed. In these cases, clinical symptoms are rarely observed, but negative effects on performance and immunity may be present. Mycotoxins that make up this group include: **gliotoxin** (produced by *Candida albicans* and *Aspergillus fumigatus*), **fusarochromanone** (produced by fungi of the *Fusarium* genus), **patulin** (produced by fungi of the *Penicillium* genus), **wortmannin** (produced by fungi of the *Penicillium, Myrothecium* and *Fusarium* genera). Other mycotoxins such as

sporidesmin (produced by *Pithomyces chartarum*) have hepatotoxic effects. The toxin generally occurs in pasture, and the most evident clinical symptoms of its presence, such as facial eczema, are caused by the photosensitization of areas of skin that are unprotected by hair. **Phomopsins A** and **B** cause lupinosis, which is characterized by severe hepatic affliction and jaundice in animals that ingest *Lupinus* species infested by different species of toxigenic fungi, such as *Phomopsis leptostromiformis*.

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8

MODERN TECHNIQUES IN MANAGING AND CONTROLLING MYCOTOXINS

MODERN TECHNIQUES IN MANAGING AND CONTROLLING MYCOTOXINS

Summary

This chapter describes the importance of sampling, and proposes a management system as well as solutions for the presence of mycotoxin. Sampling is critical because it directly affects the solutions to the mycotoxin problem. When the presence of mycotoxins is detected in a timely matter, it can be treated using Anti-Mycotoxin Adittives (AMA). In order to be considered an effective solution, these additives must meet important requirements that demonstrate their efficacy, and therefore ensure animal benefits and economical advantages for the swine producer.

Introduction

Mycotoxin management in the feed and swine industry implies a constant process of decision making which include financial decisions, and therefore requires a comprehensive knowledge of all aspects of feed production.

Managing and controlling mycotoxins entails a process comprised of a series of critical activities. Everything begins with the samples, which undergo a series of analyses and controls leading to a decision which should take into consideration the safe use of feed in order to decrease the risk of mycotoxin intoxication, so that cost/benefit is quantified, ensuring maximum animal productivity and profitability.

This chapter will describe a practical sampling system as well as the stages that make up the process. Individual adjustments that take into account the cost/benefit relation of the program are necessary because of the particularities of each producer. Therefore, it is a dynamic program, a guideline for visualization of critical points in managing mycotoxins (Figure 38).

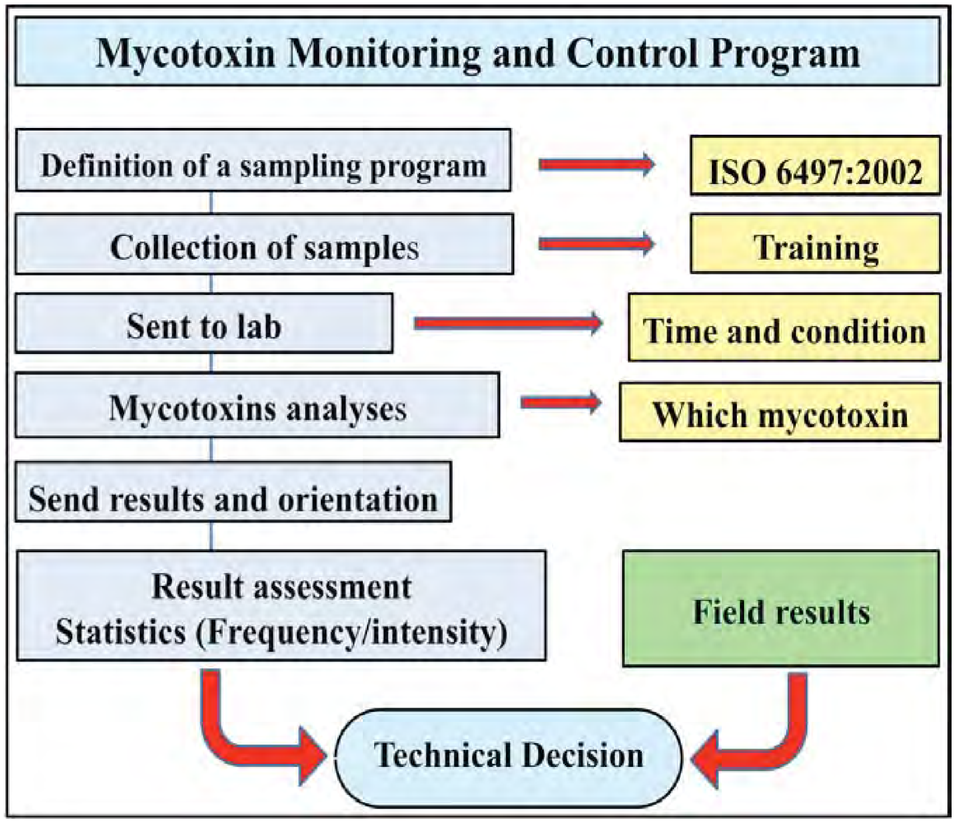
Cost of managing program = Cents/Ton produced

Sampling should take into consideration some basic aspects:

1. What is the probability that a batch of feed or feed ingredient with a certain concentration of mycotoxins will be accepted or rejected?

2. What is the error percentage in the classification of batches?
3. What is the concentration of a specific toxin in a batch that will be accepted or rejected?
4. What is the cost of sampling?

Figure 38 - Mycotoxin monitoring and control program



The presence of mycotoxins in food and feed is the result of various factors. In commercial and/or industrial conditions these aspects becomes important because of the deleterious effects on the health of animals when their diet is contaminated. In commerce, determining the concentration of mycotoxins should be considered from the perspective of the buyer as well as the seller, determining risks for both parties involved. When researching, it is important to have precise evaluations of contamination in order to obtain concrete information regarding the impact of mycotoxins in contaminated feed on experimental animals.

Sampling of grains and feeds for mycotoxin analyses

Determining the exact concentrations of mycotoxins in a large amount of feed is difficult to do because of the large number of variables associated with diagnostic procedures as well as the heterogeneous distribution of contaminated grains within a batch. For practical purposes, testing for mycotoxins consists of the following phases:

1. Collecting samples
2. Grinding samples
3. Subsampling
4. Analysis, identifying and quantifying the mycotoxin/s.

In each of these steps, there is a degree of deviation, also called "error". "Error," in this case, is defined as the degree of variability in the results. The accurate determination of mycotoxin contamination in a batch becomes a complicated process because of this factor. Therefore, a number of precautions should be taken in order to minimize the deviations of results.

Variations between results of mycotoxin analysis

The complexity of the problem of variability in the results of mycotoxin analysis is exemplified by a study carried out by Whitaker *et al*. (1972), where 10 lots of cottonseed were evaluated, using a delineation of 20 analyses per each batch. The main aspects evaluated showed a range in results (some very high, others very low) and the behavior of each given batch. Subsamples of 100 g were taken from 2.7 kg of sample. The quantification of toxins was done using the technique of densitometry in minicolumns. It was observed that the distribution of results of each lot was not symmetrical around the mean; this distribution is always positively skewed. The mean was higher than more than half of the results. If, for example, only one sample was examined in the lot, it was observed that there was a probability higher than 50% that the value found would be below the average. The deviations were greater when fewer samples were analyzed. That is, the distribution of results becomes more symmetrical when the number of analysis increases. This tendency was also observed when mycotoxin concentration increased in the batch that was analyzed.

Analysis of one sample per batch usually resulted in values lower than the mean.

The amplitude in the mycotoxin concentrations results of the various samples analyzed of the same lot is reflected in the high coefficient of variation (CV). In these evaluations, it was observed that the CV, a relative measurement expressed in percentage, seems to be an inverse function of the mycotoxin concentration in the lot.

Taking into consideration the fact that each step of this process has a determined level of error, the total variable is a result of the sum of the errors associated with each one of these steps. In other words, total variance (TV), which constitutes the error, is the result of the sum of sampling variance (SV), subsampling variance (SSV), and analytical variance (AV), as demonstrated in the following formula:

VT = SV + SSA + AV ≅ "error" = sampling + subsampling + analysis

Studies done by Whitaker *et al* (1972) using peanut and corn, suggest the following characteristics:

- All components of variability are in function of the mycotoxin concentration.
- The variances of sampling, sample preparation, and analysis increases with increasing concentration of mycotoxins.
- The variance between grain concentrations is numerically higher than the average concentration of aflatoxin in the lot.

Sampling variation

Analyzing three products (peanuts, cottonseed, and corn), demonstrated that peanut and cottonseed samples, especially small samples of the product accounted for the largest deviation factor amongst the different types of "errors." Sampling error is high because aflatoxin is generally only found in percentages smaller than 0.1% of grains in a given batch. However, the concentration of a given grain could be extremely high. Concentrations found in a single grain could be 1,000 mg/kg, 5,000 mg/kg and 400 mg/kg for peanuts, cottonseed, and corn, respectively. Variations between replicate samples from the same lot tend to be very different because of the variation in contamination between the grains of a given lot. Therefore, the sampling variance was estimated empirically as a result of:

Generally, less than 0.1% of grains in a given batch are contaminated with mycotoxins.

SV = 3.9539 A/MG

Where:

SV = Sampling variance.
A = Concentration of aflatoxin in lot (μg/kg)
MG = Mass of grains samples, in kg (in corn it is equivalent to 3.0 g/grain).

It can be observed that the sampling variance is a function of the toxin concentration in the batch (A). This variance is due to the average weight of grains per gram. If the average particle size of the sample is reduced (by grinding, for example), the sample variance for a given mass decreases. Therefore, it is always important to get the material for analysis after grinding.

Number of particles in 100 g of ground corn = number of particles in 10 kg of whole grain corn.

Subsampling variation

After a sample has been collected from a given lot, the process of subsampling takes place, which consist of obtaining the sample that will be analyzed. It is not technically or economically viable to extract mycotoxins from a large sample. Therefore, the sample destined for analysis is crushed and partitioned once again, removing only a portion, which usually corresponds to 50 g.

It is not technically or economically viable to extract mycotoxins from a very large sample.

A certain amount of homogenization of the different particles always occurs when cereals are milled. Therefore, contaminated particles are better distributed in the sample. This results in a lower variability of the mycotoxin concentration in subsamples taken from a given sample. In practice, the variability of the subsample tends to be smaller than that of the sample, due to the number of milled particles within the subsample. The variability of the subsample of corn, for example, can be estimated with the following equation:

SSV = 0.0125A/MG

Where:

SSV = Subsample variability
A = Concentration of aflatoxin in the sample in μg/kg.
MG = Mass of the milled grains in the subsample per kg

In this equation, the variance of the subsample is also proportionate to the concentration of aflatoxin in the sample.

Analytical variation

An aliquot of the ground subsample is analyzed according to the analytical procedure recommendations. Analytical methods are relatively complex procedures, which involve different phases, including extraction with solvents or solid phase columns, or immunoaffinity, centrifugation, drying, dilution, filtration, phase separation, identification, and quantification. The use of these different steps depends on the methodology used as well as the mycotoxin being analyzed. A considerable variance in results could occur when repeated analyses of a given extract are carried out.

Whitaker *et al*. (1979) determined analytical variance using the CB method to extract aflatoxin from corn, developing the following formula:

$$AV = 0.699M^2/N$$

Where:

M = Concentration of aflatoxins in the subsample (μg/kg).
N = Number of analyses.

Like the sampling and subsampling variance, the analytical variance is proportional to the aflatoxin concentration. According to some studies done using the BF method to analyze aflatoxins in peanuts, quantification by Thin Layer Chromatography (TLC) represents the largest variable within the process of analysis. Modern analytical methods significantly decrease these problems, if control standards are followed.

Reducing results variability

It is difficult to determine the real concentration of mycotoxins in a certain batch of cereal. The objective of a determination, in most cases, is to classify the lot as acceptable or unacceptable for consumption, depending on the highest or lowest concentration of mycotoxin in comparison to a predetermined limit level. An exception occurs when the concentration of a lot approaches the limits of tolerance. For example, it is easier to determine

The objective of a determination, in most cases, is to classify the lot as acceptable or unacceptable for consumption.

if a lot containing 5 μg/kg is below or above the limit of 100 μg/kg, than determining that a lot has a concentration of 5 μg/kg. The only way to obtain a true estimate of the lot is by reducing the total variance associated to the results of the tests or analyses. The three points of variance can be reduced by adopting the following procedures:

1. Increase the sample size and the number of sampling points.
2. Increase the quantity of subsampling and decrease the size of particles by grinding.
3. Analyze a larger number of subsamples and utilize automatic and precise techniques.

When the weight of a sample is doubled for analysis, the variance in the sample is reduced by half. This relation is also true to obtain a variance reduction in the process of subsampling and analysis.

Planning a mycotoxin analysis program

Analyzing food, feed, and feed ingredients for mycotoxins is essential for industries that produce products for human and animal consumption. In cases where contamination levels surpass predetermined concentration limits, measures to control these levels should be taken. This can be done through dilution or adopting measures such as the use of anti-mycotoxin additives (AMA), or more rigorous measures such as the destruction of the contaminated product.

Analyzing food, feed, and feed ingredients for mycotoxins is essential for industries that produce products for human and animal consumption.

Due to great variability of test results, two types of “errors” are associated with mycotoxin control programs aimed at helping make decisions, especially in situations that involve products of bilateral commercial transactions. The first occurs when an acceptable lot, having mycotoxin concentrations equal to or less than the established limit, is evaluated as being unacceptable and therefore, rejected by quality control. This type of error, referred to as “processor, industrial, or producer risk” generates unnecessary costs in eliminating the lot or even unnecessary AMA. The second occurs when unacceptable lots, having concentrations above permitted limits, are analyzed as being acceptable and therefore, authorized by quality control. This type of error is referred to as “consumer risk” because the contaminated product is used in food or feed, causing health risks and economic ramifications to producers and/or the industry.

In order to maintain an effective control, these risks associated with the monitoring program must be evaluated correctly, as well as taking into consideration the cost/benefit relation of the mycotoxin analysis program.

Sampling programs

Various sampling programs are used in mycotoxin analysis. They all have advantages and disadvantages determined by the heterogeneous distribution of grains contaminated with mycotoxins in the cereal mass. This variable leads to a number of difficulties when interpreting analyses. Years of accumulated experience in implementing these models in various animal producers, has led us to select the sampling system adopted by the European Union. This program, which has been used and refined in various Brazilian industries, is easily applicable if followed and monitored properly.

European legislation presents practical characteristics for taking samples from given batches. The Commission of European Union Norms (Amtsblatt der Europäische Gemeinschaften N L 102/1, Teil II, 1976 and Futtermittelrecht mit Typenliste fur Einzel - und Mischfuttermitteln, 1994) and Norm ISO 6497 (2002) that define the rules of the sampling procedure for undesirable substances with heterogeneous distribution, such as mycotoxins, starts with the definition of the following terms:

Lot or **batch** is a quantity of feed or feed ingredient, which can be accepted as having the same characteristics in terms of homogeneity, or the daily output of a production unit at a given time (shift output); the batch should not be greater than 500 MT.

Increment is the quantity taken from a given lot (normally ten samples of 100 g for each kg of collected sample).

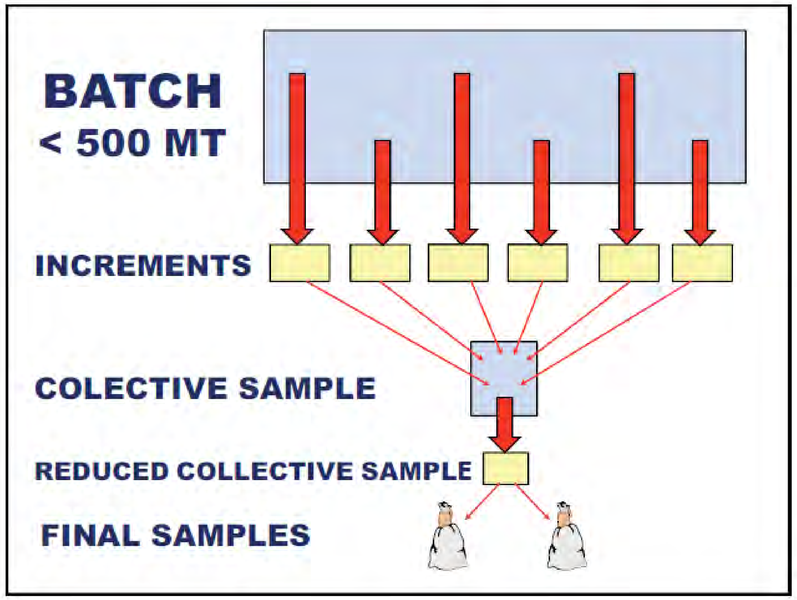
Collective sample is the result of the sum of the increments. The collective sample should be homogenized and divided with the help of a riffler, or in its absence, the cross-cut system should be used for the reduction process, which will eliminate 2/4 of the total in each homogenization step.

Reduced collective sample is a representative part of the collective sample after the quantity has been reduced.

Final sample or laboratory sample is the partial result of the reduced collective sample or of the homogenization of the collective sample, as represented in Figure 39.

The equipment recommended for manual sampling consist of shovels, shells of rectangular edges, augers or pneumatic equipment compatible with the lot size and the particles of the material being sampled. To reduce the sample mass volume of the collective sample, the use of rifflers is recommended.

Figure 39 – General outline for mycotoxin sampling methodology.



Collecting increments

In **bulk products,** taking seven samples per lots of up to 2.5 tons is recommended. For lots containing more than 2.5 tons, the number of samples taken is determined by the following equation, limited to a maximum of 40 increments: $\sqrt{20 \text{ x Tons}}$ **.** The same formula is used by the feed industry.

Bagged products should follow the recommendations outlined in Table 19.

Table 19 - Sampling bagged products.

Number (n) of bags in a lot	Minimum number of bags to be sampled in a lot
1 to 4	All
5 to 16	4
More than 16	$\sqrt{2 \times \text{bags}}$ up to a maximum of 100

Collective samples

For collective samples (result of the sum of increments) the minimum quantity should never be less than 4 kg, for both bulk and bagged products. The number of collective samples, according to European norms, is determined according to the number of metric tons (MT) in a given lot:

< 1 MT = one collective sample per lot.

> 1 and < 10 MT = two collective sampler per lot.

>10 and < 40 MT = three collective samples per lot.

> 40 MT = four collective samples per lot.

Final sample

Each collective sample, after reduction, will form part of the **final sample** or laboratory sample. These samples will always be 3, individually wrapped and labeled. The analysis of at least one collective sample is necessary, being that the quantity of the final sample should not be less than 500 g.

In order to analyze mycotoxins in very large lots (> 500 MT), these must be divided, and the number of samples taken should conform to the number determined for collective samples. If the number is greater than one, the number of samples to be taken should be the same as those described in individual samples, being divided evenly by the batch. Finally, samples of equal amounts will be taken, and each sample must be greater than 4 kg as recommended for the collective sample. The samples formed should not be mixed together.

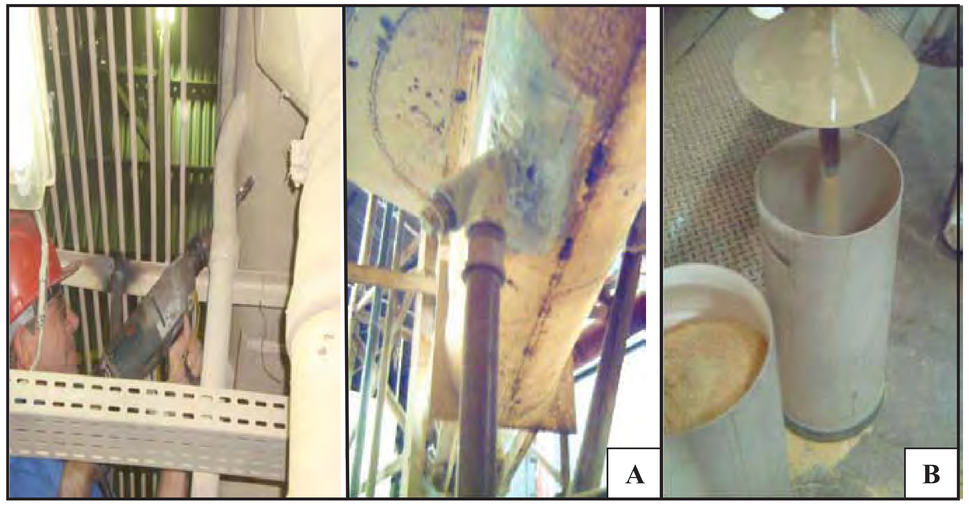
Automatic sampling

Automatic sampling is very practical and efficient, that is why it is widely used in a variety of companies. Using automatic samplers and other more simple systems such as the collection of increments already ground by holes made in pipes, especially in screw conveyor which transport feed and/or feed ingredients, ideally after grinding, significantly facilitates the sampling procedure (Figure 40).

Simples systems such as the hole in screw conveyor significantly facilitate the sampling process.

In order for this to be done, perforations varying from 8 to 15 mm in diameter are made in the pipe at a 45º angle. The material poured from the pipe is usually conducted by a 1” diameter PVC pipe to the base of the elevator, into a container large enough to collect the amount of increments resulting from the formula $\sqrt{20 \text{ x Tons}}$.

Figure 40 - Hole in screw conveyor, system of collecting samples for mycotoxin analysis.
(A) Pipe coupled to the tube transferring feed or feed ingredient at a 45º angle.
(B) Collection of feed or feed ingredient sample.



Sampling for products transported by trucks or trailers

In case of products transported by trucks or trailers, sampling can take place when grains are received. This sampling method follows the parameters set by the European Committee (EC) establish by ordinance N. 401/2006, as the values shown in Table 20.

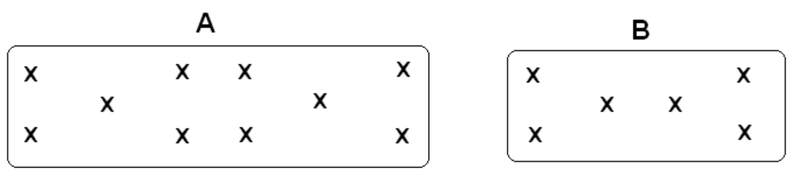
Table 20 - Number of increments and weight of collected samples according to lot weight.

Lot weight (metric tons)	Number of increments	Collective sample weight (kg)
≥ 50 and ≤ 300	100	10
> 20 and ≤ 50	100	10
> 10 and ≤ 20	60	6
> 3 and ≤ 10	40	4
> 1 and ≤ 3	20	2
> 0.5 and ≤ 1	10	1
> 0.05 a d ≤ 0.5	5	1
≤ 0.05	3	1

Source: (EC) No 401/2006.

Increments can be collected using hand probes. For example, in a 30 ton cart, 100 increments should be collected. If the probe has ten collecting holes, the truck should be probed 10 times, and the sum of those 100 increments results in 10 kg. The points of the cargo to be sampled should follow the specifications laid out in Figure 4, remembering that at each point 10 increments should be removed.

Figure 40- Sample points. Cargo with > 20 metric tons (A), and with 10 to 20 metric tons (B)



In this sampling method, the result of the increment collection of each truck or trailer results in barely one collective sample. It should be partitioned until 500 g are obtained, which will be the final sample that will be sent to the laboratory for analysis.

Sampling in cases of suspected mycotoxicosis

In certain circumstances, the outbreak of mycotoxicosis warrants an investigation. The procedures should take into account the heterogeneous distribution of mycotoxins in the feed, as well as knowledge of the toxicokinetics of mycotoxins in order for the diagnosis to be confirmed by the etiological diagnosis.

When mycotoxicosis is suspected, samples should be collected as close as possible to where animals consumed the

In cases of sampling for legal purposes, official sampling services should be used, and when that is not possible, both parties involved should be present during the sampling process.

Some basic precautions should be adopted to ensure efficient sampling:

- Collection of representative samples following a sampling plan.
- The sample should be collected as close as possible to where the intoxicated animals consumed the feed (feeders).
- The collection of blood and organs allows a retrospective analysis of some contaminations, especially in situations where feed is not available. Some mycotoxicosis, like the one caused by ochratoxin A, can be detected in blood for up to 35 days after ingesting the toxin.
- Identifying the ingredient (or ingredients) in contaminated feed, in cases where mycotoxins are detected in feed.
- Generally, material collected “blindly” can have water activity (Aw) above pre-established limits. Some precautions should be taken when shipping materials to avoid fungal growth during transport, including appropriate packaging.

Important Recommendations:

Due to the large variability associated with the analysis of aflatoxins in products in granular form, such as cereals, the concentration of mycotoxins cannot be determined with

100% accuracy. In order to decrease the variability of results, the following recommendations could prove to be extremely useful:

- Adopt a practical sampling program, implemented fully and in all samples.
- Increase the number of increments, making them systematic, but distributed randomly.
- Increase the number of particles per sampling unit. This is accomplished by grinding grains.
- Conduct the greatest possible number of analyses.
- Collect samples when the material to be sampled is in motion, during the formation of piles, and loading or unloading of silos.
- Identify samples correctly, allowing tracking of the sublot.
- Use automatic equipment to collect samples or automatic sampling by deflecting small aliquot of material flow. In cases where the subsamples are very large (40 kg), rifflers or automatic samplers should be used.

Monitoring sampling

Collecting samples entails carrying out a process while respecting the chosen sampling methodology as well as task execution, always respecting the operational standard. The operator or team responsible for the collection of samples should be properly trained to do this.

Two strategies can be adopted in the collection of samples in a feed mill: **receiving sampling** and/or **production sampling**. In both situations, risks, advantages, and disadvantages should be assessed, the cost involved, not only of the sampling process, but also the relative cost price analysis required in each situation.

One possibility is to evaluate feed ingredients when it is received (**receiving sampling**). This strategy is used in order to obtain data that will anticipate future risks, allowing one to plan ahead. Thus, possible problems can be corrected, especially when it is possible to use the information to monitor suppliers. Besides preventing inherent problems associated with intoxication, the analysis of mycotoxins becomes an important factor of quality control in the industry, even allowing it to be used for commercial purposes. In these cases, the process can be executed when receiving the raw material.

The criteria of a previously established sampling plan are employed in order to use samples for other evaluations. Apart from the large volume that makes up the lot on the scale or receiving platform, the biggest problem in this type of procedure is the difficulty of correctly homogenizing the grain sample. The volume of the sample depends on the sampling system that will be used. Special care must be taken so that the sample taken should increase its representativity. Grinding increases the number of particles, minimizing the volume of the material that will be submitted for laboratory analysis. The option of sending whole grains is accompanied by a sampling error that can be minimized by milling and partitioning all material initially collected.

A good option used to reduce costs consists of creating a material pool which should be composed of subsamples from the same origin/supplier, but without forming large lots. It is important that the dispersion of the sample is proportional to the size of the pool, thus the probability of correct detection of the contamination is proportionately diluted.

Another moment that allows efficient sampling occurs in the final processing of the production of feed (**production sampling**). This sampling permits the verification of all contamination that occurred in all the ingredients during the entire process of cultivation, harvesting, transport, drying, storage, and processing.

Receiving sampling presupposes good storage practices of the cereals, not accounting for the formation of mycotoxins during this period. Sampling should be done as close to mixing of the ingredients as possible, that is, when there is the greatest homogenization of the particles. A practical and common point for taking the increments is immediately after grinding, when the granulometry and efficiency of the process is usually controlled.

Special care should be taken to avoid the formation of water condensation in the collected sample, especially when plastic packaging and/or samples heated during processing are being used. Samples should be cooled in open packages when their temperature is above room temperature. The volume of increments taken should represent the whole lot. Homogenization is crucial, and can be obtained in Y mixers or rifflers. If this equipment is not available, the sample can be reduced through the cross-cut system until samples reach a weight of approximately 2 kg (samples and reference sample). Afterwards, material should be packaged, sealed, and kept in conditions that preserve its characteristics until reaching the laboratory for analysis.

Shipping material

The number of times samples of feed or feed ingredients are sent to the laboratory for analysis should be determined taking into account certain factors, including needs and most convenient shipping possibilities. When results are needed immediately, site analysis using semi-quantitative methods should be considered, and the stability of the material should be evaluated. Humidity and temperature are two key factors in conserving the integrity of feed. High humidity of samples favors fungal growth and promotes the formation of mycotoxins, altering the sample and the analytical results of the lot in question. When samples having temperatures higher than the average room temperature are hermetically closed, condensation inside the package can occur, promoting fungal development. Special care should be taken when sending refrigerated samples and when labeling samples, as well as taking into consideration how samples will withstand conditions in which they will be transported.

Humidity and temperature are two primary factors in maintaining food/feed integrity.

Identifying samples using a form, such as the one presented in Figure 41, streamlines the process, especially with reference to the use of the analytical result by official laboratories.

Mycotoxin analysis

Analyzing feed ingredients represents a cost factor in the employment of materials, equipment, and skilled labor. For this reason, this is a factor of the monitoring system that should always be well evaluated. Analyzing all samples for all possible mycotoxins is not viable in terms of cost/benefit.

Determining which mycotoxins should be analyzed for in a lot should be determined by preexisting epidemiological data, since these are very important and help make this decision.

Available information in each region/country helps make these decisions. In Brazil, statistics suggest that the main toxins are: aflatoxins, fumonisins, zearalenone, and deoxynivalenol, followed by other less prevalent ones such as those from the *Fusarium* genus. Seasonality, as well as the presence of toxins according to climate and harvest periods should be taken into account.

In the monitoring process, feed ingredients and feeds with greater risk of contamination are usually monitored. Not all products need to be evaluated; especially those that are rarely contaminated with mycotoxins because of the way they are processed. The main ingredients used to make swine feed, such as corn, should always be analyzed.

Analysis of results and statistics

Evaluating results of the monitoring process of mycotoxins initially includes careful observation of its epidemiological consistency. It is also very important to compare results of evaluations undertaken during the monitoring process with those obtained from existing external data banks, or form the company itself. The occurrence of contamination trends can be observed when adequate sampling methods, as described in this chapter, are implemented. This means that results normally assume a pattern, such as the historical seasonality of aflatoxin contamination during the first half of the year in Brazil.

Results should be related to the history of the sample analyzed; for example, the occurrence of one or two positive samples, from the bottom of the silo can be explained from an ecobiological fungal point of view, since it normally represents material that was stored longer or is the residue of disintegrated grains.

Evaluating the results of mycotoxin analyses should be carried out in order to interpret the intensity of contamination occurrence due to the frequency with which the phenomena occurs, as shown in the following formula:

Mycotoxin Risk Coefficient = average contamination x (contamination % x factor)

Where factor is the degree of confidence of the sampling system which is normally 1.3. The composition of the average, as well as the percentage of contaminated samples is the assembly of the results obtained in a determined amount of time; commonly, the period of a week is used when more than one sample is being analyzed.

The data is organized into a graph where the x-axis represents the unit of time (weeks), while the y-axis is the calculated risk coefficient (formula result).

The monitoring process requires a certain degree of experience and time to adjust levels obtained in order to be able to reach a decision with a high level of confidence (or with

less uncertainty). Crossing the data from field performance, with the period of production / ingestion of feed is important, helping feedback and adjusting the monitoring system. Each production unit of feed should be evaluated individually; only in this way can the real impact of contamination on animal performance be determined. This "fine tuning" needs a varying period of adaptation, depending on some factors such as:

- Following the sampling plan.
- Relative uniformity of the feed ingredient supply.
- Adequate number of samples.
- Uninterrupted flow of samples during the period.
- Intensity of contamination.

Making decisions for controlling mycotoxins

Controlling acute mycotoxicosis is fairly simple. Clinical cases display clinical symptoms that can easily be associated to intoxication by an experienced professional, and this problem disappears in a matter of days by immediately removing the contaminated feed. However, there is always some doubt as to: how severe was the mycotoxicosis? In practice, difficulties arise in subclinical contamination, associated with the heterogeneous distribution of mycotoxins in lots of feed, as has been shown in this chapter. Thus, productivity losses and damage to animal health mostly occur when toxins go undetected, especially in the absence of monitoring or when monitoring is done incorrectly. Experts in mycotoxicological analysis agree that reliable tests are rarely performed in less than 24 hours mainly because of the distance from production centers to certified laboratories. Therefore, there is a delay in information which coupled with the time it takes for the sample to reach the laboratory, can often last several days. This time period should be considered important in terms of protecting pigs of different growing stages, or when swine are more sensitive to mycotoxins, during gestation or when they are young. Therefore, when making decisions, the following considerations should always be taken into account:

- The prevalence of the mycotoxin affecting swine.
- The seasonality of the respective mycotoxin, as well as the mycotoxicological epidemiology of the region where the feed ingredients or feed comes from
- Other factors that influence swine susceptibility:

- **Management** deficiencies, which create stressful situations;
- **Genetic** specialization, which tend to be more sensitive to mycotoxin effects;
- **Sanitary program**, since some mycotoxins have immunosuppressant characteristics which increase the incidence of diseases in the herd;
- **Nutritional** deficiencies or unbalanced diet, which exacerbates the effects of many toxins, especially those that have hepatotoxic characteristics;
- **Age** and **sex** of the animal, which accounts for higher or lower susceptibility to mycotoxins.

Proper management should correctly evaluate these factors. Information generated by the sampling/analysis system should always support management decisions.

Controlling Mycotoxins

The initial premise in controlling and eliminating problems related with mycotoxins should always prioritize the use of non-contaminated feed in the feeder. The objective is to guarantee this, not only because of the losses due to ingestion of mycotoxins per se, but also because of the nutritional deficiencies that occur when fungi infest cereals.

Some programs of decontamination use chemical products such as sodium hypochlorite or radiation which are capable of controlling the development of fungi and reduce mycotoxin concentrations. However, the cost/benefit of these programs should be considered because they may not be efficient on a large scale due to high costs and questionable results.

A long standing practice used to control mycotoxins is the use of nutritionally inert material in animal diets, in order to diminish mycotoxin absorption through the animals' gastrointestinal tract. These substances were called mycotoxin absorbents, and are generically referred to as Anti-Mycotoxins Additives (AMA).

Anti-Mycotoxins Additives or Adsorbents / Binders

AMA are theoretically inert substances or combinations of inert substances with microorganisms and/or enzymes that bind irreversible mycotoxins, or biotransform them, making them unabsorbable by the gastrointestinal tract of animals, thus being eliminated naturally. Researchers have defined AMA as non-nutritional additives, which when added to

feed, have the capacity to bind to mycotoxins forming complex macromolecules that cannot transpose the intestinal barrier, eliminating toxins through the feces and thus neutralizing the negative effects that mycotoxins would have on the performance of the animals.

In the last few years some technological innovations are being used in the same ways as AMA. These products are based on yeast, its constituents, and microorganism specifically selected to neutralize mycotoxins. There are various substances or products that were evaluated as AMA, principally for aflatoxins, and according to their origin, can be classified as organic or inorganic, and within these two classifications, can be divided into natural and synthetic.

Special attention should be paid to natural variation of commercial AMA, their chemical composition, the location where the raw material was obtained, such as in the case of clay, and industrialization, especially in the cases of organic or synthetic AMA, as well as those that are composed of plant and/or enzyme derivatives.

> AMA are capable of binding mycotoxins, forming complex macromolecules that cannot be absorbed by the gastrointestinal tract.

Using AMA in swine diets

The ideal way of detoxifying feed for swine should not only reduce mycotoxins, but the substance used should also not produce toxic effects or reduce the nutritional value of the feed. Adding nutritionally inert AMA to diets has been an important tool to decrease the problem of mycotoxin contamination.

Some AMA, such as those classified as adsorbents, are highly valued as medication carriers in the pharmaceutical sector, and as catalysts in the petrochemical industry. In the feed industry, the use, selection, and processing of clays is being used more and more to inactivate/adsorb mycotoxins, with the objective of reducing its absorption by swine gastrointestinal tract.

Criteria for selecting an AMA

From a technical point of view, two criteria are considered in order for a product to be used as an AMA: the results of *in vitro* and *in vivo* evaluations. For a product to be released

for commercialization in Brazil, it must be duly registered in the Ministry of Agriculture, Livestock, and Food Supply (MAPA).

An *in vivo* test of the product that demonstrates its protective function should be done. In order to remain registered with MAPA, a report of the *in vitro* evaluations with satisfactory results similar to results from the products used *in vivo*, must be submitted each semester. *In vitro* and *in vivo* evaluations must be done in laboratories accredited by MAPA.

An ideal AMA should present the following characteristics:

- Protect the functional, morphological, and productive parameters;
- Destroy, inactivate, or eliminate the toxin;
- Does not produce toxic or carcinogenic residues;
- Maintain the nutritional value and the acceptability of the feed;
- Does not induce diarrhea or other pathologies;
- Protects mucosa;
- Does not alter important technological properties;
- Easily administered and low dosage;
- Proven cost/benefit;
- Prolonged stability;
- Suitability for periodic evaluations (*in vitro* and *in vivo*);

***In vitro* evaluations**

The main objective of *in vitro* evaluations of AMA is to determine the capability of the product to adsorb/inactivate mycotoxins present in a liquid medium, making them unavailable. The first methodologies used to do this were developed more than 20 years ago and used a hydro-alcoholic solution to test aflatoxins.

Many products may show different results when compared to assessment using gastrointestinal juices. Generally, different rates of inactivation/adsorption are observed in evaluations done with these fluids. This occurs because many types of clays lose their adsorption capacity when they come in contact with the pH of these juices, and because of the presence of other substances such as enzymes, which are not present in hydroalcoholic methodologies. This decrease in inactivation/adsorption, under these conditions, occurs due to

the geological characteristics of these clays. They often do not dissolute completely in the gastrointestinal tract.

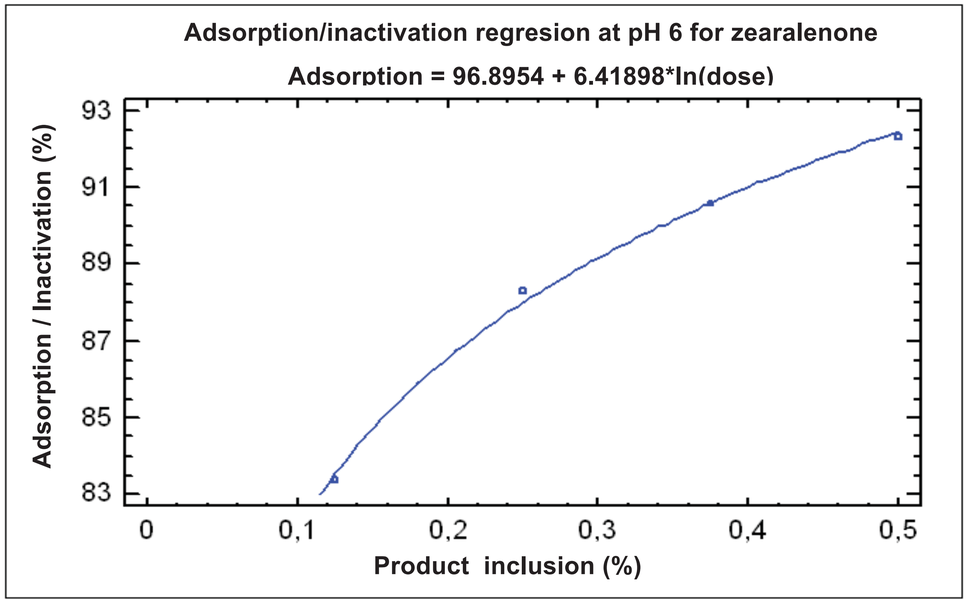
Using the gastrointestinal juices model is extremely useful for evaluating specific inorganic AMA, partly because the metabolism of aflatoxin B_1 is influenced by variables that are present in the gastrointestinal tract, such as the pH of the medium, which varies between 1.5 – 3.0 (gastric juice) and 6.0 – 7.0 (intestinal juice). Some chemical reactions can be catalyzed by AMA when tested in hydroalcoholic solutions, which does not occur in evaluations which use the gastrointestinal juices model.

In vitro evaluations seek to determine a product's capability to adsorb/inactivate mycotoxins present in a liquid medium.

With more than 24 years of experience in mycotoxin analysis and more than 17 years analyzing AMA, LAMIC uses *in vitro* analytical methodologies of these products, based on internationally accepted methodologies. The methods which use gastric and intestinal juices most accurately mimic the medium where AMA will be administered. Two solutions are prepared when using this methodology: gastric juice, with a pH of 3.0, and intestinal juice, with a pH of 6.0. Both are described in American Pharmacopeia (Pharmacopeia National Formulary, 1990). These solutions are fortified with mycotoxins and the Anti-mycotoxin Additive is later added in specific concentrations for each one.

Calculating *in vitro* adsorption/inactivation is done based on chromatographic response, using normal liquid chromatography (LC-DAD, LC-FLD), as well as modern chromatography systems coupled with mass spectrometry (GC/MS, LC/MS and LC-MS/MS) of the fluid containing the AMA and fluid not containing the additive. The product that will be evaluated is added at the maximum concentration recommended by the manufacturer, followed by 75, 50, 25, and 0% of the additive, generating a concentration curve, as shown in Figure 42.

Figure 42 - *In vitro* adsorption/inactivation curve at pH 6. Anti-Mycotoxin additive with more than 90% adsorption/inactivation for zearalenone.



The methodology for *in vitro* AMA evaluation is interpreted considering adsorption/inactivation results. Evaluations are reached based on 3 analyses at the same concentration. The valid curve is considered when the variation coefficient is less than 5%. In evaluations of aflatoxins, for example, using the maximum concentration recommended by the manufacturer, an adsorption/inactivation minimum of 90% is considered as a probable effective product *in vivo*. However, a product with more than 90% adsorption/inactivation does not always work, and the opposite may also occur.

In vivo evaluations

In order to perform *in vivo* AMA evaluations, the experimental unit used should be adapted for swine, with controlled temperature of the room where the animals are kept, an exhaust system is in place, feed and water are given "*ad libitum,*" and the flow of people is limited. The experimental standard protocol should have a minimum of four treatments. A control group, a group that will assess the safety of the product, a group that has a mycotoxin dose capable of causing clinical/enzymatic/pathological effects, and a group where the AMA has been added to the contaminated diet. The doses of the product added to the diet are those

recommended by the manufacturer. If they recommend a smaller dose, an additional treatment with lower inclusions should be added. This additional low-dose treatment should ideally present a statistical difference, but should at least show a positive effect tendency in the evaluated parameters. One example of the experimental protocol is shown in Table 21.

Table 21 - Experimental protocol used to evaluate the efficiency of a specific AMA in swine intoxicated with a mycotoxin**.**

Treatment	Mycotoxin	AMA
1	-	---
2	-	Maximum Dose (100%)
3	+	---
4	+	Maximum Dose (100%)
5	+	75% of maximum dose
...	+	…% of maximum dose

Two parameters are considered when evaluating results, productive performance, which includes weight gain, feed conversion, and feed consumption, and morphological /enzymatic, which include lesions, alteration of relative organ weight, color and size of organs, and blood analyses. The duration of the experiments vary depending on the toxin that is evaluated. Normally, it lasts at least 21 days, varying depending on the doses used.

The criteria for AMA approval evaluated *in vivo* is that the treatment of the group of intoxicated animals consuming feed containing AMA presents a statistical significant improvement from the group of intoxicated animals that were not given AMA in their feed. The effects that prove a reduction in mycotoxin absorption, involved in testing, should also be documented. Effects such as, weight gain, decrease/increase in relative weight of the specific organs involved, size of reproductive system, and mycotoxin levels used, are described in Table 22. Another criterion to be observed is the group that received AMA in a mycotoxin-free diet, which should present the same results as the control group in order to demonstrate the additive's safety.

To approve an AMA using *in vivo* testing, the AMA must significantly prevent productive performance and/or morphological damages caused by the mycotoxin being evaluated.

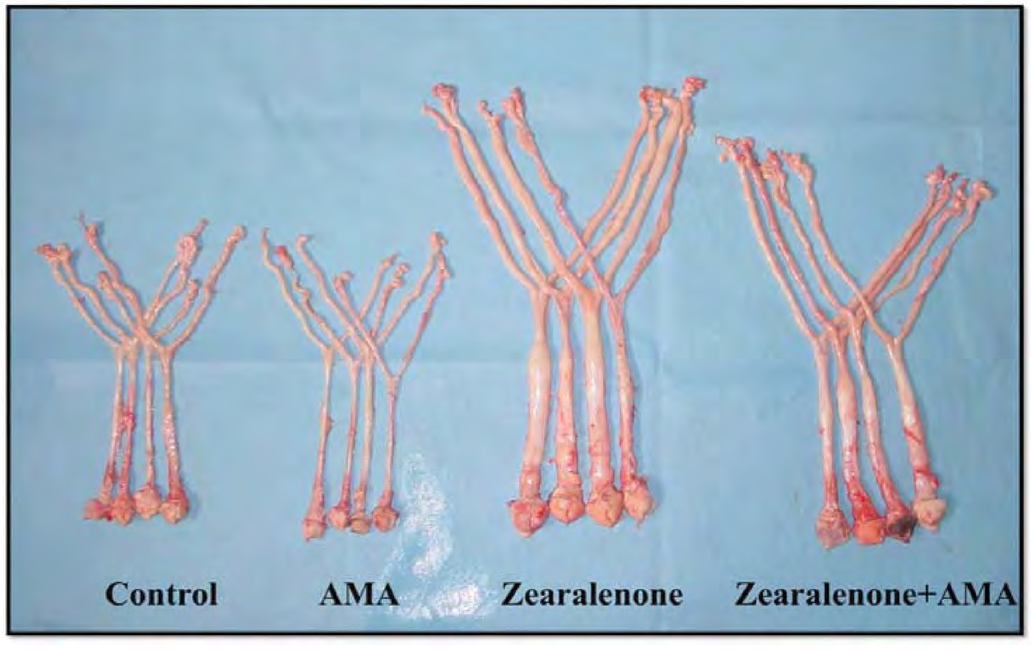
Table 22 - Criteria evaluated for *in vivo* approval of Anti-Mycotoxin Additive use in swine feed contaminated diets with mycotoxins.

Mycotoxin	Level included in diet (mg/kg)	Criteria evaluated*
Aflatoxina	1	Increment in live weight and/or average daily weight gain after 28 days. Decrease of relative weight and/or decrease in coloration difference of the liver at 28 days.
Zearalenone	2	Decrease in vulva volume after 21-28 days and/or decrease of the relative reproductive system weight.
Fumonisins	50	Decrease in relative liver and/or lungs weight at 42 days. Ratio between sphingolipids (SA/SO) similar to those of control group.

*Significant difference ($P \leq 0.05$) in Tukey or Bonferroni Test in relation to the positive control group.

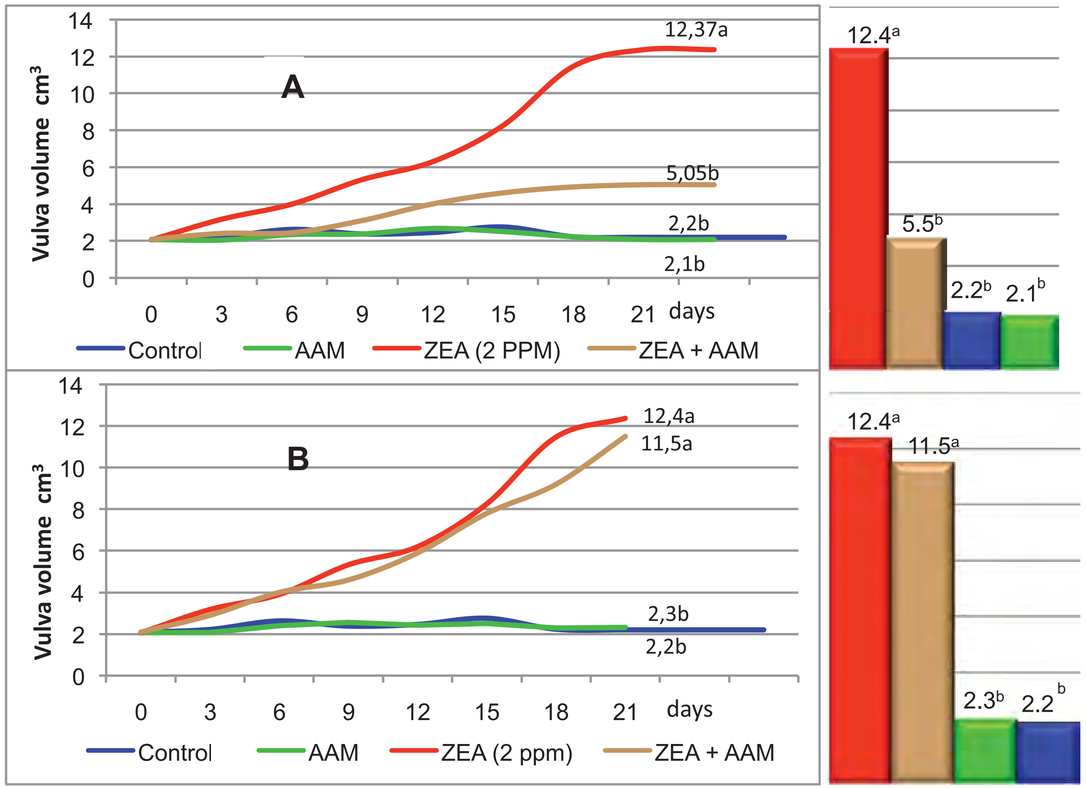
The reproductive system is always evaluated when conducting the *in vivo* study for the approval of an AMA in gilts intoxicated with zearalenone (Figure 43).

Figure 43 – Reproductive system evaluation of prepubertal gilts for *in vivo* approval of an AMA against zearalenone.



An example of comparative results of *in vivo* efficacy of two AMA against zearalenone intoxication in gilts for a period of 21 days, in different tests carried out by LAMIC, is illustrated in Figure 44. The addition of one AMA to the diet resulted in a decrease in vulva volume of the gilts compared with those that only received feed with zearalenone, as can be observed in the upper portion (A) of Figure 44. In the lower portion (B), the results of another AMA that did not cause significant difference in vulva volume, when compared to the intoxicated group, can be observed. This justifies the fact that the second additive was not approved using the *in vivo* test.

Figure 44 - Results of *in vivo* evaluations of vulva volume (cm^3) of gilts fed diets with and without zearalenone and AMA. Approved product (A), and rejected product (B).



a – b Averages in columns with different letters differ by the Tukey test (P≤0.05).

The *in vivo* approval demonstrates the protective effect of AMA in relation to mycotoxins evaluated. The AMA can be approve for more than one mycotoxin if another *in vivo* test is preformed demonstrating its effectiveness against that mycotoxin.

The suggested validity for *in vitro* tests is 6 months, and for *in vivo* tests, 2 years. The objective of these periodic evaluations is to guarantee the quality of the AMA because these products can vary, leading to major constitutional changes. It is essential that AMA always meet the approval requirements, thus, offering the consumer only those AMA that have been approved by a certified institution.

The suggested validity for *in vitro* tests is 6 months, and for *in vivo* tests, 2 years

Results of Anti-Mycotoxin Additives evaluations

In the last 24 years of performing evaluations, the Laboratory of Mycotoxicological Analysis (LAMIC) of the Universidade Federal de Santa Maria (UFSM), more than 600 AMA from different countries (Germany, Argentina, Austria, Belgium, Chile, Colombia, Cuba, Spain, Hungary, Italy, Mexico, Pakistan, Peru, Dominican Republic, and USA), besides those produced in Brazil, have been tested for aflatoxins. In analysis of more than 1,150 *in vitro* evaluations done by LAMIC during 1998 to 2010, nearly 50% of analyzed AMA presented adsorption capacities higher than 90% in *in vitro* trials.

AMA evaluations, initiated in the 1990's, began to consolidate an evaluation protocol agreed upon by the main mycotoxin researchers. In 2005 evaluations began to be completed based on previously recommended protocols. Table 5 illustrates a summary of the work done with the SAMITEC Institute. Of 144 evaluations, 70 were done on swine. 27% of the products were effective against a specific mycotoxin. Some products are effective against more than one type of mycotoxin.

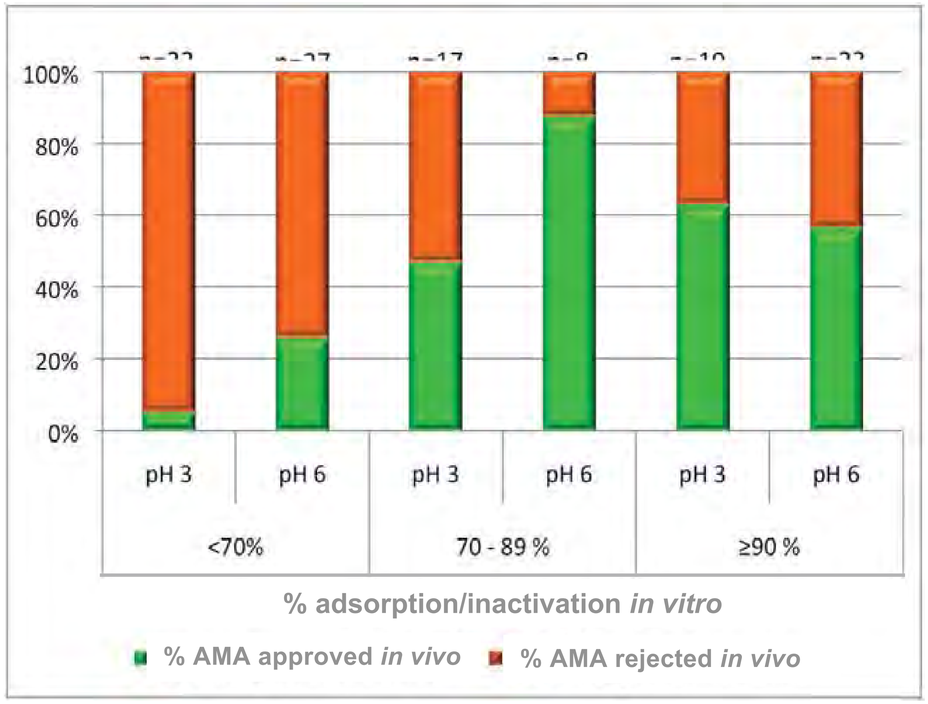
Table 23 - *In vivo* AMA evaluations in swine, done by LAMIC and SAMITEC Institute (2005-2010).

Mycotoxin	Evaluated	Approved	% of approval
Aflatoxins	13	7	54
Fumonisins	12	4	33
Zearalenone	45	8	18
Total	**70**	**19**	**27**

The relation between *in vitro* evaluation, in conditions which mimic gastrointestinal juices, and *in vivo* effectiveness cannot always be confirmed. In evaluations done on 58 AMA for different toxins and species, little more than 55% of AMA approved *in vivo,* had an adsorption/inactivation percentage greater or equal to 90% at pH 3 and 6. For AMA approved *in vivo,* more than 50% had an adsorption/inactivation percentage less than 70% at pH 3 and 6. Figure 45 illustrates the adsorption/inactivation percentages of 58 AMA evaluated *in vivo* and *in vitro* and their respective phases of adsorption/inactivation *in vitro*. Of the 19 AMA with 90% or more of adsorption/inactivation *in vitro* at pH 3 only 63% were approved *in vivo*. Of 22 AMA presenting less than 70% absorption/inactivation *in vitro*, only 5% were effective *in vivo*.

An AMA with low *in vitro* adsorption/inactivation percentage can perform satisfactorily in *in vivo* trials.

Figure 45 - *In vitro* adsorption/inactivation of 58 approved or rejected AMA *in vivo*.



There was no significant correlation between *in vitro* and *in vivo* evaluations when the data of the 58 *in vitro* and *in vivo* evaluations were submitted to a linear regression analysis. The greatest correlation found was in broiler chickens, at pH 6 with fumonisins ($P < 0.07$ and $R = -0.55$), followed by correlation for swine at pH 6 with aflatoxins ($P < 0.1$ and $R = 0.55$). It is evident from these analyses that some products that were ineffective in laboratory conditions, such as those found in *in vitro* trials, can often function satisfactorily in *in vivo* trials.

It is evident from this data that the results obtained from *in vitro* evaluations are not sufficient to prove the efficiency of an AMA. In order to approve an AMA, satisfactory results obtained from *in vivo* trials are necessary because even when reproducing the medium conditions using gastrointestinal juices, *in vitro* tests are merely a simulation and should only serve as a screening of these products. The *in vitro* test can be used as a quality control tool of different lots of AMA which have been approved using *in vivo* tests.

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