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Effects of orally administered Fumonisin B_1 mycotoxin on the fatty acid profile of the lipid classes in the renal and hepatic tissue of piglets

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Index

Abstract

The present work evaluates the effect of fumonisin B_1 (FB_1) on the fatty acid profile of the lipid classes of renal and hepatic tissues of piglets. The experiment was conducted at the University of Kaposvàr (Hungary) dividing the pigs into two groups: the group treated with 20 mg of FB_1/kg of feed and the control group. The duration of the trial was 10 days, followed by the killing of the animals. The analysis of the data showed that there were no significant differences between the groups in terms of production parameters and organs weight (liver, kidneys and lungs). The fatty acid profile proportion of kidney and liver was slightly compromised, suggesting a disturbance of lipid metabolism linked to the inhibition of the enzyme ceramide synthase. In the study, a slight change in the composition of membrane phospholipids was noted, probably linked to the lipid peroxidation action induced by treatment with fumonisins. Although pigs are one of the species most susceptible to fumonisins, this experiment has shown that exposure to fumonisins B_1 for a short period does not induce healthy and biochemical changes.

Riassunto

Il presente lavoro valuta l'effetto della fumonisina B_1 (FB_1) sul profilo degli acidi grassi delle classi lipidiche dei tessuti renali ed epatici di suinetti. L'esperimento è stato condotto presso l'Università di Kaposvàr (Ungheria) suddividendo i suini in due gruppi: gruppo trattato con 20 mg di FB_1 per kg di mangime e gruppo di controllo. La prova è durata 10 giorni seguita dall'uccisione dei soggetti. Dall'analisi dei dati è emerso che non ci sono state differenze significative tra i gruppi sia per quanto riguarda i parametri produttivi che per quanto riguarda il peso degli organi (fegato, reni e polmoni). Le proporzioni del profilo degli acidi grassi di reni e fegato è stato lievemente compromesso, suggerendo un disturbo del metabolismo lipidico legato all'inibizione dell'enzima ceramide sintasi. Nello studio è stato notato un leggero cambiamento nella composizione dei fosfolipidi di membrana, legato probabilmente all' azione di perossidazione lipidica indotta dal trattamento con fumonisine. Sebbene i suini siano una delle specie maggiormente sensibili alle fumonisine, questo esperimento ha evidenziato che l'esposizione a fumonisine B_1 per un breve periodo di tempo, non induce cambiamenti a livello biochimico e dello stato di salute.

1. Introduction

1.1 General description of mycotoxins

Mycotoxins are secondary toxic metabolites produced in nature by moulds growing on plants, which have adverse effects on humans, animals and crops that results in diseases and economical losses. Mycotoxins are termed as secondary metabolites since they have no biochemical significance in fungal growth and development. Not all moulds are toxigenic and not all secondary metabolites from moulds are toxic but one toxigenic mould can produce one or more of these toxic secondary metabolites (Hussein and Brasel, 2001). Unfortunately, in some genera there are species, for example *Fusarium verticillioides*, in which the 100% of the strains are mycotoxins producer under certain environmental conditions (Causin, 2009). Nowadays more than 400 mycotoxins are known (Zain, 2011). Mycotoxins are hard to classify because of their diverse chemical structures, biosynthetic origin, biological effects and their production (Bennett and Klich, 2003). Usually the classification is according to the training of the people doing the categorizing. Therefore, under a clinical point of view, mycotoxins are classified in relation to the affected organs: hepatotoxins, nephrotoxins, neurotoxins, immunotoxins, etc. Cells biologist used to group them in teratogens, mutagens, carcinogens and allergens. Even if no one of these is completely satisfactory, mycologist classified mycotoxins by the fungi that produce them. For example Aspergillus toxins, Penicillium toxins or Fusarium toxins (Bennett and Klich, 2003). Most of the studied mycotoxins belong to three genera of fungi: *Aspergillus*, *Penicillium* and *Fusarium.*

The most recent important epidemic was in 1960 when in England near London, there was an unusual veterinary crisis in which approximately 100,000 young turkeys died. It was discovered that this turkeys “X” disease was linked to a groundnut meal contaminated with alfatoxins, a secondary metabolite of *Aspergillus flavus* (Bennet and Klich, 2003). Since that moment, there was an increasing interest to investigate mycotoxicological effect both i*n vivo* and *in vitro.*

The major toxigenic species of fungi and their mycotoxins are presented in the following table. (Table 1)

Table 1: Moulds and mycotoxins of worldwide importance (FAO 2001)

Mould species	**Mycotoxins produced**
Aspergillus parasiticus	Alfatoxins B_1,B_2,G_1,G_2
Aspergillus flavus	Alfatoxins B_1,B_2
Aspergillus ochraceus	Ochratoxin A
Fusarium sporotrichioides	T-2 toxin
Fusarium graminearum	Deoxynivalenol Zearalenone
Fusarium verticillioides	Fumonisin B_1
Penicillium verrucosum	Ochratoxin A

Is well known that exposure to mycotoxins (by ingestion, inhalation or skin contact) can develop acute and toxic effects both in humans and in animals depending on the type of mycotoxins, level of exposure, the species, and the susceptibility of an animal within a species (Zain, 2011; Loi et al., 2017; Hussein and Brasel, 2001; Udomkun et al., 2017; EFSA, 2014; Fao/Iaea, 2001; WHO and FAO, 2012; Gowda et al., 2017; Pleadin, 2015; Zaki et al., 2012; CAST, 2003). Another main aspect to take in account is the interaction between mycotoxins named multi-toxic effect. Usually, feed is contaminated with multiple mycotoxins. For example, aflatoxin and fumonisin B_1 commonly occur together in the same grain. In 1993 the World Health Organization and the International Agency for Research on Cancer (WHO-IARC, 1993a,b) classified alfatoxins as carcinogenic to humans (Group 1), ochratoxins and fumonisins were classified as possible carcinogens for humans (Group 2B) while trichothecenes and zearalalenone were classified as not carcinogenic for humans (Group 3). Since these toxic compounds are transferred to animas with plants, and to humans with animal products, they could infect all the food chain. To protect the European consumers the European Food Safety Authority (EFSA) suggested to the European Commission, not only set the maximum level of mycotoxins in food and feed to guarantee humans and animals healthiness (Commission Regulation, 2006; European Parliament 2002/32/EC). They also gave a regulation about the sampling methods for the official control of the mycotoxins level (Commission Regulation 401/2006). However, the safety limits recommended are very different and depend on the country regulation and even species.

Focusing on the principal types of mycotoxins, alfatoxin B_1 is the most potent natural carcinogenic known (Squire, 2017). Alfatoxins are produced by different species of the genera *Aspergillus*. In farm animals, AFB_1 can cause liver dysfunction, reduction in egg and milk production and a long-term exposure, can result in an embryo toxicity; AFB_1 has also been shown to exert an immunosuppressive activity. The *Aspergillus* formation and the presence of AFB_1 in maize are favoured by hot and dry season and storage. Indeed in Croatia in 2013, an extremely warm year, 38.1% of maize samples were contaminated by AFB_1 and the 28.8% of the samples contained this toxin in levels higher than the maximum permitted level (Pleadin, 2015). Another important group of mycotoxins are the ochratoxins. In this group, ochratoxin A is the major metabolite of toxicological significance and is a frequent natural contaminant of many foodstuff like cereals, beans, fruits, fish, eggs and milk (Pleadin, 2015).

Fumonisins are a group of cancer-promoting metabolites, produced by *Fusarium spp.*, which are associated with several mycotoxicoses. In animals fumonisins damage the liver and kidneys, decrease weight gain and increase mortality rates (Akande, 2006). Fumonisin B_1 is identified as the most toxic metabolite, and the most sensitive species are pigs (pulmonary edema) and horses (equine leukoencephalomalacia) (Efsa, 2014). As *Fusarium* is among the most prevalent fungi on maize, it was noted that consumption of maize contaminated with fumonisins is associated with elevated occurrence frequency of human oesophageal cancer incidence in various parts of Africa, China and Iran but especially in South Africa (Zain, 2011).

One more kind of mycotoxins produced by Fusarium spp. are trichothecenes. The toxic actions generated by this class are necrosis of the oral mucosa and skin in contact with the toxin, acute effect on the digestive tract and decrease immune function (Schwarzer, 2009). There are two types of trichothecenes: type A included T-2 toxin (T-2), HT-2 toxin (HT-2) and diacetoxiyscirpenol (DAS); type B is represented by fusarenone-X (FUX), deoxynivalenol (DON) and nivalenol (NIV). Type B have a relatively low toxicity compared to type A trichothecenes, but this varies with species and cell types (Loi et al., 2017).

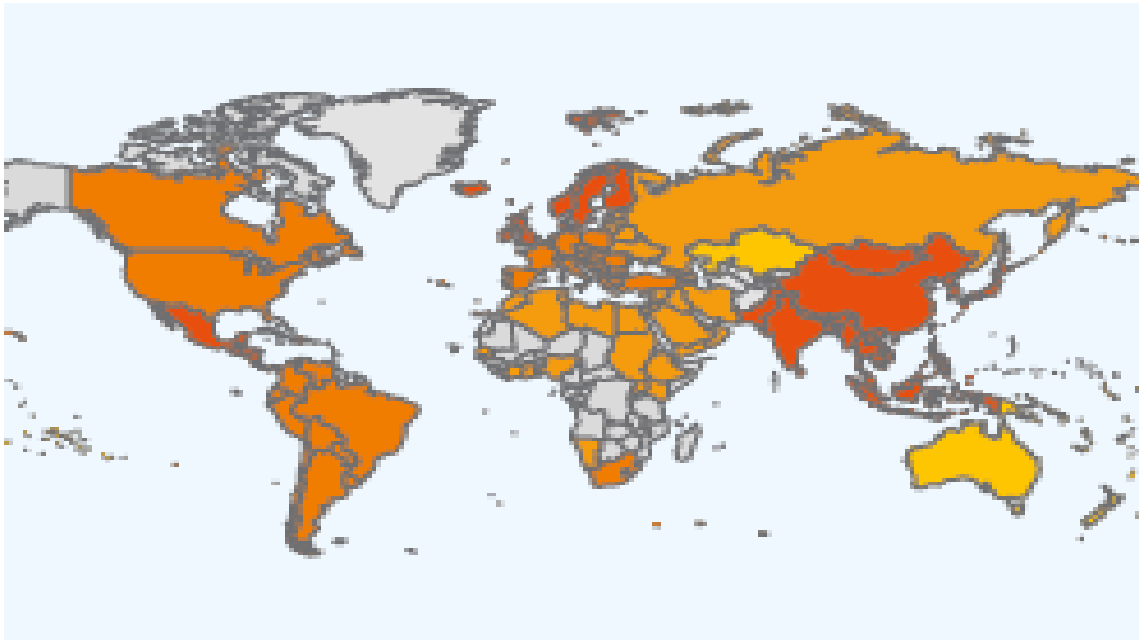
An additional mycotoxin produced by *Fusarium* moulds is zearalenone. This mycotoxin is known for its ability to inhibit the binding estrogenic hormones, resulting in fertility problems

in both humans and animals (Udomkun et al., 2017; Zain, 2011). Generally, ruminants are more resistant to mycotoxins due to the rumen microbial population. As Kiessling et al. (1984) describe, the rumen flora is able to convert some mycotoxins to less toxic metabolic compounds or even inactivate them at common exposure levels by bio-hydrogenation or deamination.

All mycotoxins are low-molecular-weight natural products. These compounds are produced in cereal grains during and after harvest (field and storage types) in various conditions and they can contaminate a wide range of commodities both pre and post harvesting. The production of mycotoxins in food and feed supply chain is a result of a multi factors combination like storage, environmental and ecological conditions. For example during the production phase in the field, temperature, water activity and insect infestation are favourable factors for fungal growth and proliferation. In the post-harvest scenario, handling and transportation systems are very important factors to keep the mycotoxins development under control (Paterson et al., 2010).

It is calculated that approximately 25% of the world's agricultural commodities are contaminated with mycotoxins (Quiroga et al., 2009). The cost produced by mycotoxins' commodities contaminations account for billion dollars every year (Wu, 2007). In CAST (2003) is reported that in the United States the mean economic annual cost of crop losses from alfatoxins, fumonisins and deoxynivalenol is 932million dollars. Even if is difficult to quantify the economic impact caused by mycotoxins, it is possible to summarize it in two categories. Direct losses, which are related to lowered crop yield, reduction of animal performance and the cost associated with the animal diseases; and indirect losses, which concern increased use of fungicide, reduction in the value of the commodities, veterinary-care cost, investments in strategies to reduce the problem (Loi et al., 2017).

As we can see from the BIOMIN mycotoxin survey (Figure 1), the contamination is spread all over the world.



Legend

- Moderate risk: 0-25% of samples above risk threshold
- High risk: 26-50% of samples above risk threshold
- Severe risk: 51-75% of samples above risk threshold
- Extreme risk: 76-100% of samples above risk threshold
- No samples tested

Recommended risk threshold of major mycotoxins in ppb

Afla	ZEN	DON	T-2	FUM	OTA
2	50	150	50	500	10

Total risk level is calculated as the proportion of samples that have at least one mycotoxin above a risk threshold level.

Figure 1: Global map of mycotoxin occurrence and risk in different regions between July and December 2016 (BIOMIN mycotoxin survey 2016)

In this survey 16.511 samples were analysed from January to December 2016 providing an overview on the incidence of the principal types of mycotoxins contained in the primary components used for feed as corn, wheat, barley, rice, soybean meal, corn gluten meal, dried distillers grains and silage (BIOMIN survey, 2016). The samples were derived from 81 different countries in the world and 63.000 analyses were performed. The assessment revealed an average of 28 mycotoxins and metabolites per sample and 9 out of 10 samples were contaminated with *Fusarium* toxins, *Aspergillus* toxins or both. Another interesting data is that the 94% of the samples contained 10 or more mycotoxins or metabolites. The survey divides the world in sub-regions and gives the opportunity to concentrate the attention on South Europe, Central Europe and North Europe. As shown in table 2 in the Southern Europe the total risk is 61% with a noticeable predominant of fumonisins (74%) followed by deoxinivalenol (46%).

Table 2: Regional overview (BIOMIN mycotoxin survey 2016)

Southern Europe Total Risk 61%		**Central Europe Total risk 60%**		**Northern Europe Total Risk 67%**	
Alfa	38%	Alfa	10%	Alfa	1%
ZEN	35%	ZEN	51%	ZEN	46%
DON	46%	DON	73%	DON	77%
T2	7%	T2	21%	T2	18%
FUM	74%	FUM	50%	FUM	12%
OTA	26%	OTA	12%	OTA	13%

Nevertheless, it is impossible to completely avoid the production of mycotoxins, but the contamination could be mitigated through strategic interventions. The "Codex Alimentarius" (WHO and FAO, 2012) contains general principles for the reduction of various kinds of mycotoxins in cereals, taking into account the different climate, crops and agricultural systems of each country. The recommendations are divided in Good Agricultural Practices (GAP's) like harvesting in the right time, crop rotation, avoid overcrowding; and Good Manufacturing Practices (GMP's) like drying to a safe moisture level, cleaning of the containers, measuring the temperature. Other studies show how to remove or inactivate mycotoxins using different chemicals like propionic acid, ammonia, copper sulfate, benzoic acid, urea, citric acid, and others. However, these ones are unpractical on food because of their potential toxic activity, poor efficacy, high costs and negative effects on the quality (Gowda et al., 2017; Loi et al., 2017). It is demonstrated that mechanical heat treatment, irradiation and use of adsorbents are able to remove contaminated fractions of food and feed. However, this approach can have some possible negative disadvantages as binding essential nutrients like minerals and vitamins and thus reducing their availability (Yadav, 2017; Loi et al., 2017). There are also biological methods, like degradation of mycotoxins into non-toxic metabolites by using fungi, bacteria or enzymes. During the biotransformation, these living microorganisms are able to metabolize, destroy or deactivate toxins resulting in less dangerous compounds. In any case, if the mycotoxins' contamination level is very high, the microorganisms can be altered or inhibited. This means that the time to rise a satisfactory decontamination level becomes longer (Loi et al., 2017).

1.2 Fusarium spp.

Fusarium spp. is a huge genus manifested worldwide. The widespread diffusion of *Fusarium spp.* is due to the ability of these fungi to grow on different kinds of substrates for example on soil, subterranean and areal plant parts, plant debris, and other organic substrates (Nelson et al., 1994). Living on dead or decaying organic matter, the major part of *Fusarium spp*. are called "saprophytes". Even if not all the *Fusarium* species are able to synthesise dangerous molecules, most of them are able to produce a large variety of mycotoxins with various action pathways. Secondary fungi metabolites adversely affect plants, animals and humans, causing toxic effects. This genus is well know as a plant pathogen that can cause rots, wilts and other diseases. *Fusarium* species infect and produce mycotoxins in wheat, corn, rice, barley, oats and other cereals and forages that are a consistent or predominant part in humans and animals diets. For example in most regions of the world, *Fusarium graminearum* is the major causal agent of head blight (or scab) in wheat and red ear rot of corn (CAST, 2003). Thus, the presence of this genus in the fields and harvest, results in an economical and sanitary consequences. Most of the contamination starts in the field due to an infection. During the storage, in order to minimize the spread of the mycotoxins, the most important factor is the control of moisture level and temperature.

There are many species, and population within species, that make *Fusarium* very difficult to classify. The variations can be morphological, cultural and physiological, that may explain the ability of *Fusarium* to be ubiquitous. It is also noted that *Fusarium spp*. require lower temperatures and higher humidity for growth and production of mycotoxins than the genus *Aspergillus*. However, *Fusarium spp*. is also common in tropical regions and is found in the desert, alpine and artic areas where severe climatic conditions prevail (Nelson et al., 1994).

The optimal temperature conditions to develop and produce fumonisins are widely different, according to the considered fungal strain. Under laboratory conditions, *Fusarium verticillioides* strain MRC 826 (the strain of *F. verticillioides* used in all experiments, originally isolated from corn in Transkei, Southern Africa 1975) grows rapidly on potato dextrose agar at 25°C in aerobic condition. It spreads in woolly or cottony colonies, as a typical filamentous fungus. In field, its development is favourite by a temperature between 22.5 °C and 27.5 °C but in some cases, it can rise up to 30°C, according to the origin area. For example, *Fusarium verticillioides* coming from a temperate zone has an optimum

temperature at 30°C. This *fungus* has a water activity (a_w) 0.98, proving that it requires a high quantity of water. In the cornfields, the infection can start in three ways: infect seeds as a spore, only apparently heathy, and ready to activate when the environmental conditions are suitable; through inoculated fungi in the soil; because of insects attacks that can damaged the plants or the seeds, facilitating the infestation. If the spreading occurs, the harvest must be destroyed or mixed with uncontaminated crop to reach an acceptable level for human or animal consumption.

It is impossible to completely eliminate the contamination caused by mycotoxins on the commodities. However is possible to prevent and reduce the occurrence of the *Fusarium* species through the adoption of good agricultural practices. These principles are illustrated in the Commission Recommendation 2006/583/EC "on the prevention and the reduction of Fusarium toxins in cereal and cereals product" (Commission Recommendation 2006/576/EC, 2006) and in the "Codex Alimentarius"(WHO and FAO, 2012). As mentioned above the fundamentals cited in these documents are generals and have to be applied in relation to the local situations (crops, climate and agronomic practices). The most important factors to take in account are: crop rotation, choice of variety/hybrid, crop planning, soil and crop management, harvesting, drying, storage, transport from storage. It is clear that the application of the good practices is not enough to contrast the development and the spread of the *Fusarium spp.* and other treatments are necessary. Physical, biological and chemical treatments can be used but with a variable degrees of success (Sweeney and Dobson, 1998). All of these procedures should inactivate, destroy or remove the mycotoxin; the process must not cause the release of toxic substances, metabolites or by-products in the food or feed; retain nutrient value and feed acceptability of the product or commodity; there must not be alterations of the product's technological properties (CAST, 2003).

To detect the presence of mycotoxins, testing of the commodities is required. This procedure starts with correct sampling. The samples must be representative of the entire lot, taking an adequate size sample. The next steps are sample preparation, extraction of the toxin into an extraction solvent and analysis. There are different methods of analysis such as chromatographic methods i.e.: thin layer chromatography (TLC), high-performance liquid chromatography (HPLC) and gas chromatography (GC); fluorescence procedure or immune enzyme test like enzyme-linked immunosorbent assay (ELISA). The decision between these

methods is based on three criteria: speed of analysis perform, technical skill level required to perform the test and if the assay afford a qualitative or quantitative result (CAST, 2003).

The main mycotoxins classes produced by *Fusarium* are: trichothecenes, zearalalenone and fumonisins (Placinta et al., 1999). In a survey of Griessler et al. (2010), over a 4.5 years period the incidence of mycotoxins was assessed in feed materials and compound feed in five Southern European countries including Spain, Greece, Portugal, Cyprus and Italy. The collected samples were analysed with two different methods (HPLC and ELISA) for the most relevant mycotoxins as alfatoxins, zearalenone (ZEA), trichothecenes A and B, fumonisins and ochratoxin A. It was discovered that the *Fusarium* mycotoxins were the major contaminants. In Italy, 72% of the samples were positive for the trichothecenes type B, 73% for the fumonisins and 35% for zearalenone. It was observed a high average fumonisins contamination on maize (especially in the north of Italy) compared to the other regions surveyed.

1.2.1 Trichothecenes

Trichithecenes is a group of mycotoxins produced by different types of *Fusarium* species. Although it is known that other genera of fungi are able to produce trichothecenes (Bhat et al., 2010; Sweeney and Dobson, 1998), the most important are *F. equiseti*, *F.graminearum*, *F. verticillioides* and *F. sporotrichioides* (Sweeney and Dobson, 1998). This large family was named after the compound trichothecin, which was one of the fist members identified. All the trichothecenes contained a sesquiterpe rings characterized by a 12,13-epoxy-tichothec-9-ene nucleus. The toxicity of this group is given by the 12,13-epoxide ring. Based on the position of their functional group, trichothecenes are classified into four groups: A, B, C and D. The most common are: type A that includes T-2 toxin (T-2), HT-2 toxin (HT-2) and diacetoxiyscirpenol (DAS); and type B represented by fusarenone-X (FUX), deoxynivalenol (DON) and nivalenol (NIV). Type A trichothecenes are more toxic than type B trichothecenes (CAST, 2003; Bhat et al., 2010). *Fusarium graminearum* produces DON and NIV. It has an optimal growth range between 24-26°C and it requires a minimum a_w of 0.90. One of the most common producer of T-2 toxin, DON and NIV is *Fusarium sporotrichioides*. It has an optimum growth range between 22.5 and 27.5°C and the minimum a_w is 0.88 (Sweeney and Dobson, 1998; Fao/Iaea, 2001). Trichothecenes are not degradable during food processing, they are stable at neutral and acid pH, and thus are not hydrolysed in the stomach after the

ingestion (Loi et al., 2017).

Trichothecenes are strong inhibitors of eukaryotic protein and DNA synthesis and are associated with several effects on animal systems such as immunologic effects, hematopoietic effects, and nephrotoxicity (CAST, 2003; Zain, 2011). Mycotoxins that belong to the family of trichothecenes are amphiphilic (both hydrophilic and lipophilic), with a low-molecular weight. This means that they can move through cell membranes passively and be easily absorbed by the gastrointestinal tract. Common symptoms are anorexia, weight loss, poor feed utilization, vomiting, bloody diarrhea, dermatitis, abortion and death. A general overview is given in table 3.

Table 3: Commodities in which mycotoxin contamination has been found and the resulting effects on animals and humans (CAST, 2003)

Mycotoxin	**Commodities found contaminated**	**Effects of mycotoxins**	
		Affected species	**Pathological effects**
Trichothecenes (T-2 toxin, DAS, NIV, DON, HT-2 toxin, FUX)	Corn , wheat, commercial cattle feed, mixed feed	Swine, cattle, chicken, turkey, horse, rat, dog, mouse, cat, human	Digestive disorder (emesis, diarrea, refusal to eat) Hemorrage (stomach, heart, intestine, lungs, bladder, kidney) Edema Oral lesions Dermatitis Blood disorders

Among type A, T-2 is the most important. T-2 is a common contaminant of grains both in Europe and the United States (CAST, 2003). It is present in wheat, rye, soybeans and maize (Kanora and Maes, 2009) and is particularly related with wet harvest (Fao/Iaea, 2001). When ingested it is metabolized by the gut microflora of mammals into other metabolites; the first metabolite is HT-2 toxin which is absorbed into the blood (Bhat et al., 2010). It is probable that T-2 toxin caused the disease called "alimentary toxic aleukia" which affected entire villages in Siberia after the Second World War. Symptoms are vomiting, fever, acute inflammation of the alimentary tract and blood anomalies. T-2 is the most toxic compound that causes dermal toxicity. It was demonstrated that after 24 h from the application it causes an edema. In guinea pigs, T-2 toxin caused erythema on the shaved backs at dose of 5ng (CAST, 2003). In poultry, T-2 is associated with mouth and intestinal lesions, decrease of feed consumption, weight loss and decrease of eggs production (Zaki et al., 2012). T-2 is the most harmful Trichothecene on the reproductive system of pigs. In one study, a low concentration of this toxin in the feed (1-2 mg/kg) of pregnant sows during the last third of gestation was sufficient to induce an inhibitory effect on the ovaries, histological degeneration and atrophy.

DON is a notable contaminant of corn, barley and wheat (CAST, 2003). Vomitoxin, the other name of DON due its emetic effect, is highly stable and can survive at various food-processing methods as milling and powdering (Bhat et al., 2010). In animals it causes neurotoxic effect demonstrated by vomit at high doses (20 mg DON/kg feed) and feed refusal at low doses (12 mg DON/kg feed), resulting in a loss of productivity and immunosuppression (CAST, 2003; Bennett and Klich, 2003; Pierron et al., 2016). Concerning farm animals, monogastrics are more sensitive to DON especially swine, followed by poultry and ruminants. Indeed in the (Commission Recommendation 2006/576/EC, 2006) concerning the presence of some mycotoxins in products intended for animal feeding, the amount of DON in complementary and complete feeding stuffs for pigs is 0.9 ppm instead of 5 ppm. In a meta-analysis (Andretta et al., 2012) it is reported that DON reduced feed intake and weight gain by the 26% in pigs.

1.2.2 Zearalenone

Zearalenone is a mycotoxin produced primarily from *Fusarium graminearum*, which is naturally present in high-moisture corn. High humidity and low temperatures promote the mycotoxin biosynthesis on food and feed stuffs. Zearalenone is a common contaminant of corn, wheat, barley and grain sorghum (CAST, 2003). Zearalenone is difficult to be eliminated or decomposed during food and feed processing because it is heat-stable (Zaki et al., 2012). Usually it is present with others mycotoxins such as trichothecenes and in particular DON. The most notable effect of this mycotoxin on animal is on the reproductive system, but it also can cause teratogenic effects (CAST, 2003). When zearalenone is ingested, it is absorbed in a very high ratio (in pig 80-85% was estimated (Zinedine et al., 2007)). It is processed in the liver where is hydroxylated and results in two different metabolites: α-zearalenol and β-zearalenol. Zaralenol, the reduced form of zearalenone, increase estrogenic activity and was used as an anabolic agent for both sheep and cattle. In 1989 it was banned from the European Union, but is still used in other parts of the world (Bennett and Klich, 2003). Zearalenone and its metabolites induce reproductive disorders, mimicking the effect of estrogens due to the structural similarity with that molecule. Low dose of ZEN results in feminization while at high concertation ZEA interferes with ovulation, conception, implantation and foetal development (CAST, 2003). In pregnant animals, it is associated with embryonic death and abortion. Swine are particularly sensitive to ZEA, showing a physiological response when the level of this mycotoxin exceeds about 1 mg/kg of feed (CAST, 2003). Symptoms of hyperestrogenism caused by ZEA are swelling of the vulva, and enlargement of the mammary glands, especially in prepubescent gilts. In young male pigs, zearalenone induces feminization by causing testicular atrophy, swelling of the prepuce and mammary glands enlargement. Young piglets can be affected through sows' milk, provoking estrogenism. Other effects induced by ZEA are anestrous, nymphomania and pseudo-pregnancy. In dairy animals zearalenone promotes reduced feed intake, decreased milk production, vaginitis, increase of vaginal secretion, low reproductive performance and mammary glands enlargements (Zinedine et al., 2007). In the (Commission Recommendation 2006/576/EC, 2006) the maximum level permitted in feed materials is 2 ppm (exception for the maize by-product 3 ppm) but the quantity in complete and complementary feedstuffs for the most sensitive animals is low. For example in piglets and gilts, the level of ZEA is 0.1

ppm; sows and fattening pigs have the limit at 0.25 ppm, and in feed for calves, dairy cattle, sheep and goats the limit is 0.5 ppm. A general overview is given in table 4.

Table 4: Commodities in which mycotoxins contamination have been found and the resulting effects on animals and humans (CAST, 2003)

Mycotoxin	**Commodities found contaminated**	**Effects of mycotoxins**	
		Affected species	**Pathological effects**
Zearalenone	Corn , moldy hay, pelleted commercial feed	Swine, dairy cattle, chicken, turkey, lamb, rat, mouse, guinea pig	Estrogenic effects (edema of vulva, prolapse of vagina, enlargement of uterus) Atrophy of testicles Atrophy of ovaries, enlargement of mammary gland Abortion

1.3 Fumonisins

The most important mycotoxins relatively to this study are fumonisins. It is proved that fumonisin mycotoxins are responsible for the major toxicological and carcinogenic effects caused by corn cultures in experimental animals.

1.3.1 Isolation, Identification and Structure

Fumonisins are produced by fifteen *Fusarium* species but the most important producers are *F. verticillioides* and *F. proliferatum* because of their worldwide distribution, particularly on maize. In order to detect cancer-promoting compounds, Gelderblom et al. (1988) firstly isolated and purified fumonisin mycotoxins from culture material of the most studied strain of *Fusarium moniliforme* (formerly= verticillioides) MRC 826. Gelderblom et al. (1988) known that the culture material of *F. Moniliforme* MRC 826 is hepatocarcinogenic in rats and cause LEM in horses. They established a bioassay to understand if fractions isolated from culture material of each toxic strains were correlated with the cancer-promoting activity by incorporating them in the rat feed. Rats were initiated with a diethylnitrosamine (DEN) treatment (as a cancer initiator) and the induction of γ-glutamyltranspeptidase positive (GGT+) foci (as end point). After four weeks of treatment, rats were killed and their livers were subjected to histochemical analyses. Culture material was extracted with ethyl acetate and CH_3OH-H_2O (3:1) by blending and filtering. The extract was evaporated and partitioned between CH_3OH-H_2O (1:3) and $CHCl_3$. The CH_3OH-H_2O fraction was chromatographed on an Amberlite XAD-2 column, and the active part was eluted with CH_3OH. This fraction was chromatographed on a silica gel column with $CHCl_3$-CH_3OH-CH_3COOH (6:3:1) and later purified on a C18 reverse-phase column. Two compounds were obtained in pure form and were chemically characterize by nuclear magnetic resonance and mass spectroscopy: fumonisin B_1 and fumonisin B_2 (Figure 2).

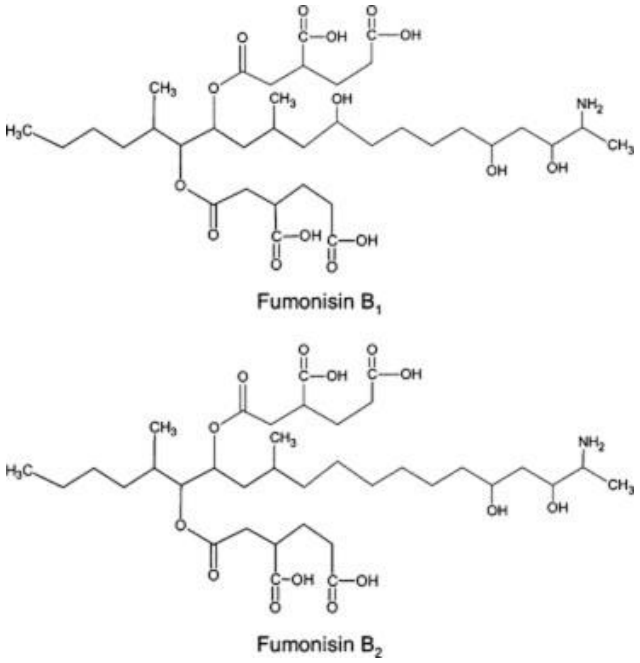


Figure 2: Chemical structure of Fumonisin FB_1 and FB_2

Since than, 28 fumonisins analogs have been characterized. Fumonisins are divided in four main groups: A, B, C and P series, which contain two tricarballylic acid moieties. The FB analogs are the most abundant naturally occurring fumonisins (Rheeder et al., 2002) and in particular FB_1 is found at the highest level (from 70% to 80% of the total fumonisins produced) (Marasas, 1996).

1.3.2 Natural occurrence

The two major fumonisins FB_1 and FB_2 occur mainly in maize and maize products at a global level (Visconti et al., 1995; Sydenham et al., 1990; SCOOP TASK, 2003; Griessler et al., 2010; Zinedine et al., 2007; Waśkiewicz et al., 2012; Zaki et al., 2012; Shephard et al., 1996). The first conclusive report of the natural occurrence of FB_1 in corn is the study of Sydenham et al. (1990). In this study, they collected home-grown corn from the Transkei area, in Southern Africa, in order to analyse the presence of the fumonisin mycotoxins. Using two high performance liquid chromatography procedures and a capillary gas chromatographic-mass spectrometric procedure, they confirmed the presence of fumonisin B_1. This evidence suggested that humans living in that area may well be exposed to the cancer-promoting activity of the fumonisins.

The occurrence of fumonisins in Europe was assessed many times through different studies.

For example Visconti et al. (1995) summarize in their article two consecutive studies ordered by The European Commission, Community Bureau of Reference and Measurement and Testing Programme in which they wanted to improve the quality of fumonisins analysis at European level. In this study they confirmed that the highest production of fumonisins was obtained with isolates from maize (1,259 µg FB_1/g), followed by isolates from wheat (769 µg FB_1/g) and barley (320 µg FB_1/g). Furthermore, they investigated the natural occurrence of FB_1 and FB_2 in different countries and in different inbred lines of maize, calculating the incidence and the levels of fumonisins. The highest contamination was found in Italy and Portugal with 100% of incidence and fumonisins levels up to 2.85 and 4.45 µg/g, respectively. However, in countries like Croatia, Poland and Romania very low levels of contamination were found. This suggests that the environment of a specific area of cultivation and storage conditions plays a role in the formation of fumonisins in maize.

An interesting data for the Italian population is the high contamination levels found in maize flour or polenta. About 85% of the samples collected in Italy, showed fumonisin levels higher than 1 mg/kg, the maximum level admitted. This implies that FB_1 is stable during most types of processing, even if the concentration is reduced during the manufacture of corn-starch by wet milling because FB_1 is water-soluble. Particular attention should be given to the link between the increased consumption of maize based products in the north-east of Italy and the increased risk of incidence of human oesophageal cancer.

Another report was conducted in 2003 (SCOOP TASK, 2003) to provide information on the exposure of the population of EU Member States to fumonisins. This report confirmed that cereals, in particular corn and wheat, are the major source of intake of FBs.

In 2016, BIOMIN (2016) conducted their annual survey to monitor the spread of mycotoxins in the primary components used for feed in the world. In 2016, fumonisins were the second source of contamination in corn in Europe: 67% of samples contaminated with fumonisins had an average concentration of 2057 mg/kg.

1.3.3 NOEL, TDI

The no-observed-adverse-effect-level (NOAEL) is the level of exposure of an organism that has no biologically or statistically significance when compared to its appropriate control (www.wikipedia.org). The NOAEL depends on the time of exposure to the mycotoxins, in

this case to FB_1. Acute toxicity is studied in one dose; subacute toxicity concerns a repeated dose (14-28 days); sub-chronic toxicity implies that the administration involves 10-25% of the life of the animal while chronic toxicity regard more than 50% of the life of the animal. Different studies have been conducted to different animals in order to determine the limit of exposure to FB_1. Anyhow, cell apoptosis is one of the observation to determine the NOAEL in experimental animals. The dose level causing apoptosis is quite different according to the duration of exposure, the form of the fumonisins (pure or fungal culture) and the species. For example in pigs and horses, some typical effects are porcine pulmonary edema (PPE) and equine leukoencephalomalacia (ELEM). The NOAEL for PPE in pigs fed with FB_1 is lower than 50 mg FB_1/kg BW/day. The NOAEL for ELEM in horses fed with FB_1 is lower than 0.2 mg FB_1/kg BW/day. Some examples of NOAEL are shown below (Table 5).

Table 5: NOAELs from different studies expressed as mg FB_1/kg BW (Scientific Committee on Food, 2000)

Species	Duration	Target organ	NOAEL	Reference
Rat	Short-term	Liver	<0.75	Gelderblom et al, 1994
Pig	Short-term	Lung/PPE	<4.5	Motelin et al, 1994
Horse	Short-term	Brain/ELEM	0.2	Ross et al, 1994
Mouse	Sub chronic	Liver	1.8	Voss et al, 1995
Rat	Sub chronic	Kidney	0.2	Voss et al, 1995
Rat	Chronic	Liver	1.25	Gelderblom et al, 1995
Mouse	Chronic	Liver	0.6	NTP, 1999

The tolerable-daily-intake is an estimate of the amount of a contaminant in food or water that can be ingested daily throughout life without causing any appreciable risk to health. In the WHO/IARC (2002) they evaluated the risk of human exposure to FB_1 and they confirmed the previous evaluation, made in 1993, that there is inadequate evidence of carcinogenicity of

fumonisins, so FB_1 is possibly carcinogenic to humans (Group 2B). However, there is sufficient evidence in experimental animals for the carcinogenicity of FB_1. The Scientific Committee on Food of the European Commission concluded that the TDI for FB_1, FB_2 and FB_3, alone or in combination is 2 µg/kg BW per day (SCOOP TASK, 2003; Commission Regulation 1881/2006, 2006; http://ec.europa.eu/food/fs/sc/scf/out185_en.pdf).

Taking into account the maximum tolerable level of mycotoxins ingested by livestock, the European commission recommended guidance values. Deoxynivalenol, zearalenone, and fumonisins FB_1 and FB_2 are transferred at an only limited extent from feed into meat, milk and eggs and so food from animal origin can contribute only marginally to the total human exposure to these toxins (Commission Recommendation 2006/576/EC, 2006). The limit of human food contamination is used as a reference also for animal feedstuffs. The maximum levels for certain contaminants in foodstuff is regulated by the (Commission Regulation 1881/2006, 2006). However, the European Commission has set a maximum level of contamination in products intended for animal feeding as shown in the following table (Table 6).

Table 6: Guidance Values in products intended for animal feeding (Commission Recommendation 2006/576/EC)

Mycotoxin	Products intended for animal feed	Guidance value in mg/Kg (ppm) relative to a feed stuff with a moisture content of 12%
Fumonisin B_1+ B_2	Maize and maize products	60
	Complementary and complete feedstuffs for:	
	✓Pig, horse, rabbits and pet animals	5
	✓Fish	10
	✓Poultry, calves and lambs	20
	✓Adult ruminants and mink	50

1.3.4 Mode of action

In all animal studied, including pigs, fumonisins are rapidly adsorbed but the quantity of FB_1 detected in tissues after oral administration is very low (< 4% of the dose). This denotes that the accumulation of fumonisins is less than expected based on the relative amounts in the feed (WHO and FAO, 2012; Riley and Voss, 2006).

The toxicity of fumonisins is due to the structural similarity to the cellular sphingolipids, and this similarity causes the disruption of lipid metabolism. The disruption of lipid metabolism implies alterations of lipid composition of cell membranes, changes in intracellular and extracellular concentrations of lipid mediators, alteration in the expression and activity of signalling and regulatory pathways that control physiological processes critical for cell growth, differentiation and normal cell function (FAO/WHO, 2012). The mode of action of fumonisins is firstly explained by the interference with the *de novo* synthesis of sphingolipids, in particular because of the structural similarity with sphingosine (So).

Sphingolipids are a class of lipids found in all eukaryotic cells as well as some prokaryotic organisms and viruses. Nowadays over 300 sphingolipids have been identified, and the common trait is a long chain base backbone (sphingoid base). The most common sphingoid base in mammals is sphingosine but other frequently occurring sphingoid bases are sphinganine and 4-hydroxysphinganine. The functions of sphingolipids are multiple because they are involved in the regulation of cell growth, differentiation, neoplastic transformation, participation in cell-cell communication and cell-substratum interactions and interactions with cell receptors and signalling system (Wang et al., 1991). The first step of the sphingolipid biosynthesis started with the condensation of the amino acid L-serine and a fatty acid as the palmitoyl-CoA, covered by the catalysed enzymatic reaction from the enzyme serine-palmitoyl-transferase. From the reaction a molecule of CO_2 and 3-ketosfinganine fatty acid are released. In the next step, the 3-ketosfinganine is reduced in D-erythro-sphinganine or sphinganine (Sa) by the enzyme 3-ketosfinganine reductase NADPH-dependent. The sphinganine undergoes an acylation with a molecule of acyl-CoA, catalysed by sphinganine N-acyltransferase, producing dihydroceramide or D-eritro-dihydroceramide. The latter is converted to ceramide by dihydroceramide desaturase. Thus, ceramide can be used for the biosynthesis of sphingomyelin, glycosphingolipids or phosphorylated sphingolipids such as sphingosine 1-phosphate. This reaction takes place in two successive phases. In the first phase, the cellular enzyme ceramidase by deacylation of ceramide produces a sphingosine molecule (So). In the second phase, the enzyme sphingosine kinase catalyse the phosphorylation of the sphingosine giving sphingosine 1-phosphate. The sphingosine 1-phosphate can be reconverted into sphingosine through sphingosine 1-phosphate phosphatase, or can be divided by sphingosine 1-phosphate lyase into ethanolamine 1-phosphate and into a C16-acyl-aldehyde fatty acid (Merrill and Sandhoff, 2002).

As shown in the figure below (Figure 3), fumonisin B_1 inhibits a key enzyme in the *de novo* biosynthesis of sphingolipids known as ceramide synthases (sphingoid base N-acyltransferase) (Enongene et al., 2000; WHO/IARC, 2002). The explanation for why fumonisins inhibit the *de novo* ceramide biosynthesis is because they have a primary amino group at the C_2 position which bind ceramide synthase and prevent the CoA-dependent acylation of sphinganine (Sa) (Voss et al., 2011). Most notably the recent discovered 1-deoxysphinganine which, like fumonisin, lacks a hydroxyl group on C_1 and is found in large

quantities in liver of mice fed diets with FB_1 (FAO/WHO, 2012).

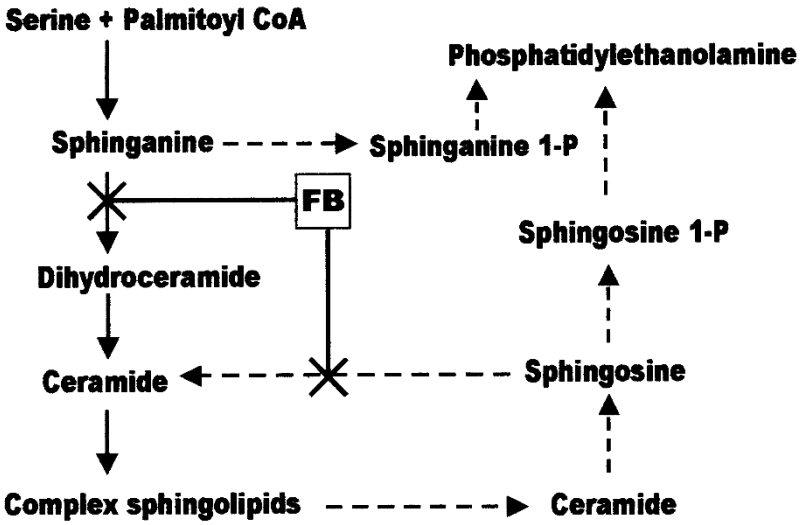


Figure 3: Outline of the de novo sphingolipid biosynthetic pathway and modality of the activity of FB_1 (Enongene et al., 2000)

The major biochemical and cellular consequences of fuminisin inhibition of ceramide synthases are the accumulation of free sphingoid bases and their 1-phosphates in tissues, serum and urine. The increase of sphingosine (So), although to a lesser extent than sphinganine, is caused by the inhibition of ceramide synthase, which promotes the acylation of sphingosine. Another consequence is the depletion or alteration of more complex sphingolipids and decreased ceramide and ceramide 1-phosphate biosynthesis (Voss et al., 2007; Voss et al., 2011; FAO/WHO, 2012; Gelderblom et al., 1997). Free sphingoid bases and ceramide can induce cell death, so the inhibition of ceramide synthase can inhibit cell death. On the other hand, redirection of sphinganine into sphinganine 1-phosphate is a potential survival signal stimulating cell proliferation. For this reason, fumonisins have been used to demonstrate the role of sphingosine 1-phosphate, ceramide and more complex sphingolipids in processes that control diverse cellular responses. Furthermore, the increase of free sphinganine is a biomarker for farm animals to evaluate the exposure to toxic levels of fumonisins. In animals, consumption of FB_1 increases the levels of free sphinganine (Sa) in liver, kidney, serum and/or urine (Enongene et al., 2000) which is thought to be largely responsible for the toxicity and carcinogenicity of this mycotoxin (Yoo et al., 1992; Humpf

et al., 1998). Accumulation of sphingoid bases and the increasing Sa:So ratio after fumonisins exposure have been demonstrated in multiple studies (Enongene et al., 2000).

1.3.5 Studies in animals and main target organs

It is proved that fumonisins have diverse effects on animals (localisation of lesions, target organs), according to sex, strain, and species (FAO/WHO, 2012). Even if certain organs appear to be constantly affected to a greater or lesser degree, the main target organ are different in each species: the brain in horses, liver and heart in rats, the lung in pigs, the kidney and liver in sheep (Kriek et al., 1981). In particular, FB_1 is toxic to the liver and kidney in all species, causing apoptosis and subsequent mitosis in the affected tissue. FB_1 is toxic to the cardiovascular system in pigs and horses as well (Voss et al., 2007). Anyway is noted that nutritional factors, such as folate and antioxidants, modulate fumonisin toxicity.

Horses

Fumonisin B_1, produced by *F. moniliforme* is the cause of equine leukoencephalomalacia (ELEM) when repeated injections of 125 µg/kg BW induced brain and liver lesions (Marasas et al., 1988). Symptoms are ataxia, head pressing, paralysis of the lips and tongue and convulsion. At the necroscopy, focal malacia and liquefaction of cerebral white matter with peripheral haemorrhage are found. The mechanism by which fumonisins cause this disease are apparently linked to their ability to inhibit sphingolipid synthesis (CAST, 2003). The rate of mortality is near the 100%, makes horses one of the most sensitive specie to fumonisins (Voss et al., 2011).

Pigs

Another sensitive farm animal is swine. The first study reported severe toxicosis, called porcine pulmonary edema (PPE) was (Kriek et al., 1981). In 1989 in the Midwestern and Southwestern parts of the United States thousands of pigs died from PPE after ingesting corn contaminated with fumonisin B_1 (from 155 to 92 mg/kg). A hot dry summer in those areas of United States were responsible for the very high levels of fumonisin. Physiologically the two main causes of pulmonary edema are left ventricular failure, which increases pulmonary capillary hydrostatic pressure, and increased vascular permeability after injuries to the alveolar capillary endothelium or alveolar epithelium (Colvin et al., 1993). To develop PPE, pigs have to be fed with feed containing concentrations of $FB_1 \geq 92$ ppm or ≥ 16 mg/kg

BW/day. Equally, lethal PPE occurs within 7 days after daily intravenous administration of FB_1 (Harrison et al., 1990). In addition, hepatic lesions consisting in apoptosis, necrosis and hepatocyte proliferation are observed.

The pharmacokinetics of FB_1 in swine indicates a poor bioavailability (3-6% of absorption). Fumonisin are firstly excreted in the faeces (60%) and secondly in the urine (20%) with enterohepatic recycling. After 72 hours from the administration of fumonisin residues are greatest in liver than kidney, large intestine, brain, lung, heart, spleen.

Low doses of fumonisin B_1 at 0.5 mg/kg BW/day can reportedly increase susceptibility of pigs to pathogenic *E. coli* strains, *Pseudomonas aeruginosa* and *Pasteurella multocida.* Fumonisins are also known to increase the susceptibility of pigs to Porcine Reproductive and Respiratory Syndrome. Another specific effect of fumonisin in swine is the accumulation of membranous material in pulmonary capillary endothelial cells. Moreover fumonisin induced cardiovascular changes due to the inhibition of L-type calcium channels by increased sphingosine and/or sphinganine concentration (Colvin et al., 1993). Because of the similarity between swine and human cardiovascular system, the mechanism of fumonisin toxicosis in swine must be studied to permit assessment of its potential toxicity in humans. In fact, it is well known that the association between high level of fumonisin ingestion and esophageal cancer caused by neural-tube defects linked to fumonisin-induced folate deficiency (CAST, 2003).

Ruminants

Ruminants are less sensitive to mycotoxins, which may be explained by the fact that fumonisin is unmetabolized in the digestive tract and excreted in the faeces. However, the production of milk and beef meat, reproduction and growth can be altered if animals are fed with mycotoxin-contaminated feed for a long period (Pierron et al., 2016; Hussein and Brasel, 2001).

Rats

Fumonisins have been shown to act as liver cancer promoters in rats. Gelderblom et al. (1991) found hepatocarcinomas in male rats fed 50 mg of fumonisins B_1/ kg for 26 months. After using purified (>90%) FB_1 and a single dose of 0, 5, 50, or 500 µg/kg BW in male Wistar

rats, a significant increase in the number of apoptotic cells in liver were observed after 24 hours at 5 µg/kg BW. At 50 and 500 µg/kg BW the number of apoptotic cells in liver was significantly increased from 4 to 48 hours after treatment, and necrotic cells were observed at all dose levels and times. This implies that the single-dose NOAEL for apoptosis in male Wistar rat liver would be below 5 µg/kg BW.

2. Experimental Part

2.1 Aim of the study

The main objective of the study was to test the effects of 20 mg/kg feed dose of FB_1 mycotoxin on the lipid composition of growing piglets, following a 10-day exposure. The basic hypothesis was that via the ceramide synthesis blockade FB_1 might induce a disturbance in the fatty acid metabolism of the liver and the kidney. This has been approached by analysing the two most abundant and important lipids fractions' fatty acid profile, namely phospholipids and triglycerides.

2.2 Material and Methods

2.2.1 Experimental Plan

To investigate the short-term effect of Fumonisins B_1 on the lipid (fatty acids) metabolism in swine, a 10 days long experiment was set up by the University of Kaposvàr (Hungary). The experiment was set at the Agricultural Department of the University in an experimental piggery area. The target organs were: liver and kidneys.

Fourteen weaned barrows of the same genotype were used in the experiment. The animals were placed in metabolic cages (80x80cm), the water was given *ad libitum* and the temperature of the room was in accordance to the needs of the weaned piglets. The feed was given twice a day, at 8:00 a.m. and 15:00 p.m., in equal portions. The feed that was not consumed was measured back at the end of the second ration. Seven days after the beginning of the experiment, the piglets were weighed (average=11.44 kg; DS=1.29 kg). During the first twelve days, the total amount of feed offered was 400 g/day/pig and it did not contain mycotoxins. After this adaption period, the amount of the feed was raised to 500 g/day/pig for 4 days still without mycotoxins. The feed during this period was a basic ration with a composition corresponding to their age (Platinum-PF Starter piglet feed mix) (Tables 7, 8).

Table 7: Tests carried out with Weende Analysis

Laboratory samples number	Examined Parameters	Standard Methods
170627	Dry matter	MSZ ISO 6496:2001
	Crude protein	MSZ EN ISO 5983-2:2009
	Ether extract	MSZ 6830-19:1979
	Crude fibre	152/2009/EK III/I
	Crude ash	MSZ 5984:1992

Table 8: Test results (% as is)

Sample name	Laboratory samples number	Water	Protein	Fat	Crude fibre	Ash
Swine	170627	9.2	17.9	5.3	2.9	4.6

On the seventeenth day, the mycotoxin administration started and the animals were divided in two groups: control group n=7 and experimental group n=7. The duration of the trial was ten days. During this period, the experimental group was fed with the same fodder but 20 mg/kg feed of FB_1 was added to provide a 10 mg of FB_1 daily intake. At the end of the experiment the animal were weighed to note some differences in the body weight (Table 9).

Table 9: Weight of pigs (Kg)

Pigs	**Live Weight (kg)**		**Weight gain (kg)**
	21/03/2017	*09/04/2017*	
1 /C	10.1	14	3.9
2 /C	11.2	15.3	4.1
3 /C	13.1	18	4.9
4 /C	10.2	14.8	4.6
5 /C	12.6	18.4	5.8
6 /C	10	12.6	2.6
7 /C	13.1	18.5	5.4
8 /T	13	18	5
9/T	9.6	13.4	3.8
10 /T	12.8	17.8	5
11 /T	10.7	16	5.3
12 /T	10.4	14	3.6
13 /T	11.7	15	3.3
14 /T	11.7	16.6	4.9
Average	*11.44 ± 1.29*	*15.89 ± 2.01*	*4.44 ± 0.91*

At the same time, the piglets were used in a Ph.D. thesis to analyse the effect of Fumonisin B_1 on the caecal microbiota.

After the dosing test, the piglets were killed by exsanguination after sedation.

2.2.2 Determination of mycotoxin content in feed

Toxin production

Fusarium verticillioides NRRL 20960 (MRC 826) fungal culture (7 days old) was grown on 0.5 strength potato dextrose agar (PDA; Chemika-Biochemica, Basle, Switzerland). Agar discs (5 mm) were prepared with cork borer (Boekel Scientifica, Pennsylvania, USA), which were then stored at 10 ºC in darkness in test tubes containing sterile distilled water (10 discs/10 ml).

For toxin production, maize (40 g) was soaked in distilled water (40 ml) at room temperature for 1 hour in Erlenmeyer flasks (500 ml), closed with cotton wool plugs. This was followed by the addition of the inoculated agar discs (10 agar discs per flask) to the two-times autoclaved (20 min.) matrix. The cultures were then stored and incubated at 24 ºC for 3 weeks, respectively. The flasks were shaken twice every day during the first week of incubation. When the incubation time was complete the fungus-infected cereal was dried at room temperature and ground.

LC-MS Analysis

LC-MS analysis was performed by a Shimadzu Prominence UFLC separation system equipped with a LC-MS-2020 single quadrupole (ultra-fast) liquid chromatograph mass spectrometer (Shimadzu, Kyoto, Japan) with electrospray source.

Samples were analysed on a Phenomenex Kinetex 2.6µ X- C18 column (100 mm × 2.1 mm). The column temperature was set to 50ºC, the flow rate was 0.3 ml/minute and the injection volume was 1 µl. The gradient elution was performed using double distilled water (eluent A) and methanol (eluent B), both acidified with 0.2% formic acid; initial condition at 60% A, 0-2 minutes isocratic step, 2-6 minutes linear gradient to 70% B, 6-13 minutes linear gradient to 100% B, 13-15 minutes isocratic step at 40% B. Total analysis was 15 minutes. MS parameters: source block temperature 90º C; desolvation temperature 250º C; heat block temperature 200º C; drying gas flow 15.0 l/minute. Detection was performed using SIM mode.

The mass spectrometer was operating in the selective ion monitoring mode, at m/z 722.4 for FB_1.

Calibration curves using FB_1 standard in the range of 10-500 µg/kg were prepared. The limit of detection (LOD) for FB_1 was 3 µg/kg, while the limit of quantification was (LOQ) 10 µg/kg.

The homogenized fungal cultures contained FB_1 at concentration of 916 mg/kg (FB_2: 185 and FB_3 79 mg/kg).

2.2.3 Blood and Tissue sampling

Blood sampling was performed according to the method of Shanmugasundaram et al. (1992).

Fresh venous blood was sampled into heparinized (20 IU/mL whole blood) tubes (lithium salt) and was centrifuged for 10 minutes at 1,000 g, at the end of the tenth day. Plasma and the buffy coat (mix of platelet and leukocytes) were removed and the erythrocyte bulk was washed three times with a common biological buffer TRIS-HCl (0.1M; pH=7.4) solution at 4°C. This isotonic solution permitted to remove the remaining plasma and adhered surface proteins. After each wash, the buffy coat and the washing medium were siphoned. Subsequent of the third wash, hypotonic TRIS-HCl (15mM; pH=7.4) solution was used to make the red blood cell porously. This ice-cold and more diluted buffer allowed the haemoglobin to enter the solution. Erythrocyte lysate (cell membrane pellet) was centrifuged at 10000 g for 10 minutes at 4°C for seven times. The supernatant contained the haemoglobin, was discarded after each centrifugation. After the washing medium was colourless, it was washed the last time with distilled water.

Stored at -20°C.

After exsanguinations the liver, kidney, spleen, lung and heart were immediately dissected and weighed. (Table10)

All samples were stored at -20°C until analysis.

Table 10: Weight of the organs (g)

Animal	Liver (g)	Kidney (g)	Spleen (g)	Lung (g)	Heart (g)
8 /T	390.3	69.4	47.1	197.3	110.8
9 /T	312	75.4	32.5	162.3	103.1
10 /T	4431	85.9	35.7	267	115.2
11 /T	349.1	85.4	34.7	174.6	123.4
12 /T	273.3	73.7	31.6	155.4	88.2
13 /T	350	75.7	34.4	172.7	93
14 /T	392.8	1033	36.7	195.2	112.5
1 /C	255.8	79.3	43.2	242	108.3
2 /C	329.8	96.9	37.3	180.7	87.4
3 /C	393.3	84.6	38	220.8	118
4 /C	299.9	90.8	29.6	156	85.7
5 /C	475	96.5	40.6	205.8	97.3
6 /C	322.6	70.7	39.6	155	82
7 /C	439	77.6	40	224.2	115.8

2.2.4 Total lipid extractions: liver, kidney and blood cell membranes

After slaughter, small pieces from liver and kidney were taken and stored at - 20°C (Figure 4). For the determination of total lipid content in these animal tissues and in the red blood cell membrane, the method of Folch et al. (1957) was used. The present method has the advantage to be simple and to decrease the losses of lipids due to the washing process.

To start the lipid extraction small tissue pieces (0.2 g) were cut. The portion was placed into a 15ml centrifuge plastic tube and 5 ml of solvent were added as to obtain an excess of 25-fold. To determine the total membrane lipids, 50 µl of washed red blood cell membrane were taken. The solvent was a 2:1 chloroform/methanol (v/v) mixture (chl/meth) + 100 mg/L butylated hydroxytoluene (BHT) which is an antioxidant and prevents the fatty acid (FA) oxidation. Chloroform acts as an extractor of the neutral lipids (non-polar) while the methanol extracts the polar lipids like the phospholipids.

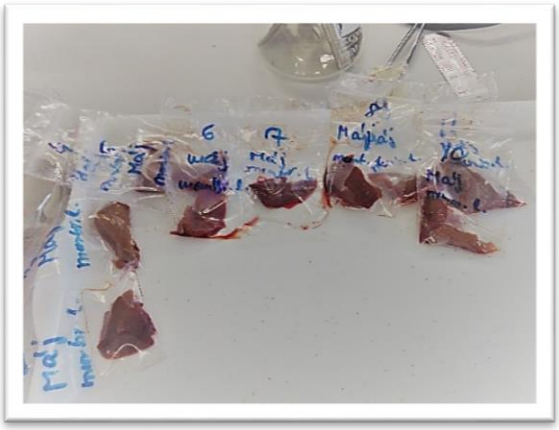


Figure 4: Samples of kidney and liver tissues

Solvent and tissues were homogenized with ULTRA-TURRAX (IKA T25 digital) (Figure 5). After each sample, the grinder was immersed into the same solution of chl/meth and any residue of tissues were removed.

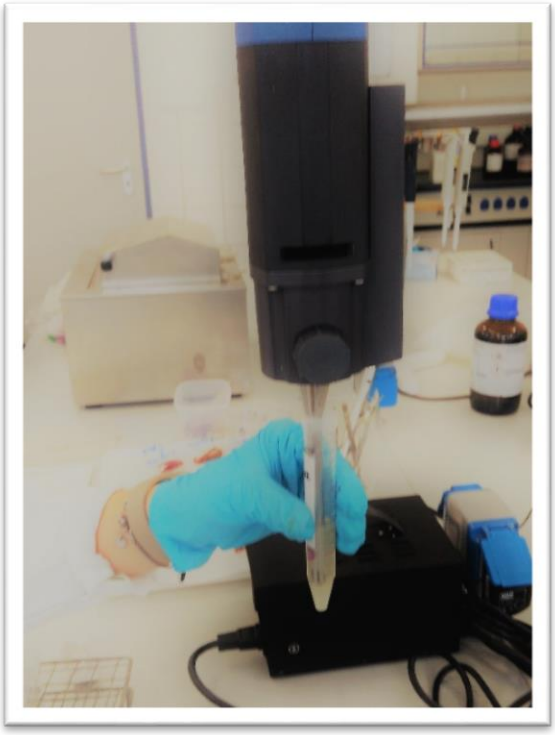


Figure 5: The figure shows the ULTRA-TURRAX (IKA T25 digital) machine

The homogenate was filtered through a fat-free filter paper (black code; quantitative type; MN 640 W; diameter 125 mm; retention pore 12-15 µm). To insure a complete removal of

the homogenate the tube was washed with an additive of 2 ml of chloroform/methanol (chl/meth) + BHT.

When the liquid dripped and the dry matter was retained in the filter, 2.5 ml of physiological saline (0.9%) were added with a pipette into the tube. Physiological saline allows the separation of the two different phases: the upper phase contains all the non-lipid substances; the lower phase contains all the lipids. The acidic lipids are present in the upper phase in dissociated forms and in the lower phase in un-dissociated forms. The addition of mineral salt decreases the dissociation of the acidic lipids by a mass action effect with a consequent shift of lipids to the lower phase (Folch et al., 1957).

In order to achieve complete separation the samples were centrifuged for 2 min at 2000 g. The upper layer (40% of the total volume of the system) was composed by water and methanol, while the lower layer (60% of the total volume of the system) contained the chloroform and the lipid fraction. Between the two layers a thin interface layer was formed which was consisted of the protein content of the tissues (Figure 6).

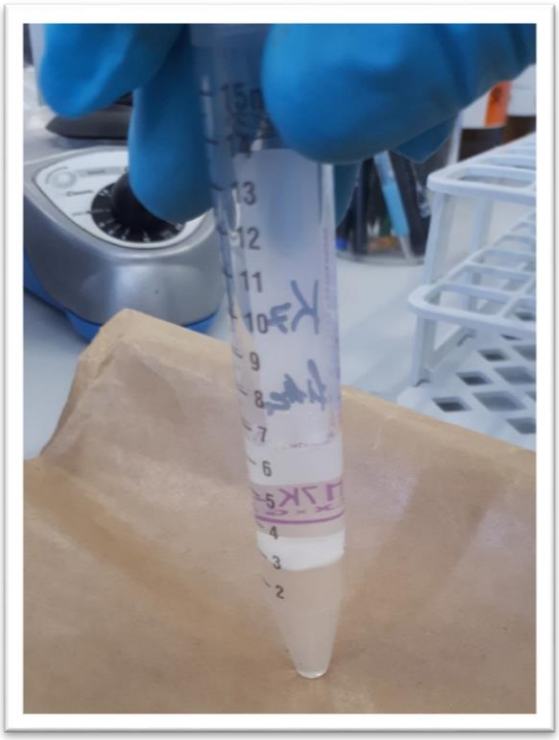


Figure 6: In the figure is possible to see the separation of the two layers

The non-lipid fraction along with the protein fraction were aspirated with a pipette and were discarded.

The lipid fraction was poured into a glass flask, previously weighed, and a wash with the solution (1-2 ml) permitted the removal of the lipids.

The flask was posed in a rotary-evaporation (62 °C; 90 r.p.m.) (Figure 7). When the flask became dry, an air stream was applied in order to eliminate any remaining traces of solvent.

Figure 7: In the figure is possible to see the separation of the two layers

The flasks were weighed after they cooled down. The difference in terms of weight was represented by the total lipid content.

Whit a pipette 3 ml of the solvent mixture the flask was washed to permit the transfer of the lipids to glass vials, than samples were stored in the fridge at -20°C

2.2.5 Separation of lipid fractions by Silicagel Column Chromatography

The general procedures for the separation of lipids fractions by Cyberlipid Center was followed (http://www.cyberlipid.org/cyberlip/home0001.htm). A small piece of cotton was placed at the bottom of a glass Pasteur pipettes in order to keep silicagel in the tube as a filter (Figure 8).

Figure 8: Silicagel Column Chromatography

Before starting the procedure 100 mg of silica gel were weighed into a micro centrifuged tube. The silica gel and chloroform were mixed and were transferred into the column in order to form a layer. This separation was based on differences in degree of adsorption of lipid components into a solid phase. The solid phase was polar and so the lipids were eluted by increasing polarity solvents.

According with to measurement obtained from the total lipid extraction expressed in mg, a proportion was made (3 mg: 1 ml= (known weight) mg: X ml). This calculation permitted to know how many ml should be put in the column for each sample. The lipid sample was transferred in the glass column to the top of the gel. When the solvent (chl/meth) dripped the separation of the lipid fraction started.

Previously labelled and weighed plastic tubes were positioned under the columns. The first fraction was obtained after adding 3 ml of chloroform in each column in order to elute the triglycerides (neutral lipids in general e.g. fatty acids, hydrocarbons, sterols, waxes, etc.). After that first sample was drained, the second fraction was obtained by adding 4.5 ml of acetone/methanol (9/1 vol/vol) that allowed the extraction of sphingolipids (glycolipids and ceramide). The fraction of the phospholipids, the third one, was eluted by adding 3 ml of methanol. Using this method, three different fractions of lipids from liver and kidney were obtained. After separation samples were stored at -20°C.

2.2.6 Lipids transmethylation for Gas Chromatography

The triglycerides, the total phospholipids and the total membrane lipids were transmethylated with a base-catalysed sodium-methoxide method of Christie (1982). This method (methyl esterification) was necessary to make the fatty acids volatile and "readable" by Gas Chromatography.

The fractions were moved in a previously weighed flask. The fractions were evaporated using a rotary-evaporator machine (62°C; 90 r.p.m.). After the cooling, the flasks were weighed again. The difference in terms of weight represented the triglycerides, phospholipids and red blood cells.

The obtained fractions were dissolved in 1.1 ml of ether (medium). The solution was briefly agitated to ensure the mixing. The solution was put into a glass tube with a pipette and 20 µl of methyl-acetate were added to avoid the hydrolysis of fatty acids. Then 20 µl of sodium methylate ($NaOCH_3$ 1M sol. in methanol) were summed. The solution immediately became cloudy due to the precipitation of the sodium-glycerol derivatives. Sodium methylate was the donor of the methyl group during the process of methyl esterification of the fatty acids. The methyl esterification was necessary to make the fatty acid volatile and readable by Gas Chromatography.

After 5 minutes at room temperature, 30 µl of oxalic acid in ether were added in order to stop the reaction. The mixture was centrifuged for 2 minutes at 1500 g to precipitate sodium oxalate (sodium-oxalate precipitate).

The ether was removed by evaporation under an air stream but the fatty acid methyl esters remained in the tube because their temperature of evaporation is around 260°C (Figure 9).

Figure 9: In the figure is possible to see the opaque layer composed by FA methyl esters

Approximately 200 mg of sodium sulphate (Na_2SO_4) and 300 µl of hexane were then added to the sample. The sodium sulphate needed to eliminate possible traces of water from the sample. To permit the precipitation of the sodium oxalate and the sodium sulphate the samples were running in the centrifuge for 2 minutes at 1500 g. The last step was aspiration of the hexane in which the fatty acid methyl esters were dissolved.

Stored at -20°C.

2.2.7 Thin Layer Chromatography

The complex lipids which were extracted from the liver and kidneys were separated to perform the thin-layer chromatography (TLC) using the protocol of Skipski et al. (1964). This method permitted the qualitative separation of phospholipids, obtaining the determination of phosphatidylcholine (PC), phosphatidylinositol (PI) and phosphatidylserine (PS).

TLC silica gel G60 plates were signed with a line made by a pencil. Following this line, the samples (50 µl or less) were applied with a micropipette. Repeating for three times to have a high amount of lipids.

The silicagel is polar and it represented the stationary phase. Chloroform/methanol/water 65:35:7 (vol/vol/vol) was the mobile phase and it was poured in a closed elution chamber (the amount of the solvent needed to be under the start line of the plate).

The plate was put into the TL development chamber and the moving phase started following the principle of capillary reaction (moving phase) (Figure 10). After the samples were eluted by the solvent, the plate was removed from the chamber and it was left to dry for some minutes at room temperature.

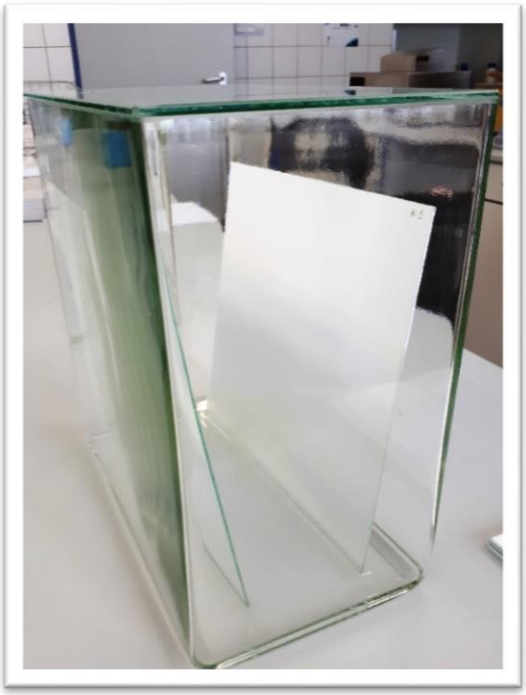


Figure 10: TLC development chamber

The plate was sprayed Primuline (5 mg/100ml). The dye permitted to visualise the spots of the different phospholipids under a UV lamp (346 nm). The separations of the lipids happened due to the different polarity of the components (Figure 11).

Figure 11: Spots of phospholipids fractions (PC, PI, PS)

Polarity: PC>PI>PS

Each fraction was removed from the plate and put in a labelled glass tube and 3 ml of ether were added. The solution was centrifuged for 2 min at 1500 g. The supernatant was aspirated with a pipette and was transferred into a clean glass tube previously labelled. Subsequently 20 ml of methyl-acetone and 20 ml of sodium methylate were added. After five minutes 30 ml of oxalic acid were added to stop the reaction.

The phosphatidylcholine (PC) fraction on the thin layer was confirmed by a PC standard which was purchased Sigma-Aldrich (Schnelldorf, Germany).

The solvent was evaporated under an air stream and 200 mg of sodium sulphate were put in the glass tube with 300 µl of hexane.

The samples were centrifuged for 2 minutes at 1500 g and the supernatant was transferred and put in a small glass vials with a pipette, for subsequent GC analysis. Until than, the samples were stored at -20°C.

2.3 Ethical Statement

Experimental protocol was authorized by the Food Chain Safety and Animal Health Directorate of the Somogy County Agricultural Office, under allowance number: XV-I-31/1509-5/2012.

2.4 Statistical analysis

To determine whether statistical differences among groups an independent samples t-test was used, using IBM SPSS Statistics Data Editor.

The level of significance was set to $P<0.05$

3. Results

3.1 Productive parameters and Organ weights

During the ten days of the trial, animals were observed to notice changings in their behaviour. The pigs fed with the experimental diet did not show clinical signs of disease. The consumption of the contaminated feed was regular during the experiment and no feed refusal was noted. Only a single animal from the toxin group, after four days of treatment, demostrated to be quiet and inactive.

In terms of productive parameters, no significance difference was observed between the two groups. There was no statistical difference in terms of body weight gain between the control and the toxic groups (Table 11).

A comparison of the organ weight values (group means) was made between the two groups. No statistical difference has been induced by the mycotoxin treatment. Kidney, liver and lungs weights have been analysed as shown in table below (Table 11).

Table 11: T-test of liver weight (g), kidney weight (g), lungs weight (g) and body weight gain (kg)

T-test							
	Control			**FB_1**			**Significance**
	Mean	±	SD	Mean	±	SD	
Kidney weight (g)	85.2	±	10.0	81.3	±	11.45	n.s.
Liver weight (g)	359.3	±	78.9	358.6	±	56.16	n.s.
Lungs weight (g)	197.8	±	34.43	189.2	±	37.65	n.s.
Body Weight Gain (kg)	4.5	±	1.06	4.4	±	0.81	n.s.

3.2 Kidney phospholipids

Kidney total phospholipids were analyzed (Table 12). As noted in the following table alpha-linolenic acid (C18:3 n-3) decrease in the mycotoxin treated group. Behenic acid (C22:0) and lignocerid acid (C24:0) had a similar reduction in the toxin-treated group. The average chain length (ACL) and the total saturated fatty acids (SFA) were the only that provided an increase in the toxin treated group.

Table 12: The FA profile (% of total FAME) of the kidney phospholipids (data represent means and SD of 7 individuals/group)

Kidney phospholipids						
Group	**Control**			**FB_1**		**Significance**
	Mean	±	SD	Mean	± SD	
C14:0	0.18	±	0.02	0.17	± 0.04	n.s.
C15:0	0.11	±	0.03	0.11	± 0.03	n.s.
C16:0	17.3	±	1.25	16.2	± 1.04	n.s.
C16:1n-7	0.28	±	0.05	0.27	± 0.08	n.s.
C17:0	0.39	±	0.07	0.48	± 0.16	n.s.
C18:0	22.3	±	0.33	22.1	± 1.00	n.s.
C18:1n-9	9.77	±	1.03	10.2	± 1.41	n.s.
C18:2n-6	22.0	±	1.13	21.0	± 1.24	n.s.
C18:3n-6	0.14	±	0.06	0.15	± 0.05	n.s.
C18:3n-3	**0.71**	**±**	**0.06**	**0.62**	**± 0.10**	**0.054**
C20:0	0.12	±	0.02	0.10	± 0.01	n.s.
C20:1n-9	0.19	±	0.05	0.22	± 0.06	n.s.
C20:2n-7	0.87	±	0.16	0.86	± 0.13	n.s.
C20:3n-6	2.00	±	0.51	2.54	± 0.92	n.s.
C20:4n-6	16.6	±	0.96	17.9	± 2.10	n.s.
C20:3n-3	0.31	±	0.08	0.28	± 0.05	n.s.
C20:5n-3	5.09	±	1.06	5.05	± 1.11	n.s.
C22:0	**0.08**	**±**	**0.01**	**0.05**	**± 0.01**	**0.0001**
C22:5n-3	1.50	±	0.33	1.44	± 0.12	n.s.
C24:0	**0.16**	**±**	**0.02**	**0.12**	**± 0.02**	**0.002**
SFA	**40.6**	**±**	**0.77**	**39.8**	**± 0.87**	**0.043**
MUFA	10.2	±	1.12	10.7	± 1.54	n.s.
PUFA	49.2	±	1.89	49.8	± 1.58	n.s.
n-6	40.7	±	1.42	41.7	± 1.05	n.s.
n-3	7.62	±	0.98	7.39	± 1.26	n.s.
n-6 / n-3	5.42	±	0.75	5.78	± 0.99	n.s.
Odd chains	0.50	±	0.09	0.59	± 0.19	n.s.
unsat. index	236.9	±	11.38	245.0	± 12.28	n.s.
ACL	**18.2**	**±**	**0.05**	**18.3**	**± 0.06**	**0.052**

3.3 Kidney triglycerides

Kidney triglyceride fatty acid profile was non-responsive towards the FB_1 treatment applied (Table 13).

Table 13: The FA profile (% of total FAME) of the kidney triglycerides (data represent means and SD of 7 individuals/group)

Kidney triglycerides							
Group	**Control**			**FB_1**			**Significance**
	Mean	±	SD	Mean	±	SD	
C14:0	0.56	±	0.10	0.63	±	0.30	n.s.
C15:0	0.12	±	0.03	0.11	±	0.02	n.s.
C16:0	13.3	±	2.77	13.9	±	4.01	n.s.
C16:1n-7	0.47	±	0.13	0.96	±	1.07	n.s.
C17:0	0.29	±	0.05	0.30	±	0.04	n.s.
C18:0	16.3	±	1.56	14.1	±	3.33	n.s.
C18:1n-9	9.93	±	1.84	14.0	±	8.09	n.s.
C18:2n-6	26.9	±	2.90	28.9	±	2.30	n.s.
C18:3n-6	0.13	±	0.08	0.15	±	0.06	n.s.
C18:3n-3	1.07	±	0.21	1.25	±	0.29	n.s.
C20:0	0.24	±	0.05	0.20	±	0.08	n.s.
C20:1n-9	0.32	±	0.09	0.40	±	0.14	n.s.
C20:2n-7	2.03	±	0.32	1.89	±	0.46	n.s.
C20:3n-6	1.45	±	0.44	1.66	±	0.76	n.s.
C20:4n-6	18.4	±	2.51	14.8	±	6.83	n.s.
C20:3n-3	1.02	±	0.21	0.93	±	0.28	n.s.
C20:5n-3	5.38	±	1.07	4.21	±	2.29	n.s.
C22:0	0.13	±	0.04	0.10	±	0.08	n.s.
C22:5n-3	1.66	±	0.24	1.37	±	0.48	n.s.
C24:0	0.22	±	0.07	0.17	±	0.10	n.s.
SFA	31.1	±	2.98	29.5	±	1.33	n.s.
MUFA	10.7	±	2.00	15.4	±	9.27	n.s.
PUFA	58.0	±	4.64	55.1	±	10.1	n.s.
n-6	46.9	±	3.81	45.5	±	7.28	n.s.
n-3	9.13	±	1.17	7.75	±	2.77	n.s.
n-6 / n-3	5.19	±	0.60	6.46	±	1.96	n.s.
Odd chains	0.41	±	0.08	0.41	±	0.04	n.s.
unsat. index	267.7	±	19.5	241.5	±	59.45	n.s.
ACL	18.3	±	0.11	18.2	±	0.33	n.s.

3.4 Liver phospholipids

In the liver phospholipid fatty acid profile it was determine by GC (Table 14). Alpha-linolenic acid (C18:3 n-3) proportion decreased as the decosapentaenoic acid (C22:5 n-3) decreased as well. An increase was found in the proportion of arachidonic acid (C20:4 n-6). The total saturation level decreased. The total level of omega-3 fatty acids (FAs) decreased significantly. An increasing n-6 / n-3 FA ratio was detected.

Table 14: The FA profile (% of total FAME) of the liver phospholipids (data represent means and SD of 7 individuals/group)

Liver phospholipids						
Group	**Control**			**FB_1**		**Significance**
	Mean	±	SD	Mean	± SD	
C14:0	0.15	±	0.03	0.17	± 0.03	n.s.
C15:0	0.14	±	0.07	0.18	± 0.11	n.s.
C16:0	16.9	±	0.68	16.2	± 0.91	n.s.
C16:1n-7	0.32	±	0.07	0.41	± 0.17	n.s.
C17:0	0.79	±	0.25	1.16	± 0.68	n.s.
C18:0	31.7	±	1.03	31.6	± 1.95	n.s.
C18:1n-9	6.27	±	0.56	6.80	± 1.19	n.s.
C18:2n-6	22.5	±	1.16	22.1	± 0.80	n.s.
C18:3n-6	0.15	±	0.03	0.19	± 0.05	n.s.
C18:3n-3	**0.23**	**±**	**0.05**	**0.19**	**± 0.04**	**0.082**
C20:0	0.08	±	0.01	0.09	± 0.02	n.s.
C20:1n-9	0.13	±	0.02	0.13	± 0.02	n.s.
C20:2n-7	0.72	±	0.09	0.67	± 0.07	n.s.
C20:3n-6	1.46	±	0.39	1.38	± 0.28	n.s.
C20:4n-6	**13.2**	**±**	**1.47**	**14.3**	**± 0.40**	**0.077**
C20:3n-3	0.15	±	0.04	0.13	± 0.02	n.s.
C20:5n-3	1.47	±	0.60	1.47	± 0.35	n.s.
C22:0	0.03	±	0.00	0.04	± 0.01	n.s.
C22:5n-3	**3.32**	**±**	**0.14**	**2.54**	**± 0.33**	**0.0001**
C24:0	**0.38**	**±**	**0.04**	**0.28**	**± 0.05**	**0.002**
SFA	**50.1**	**±**	**0.74**	**49.7**	**± 1.10**	**0.045**
MUFA	6.7	±	0.61	7.3	± 1.34	n.s.
PUFA	43.2	±	1.00	43	± 0.94	n.s.
n-6	37.2	±	0.88	37.9	± 0.64	n.s.
n-3	**5.17**	**±**	**0.63**	**4.32**	**± 0.36**	**0.009**
n-6 / n-3	**7.29**	**±**	**0.82**	**8.83**	**± 0.67**	**0.002**
Odd chains	0.93	±	0.31	1.33	± 0.79	n.s.
unsat. index	189.8	±	8.75	187.9	± 3.97	n.s.
ACL	18.1	±	0.04	18.1	± 0.04	n.s.

3.5 Liver triglycerides

In contrast to the non-responsive liver phospholipids, the liver triglycerides (Table 14) provided slight compositional alterations. The proportion of stearic acid (C18:0) increased in the toxin treated group, as well as the proportion of arachidonic acid (C20:4 n-6). A slight decrease was found for lignoceric acid (C24:0). The total n-6 / n-3 fatty acids increased.

Table 15: The FA profile (% of total FAME) of the liver triglycerides (data represent means and SD of 7 individuals/group)

Liver triglycerides						
Group	**Control**			**FB_1**		**Significance**
	Mean	±	SD	Mean	± SD	
C12:0	0.04	±	0.01	0.08	± 0.12	n.s.
C14:0	0.38	±	0.15	0.28	± 0.09	n.s.
C15:0	0.11	±	0.05	0.13	± 0.06	n.s.
C16:0	13.5	±	2.30	12.1	± 0.75	n.s.
C16:1n-7	0.61	±	0.32	0.41	± 0.15	n.s.
C17:0	0.69	±	0.24	0.95	± 0.65	n.s.
C18:0	**29.3**	**±**	**2.09**	**31.2**	**± 1.39**	**0.067**
C18:1n-9	8.96	±	2.98	7.20	± 0.95	n.s.
C18:2n-6	20.3	±	2.17	19.6	± 0.88	n.s.
C18:3n-6	0.16	±	0.09	0.20	± 0.05	n.s.
C18:3n-3	0.61	±	0.32	0.44	± 0.14	n.s.
C20:0	0.14	±	0.07	0.12	± 0.01	n.s.
C20:1n-9	0.16	±	0.12	0.21	± 0.08	n.s.
C20:2n-7	0.77	±	0.12	0.70	± 0.05	n.s.
C20:3n-6	1.42	±	0.43	1.45	± 0.28	n.s.
C20:4n-6	**17.1**	**±**	**1.91**	**19.5**	**± 1.16**	**0.015**
C20:3n-3	0.23	±	0.07	0.21	± 0.05	n.s.
C20:5n-3	1.58	±	0.64	1.68	± 0.29	n.s.
C22:0	0.11	±	0.09	0.06	± 0.02	n.s.
C22:5n-3	3.45	±	0.39	3.15	± 0.28	n.s.
C24:0	**0.42**	**±**	**0.08**	**0.36**	**± 0.04**	**0.072**
SFA	44.7	±	2.58	45.3	± 1.29	n.s.
MUFA	9.7	±	3.38	7.8	± 1.14	n.s.
PUFA	45.6	±	4.68	46.9	± 1.13	n.s.
n-6	38.9	±	3.72	40.7	± 0.97	n.s.
n-3	5.87	±	1.09	5.48	± 0.35	n.s.
n-6 / n-3	**6.74**	**±**	**0.79**	**7.46**	**± 0.47**	**0.063**
odd chains	0.80	±	0.28	1.08	± 0.70	n.s.
unsat. index	216.6	±	20.29	225.0	± 5.89	n.s.
ACL	18.3	±	0.11	18.3	± 0.04	n.s.

4. Discussion

Fumonisin B1 has practically no effect on the kidney weight. This is rather interesting, since it is known that FB_1 is generally weakly absorbed (4-6% of the total dietary intake), but once entering the circulation, its elimination happens via the renal filtration (Fodor et al., 2008). In the cited study, 45 mg/kg mycotoxin dose was applied for 10 days, and this did not induce any alteration in the renal weight of weaned piglets.

The weight alteration of liver was based on the hypothesis that FB1 is providing slight hepatotoxicity in rodents. This effect is mainly attained via immediately impairing the integrity membrane phospholipids, even before the enzymatic antioxidant defence is activated (Szabó et al., 2016). It seems that piglets are much less sensitive towards this effect, most probably due to the lack of coprophagy, characteristic for the rodents. Anyway, in a study conducted by Abel and Gelderblom (1998) in which rats were fed with a dietary levels of FB_1 of 10, 50, 100, 250, 500 mg/kg diet for 21 days, the relative liver weight of the first three groups did not have significant effects. Only the relative liver weight of the 250 mg FB_1/kg diet group had a significantly reduction.

We similarly did not detect any difference in the weight of spleen and heart between groups, but detailed analysis of this dataset is not reasoned, since immunotoxic or sacolemma damaging effects have never been reported for FB_1. Alteration in terms of mycotoxin feeding has been expected since pulmonary edema (interstitial) is a *differentia specifica* of FB_1 toxicosis of piglets (Kriek et al., 1981). Since FB_1 has a rather strong effects as well on the cation exchange in mammals (Szabò et al., 2014), edema development has been hypothesized, but again, not proven.

Our results are in agreement with the study of Andretta et al. (2012), which asserts that fumonisins do not influence the weight of kidneys and spleen, but it increased the relative weight of liver, heart and lungs. Probably in this study, we did not notice the increase in weight of liver and heart because of the short-term and low-dose trial. Thus, summarized, we hypothesize that FB_1 at a concentration of 20 mg/kg of feed for weaned piglets may affect membrane integrity to a slight extent, but does not have major effects, at least not at a level that may exert harmful effects on the organs in study.

Similar results of productive parameters were reported by Riley and Voss (2006) in a study with rats. Rats were fed diets contaminated with fumonisins at concentrations 1.1, 13.5 and 88.6 mg/kg of feed for 10 days. After the trial no significant statistically difference was observed in body weight or feed consumption among groups. Dilkin et al. (2003) conducted an experiment with crossbreed piglets that were exposed to dietary FB_1 at concentrations of 10 mg/kg or 30 mg/kg of feed for 28 days. In this case, a statistically significant difference in feed consumption and weight gain were noticed in the group fed the 30 mg of FB_1/kg diet compared with the 10 mg of FB_1/kg diet and can be attributed to the longer exposure period. It should be highlighted that one of the animals consuming the high-dose diet died of pulmonary edema. It was concluded that 10 mg of FB_1 per day into diet (500g), equivalent to ca. 0.7 mg/kg BW per day is safe for swine. A meta- analytical study of productive and nutritional interaction of mycotoxins in growing pigs demostrated that 87% of variance in weight gain was due to the feed intake ($P<0.01$) and only 4% was due to the presence of mycotoxin in diets ($P<0.01$) (Andretta et al., 2012). In the same meta-analysis, Andretta et al. (2012) concluded that fumonisins and zearalenone do not alter ($P>0.05$) the pig feed intake. However, young animals are usually more sensitive to mycotoxins suggesting a less efficient elimination metabolism, which increase the resistance with maturity.

Since liver and kidney are target organs for fumonisins, the fatty acid composition analysis of kidney and liver phospholipids and triglycerides was performed to test the effect of low dose of FB_1 on piglets. Fatty acid composition was expressed as weight % of total FA methyl esters. Due to the lack of experiments in swine, the alterations in FAs profiles were compared only with studies on other species or doses.

Kidney total phospholipids were analyzed. Alpha-linolenic acid (C18:3 n-3) provided a proportional decrease associated with mycotoxin treatment. In the study of Klaric et al. (2006) lipid peroxidation was tested on porcine kidney epithelial cells PK15. Cells were treated with 0.05, 0.5 and 5 μg/ml of FB_1 and the lipid peroxidation was calculated as a concentration of thiobarbituric acid-reacting substances (TBARS). The level of TBARS increased its concentration following a time-dependent rate demonstrating that porcine kidney cells are sensitive to fumonisin B_1. Thus, lipid peroxidation could be the cause of the α-linolenic acid decrease. Moreover, we as well recorded the daily feed intake. Since alpha-linolenic acid is essential, its only source in the body lipids can be the diet. Thus, by unchanged dietary intake

its augmented damage may be the only reason of its decreased phospholipid level. From the saturated fatty acids, behenic acid (C22:0) and lignocerid acid (C24:0) showed similar alterations, namely lower proportions in the toxin treated animals. Thus, as a result of the latter two items, the total saturation decreased as well. Similar decrease of behenic acid was found in the study of Szabò et al. (2014) in rabbit tissues. The study analyzed the treatment of FB_1 at 10 mg/kg of feed on red cell membrane fatty acid composition in weaned rabbits. The author suggested that this long chain fatty acid is an important component of sphingomyelins which is hydrolyzed by the enzyme sphingomyelinase to phosphorylcholine and ceramide. More recently in a study of Szabó et al. (2018), behenic acid depletion has been described again in the liver of FB_1 treated rats. Ceramide is converted by the enzyme ceramidase into sphingosine, thus the inhibition of ceramide synthesis refers indirectly the decrease of the behenic acid (C22:0) proportion.

As a single item form the indices comprising multiple toxin data the average chain length (ACL) was the only that provided increase in the toxin treated group. Most probably this is attributed to the fact that proportional changes happened mostly in case of fatty acids having C 20 chains (C20:4 n-6 and C20:3 n-6), strongly affecting the ACL value.

The fatty acid composition of liver phospholipids was defined. However, FB_1 was not a very potent modulator/disruptor of hepatic total phospholipid FA composition, neither inducing liver weight alteration. The dose applied (20 mg/kg feed) is a limit value for the pigs. Since this was not a very drastic treatment, gross pathological alterations were not expected. Indeed, the primary aim of the study was to attain FB_1-associated effects on the intestinal microbiota. Thus, organ level alterations were monitored as a second goal. As a practical basis a 50 mg/kg rat study was used, in which only 5 days were enough to detect hepatic effects of FB_1 (Szabó et al., 2016). However, piglets are generally more sensitive on FB_1. Likewise in the kidney, alpha-linolenic acid (C18:3 n-3) proportion decreased as a result of lipid peroxidation (Gelderblom et al., 2001). Similarly, the long chain polyunsaturated n-3 FA, docosapentaenoic acid (C22:5 n-3) decreased significantly. In a recent study Szabó et al. (2016), docosapentaenoic acid proportion was decreased significantly in FB_1 treated rabbits liver phospholipids. According to Abel et al. (2001), the proportional decrease of C20-C22 FAs is a factor to control/improve antioxidant properties.

The proportional reduction of alpha-linolenic acid is probably due, like in the kidney, to the lipid peroxidation activity induced by the mycotoxin. Since alpha-linolenic acid is a precursor of decosapentaenoic acid, its reduction is associated with the absence of the precursor. Another change in the alteration of the lipid profile of phospholipids caused by FB_1 is an increase in the proportion of arachidonic acid (C20:4 n-6). This result has no explanation since this alteration is confirmed only in *in vitro* studies in primary hepatocytes (Gelderblom et al., 2001). The increase of arachidonic acid could be caused by the disruption of prostaglandin biosynthesis by inhibiting the cyclooxygenase enzyme. It was shown, *in vitro*, that the mitoinhibitory effect of FB_1 was contrasted by the addiction of prostaglandin E_2. The total saturation level decreased. This is probably associated with the proportional increase of arachidonic acid and the proportional, but not significant, increase of the total mono-saturated fatty acids. Increase in mono unsaturated fatty acid was also observed in Szabó et al. (2016) and Gelderblom et al. (2002). In parallel, the total level of omega-3 fatty acids (FAs) decreased significantly. The increase of arachidonic acid proportion and the decrease of the n-3 FAs led to an increasing n-6 / n-3 FA ratio.

The triglycerides were analysed as well in order to detect changings in FA composition. In the present study, kidney triglycerides did not response to the fumonisin B_1 treatment. On the other hand, liver triglycerides denoted some proportional changes between the two groups.

The proportion of stearic acid (C18:0) increased in the toxin treated group, as well as the proportion of arachidonic acid (C20:4 n-6), in the liver. The same proportional increase of arachidonic acid was observed in the analysis of liver phospholipids. Probably this acid being a precursor of the prostaglandin H_2, which can be converted in prostaglandins, is involved in the contrasting of inflammatory processes caused by FB_1. A slight decrease was found for lignoceric acid (C24:0). This result was obtained also in liver and kidney phospholipids. Unfortunately, we were not able to find any contradictive or supportive literature data for this finding. Most probably due to the alteration of arachidonic acid, the total omega-6 / omega-3 fatty acids increased. However, the compositional changes of liver triglycerides are seldom reported and are generally a part of energy metabolism. Triglycerides stores have less other function than energy supply, and are thus mostly reflecting the needs of specific FAs at an extrahepatic site. Since we only registered minor changes, our triglycerides dataset may be handled as a secondary one.

5. Conclusion

In this study the effect of a naturally contaminated diet with fumonisin B_1 at 20 mg/kg of feed in a short period of 10 days in weaned piglets was assessed. The results showed a non-response treatment on behaviour, productive parameters and organ weight values. Membrane fatty acid composition of liver and kidney was slightly responsive, suggesting ceramide synthesis disturbance. The most probable reason might be the low dose and short period of exposure to the toxin, even if swine are the most sensitive farm animal to this toxin. The slight changes of phospholipid fatty acid proportions observed in liver and kidneys, is maybe a consequence of the increased susceptibility to lipid peroxidation (Abel and Gelderblom 1998; WHO 2005). It would be interesting to analyse if the anti-oxidant defence system (non-enzymatic, and even enzymatic) was activated even at a low contamination and in a short period. Results of the present study indicate that low, but realistic FB_1 exposure for a limited time-frame has no drastic health or biochemical consequences, but is already detectable with sensitive techniques. However, production has not been compromised at the dose and exposure applied.

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