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Perils of the Fungal Kingdom: Mycotoxins in Food and Feed

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PERILS OF THE FUNGAL KINGDOM: MYCOTOXINS IN FOOD AND FEED

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Masters of Science

in

The Department of Environmental Sciences

by

Adrianna Isobel Pribil
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Abstract

Humans and fungi have a complex relationship, especially in regards to the many fungal secondary metabolites that can be produced. When secondary metabolites are toxic to animals and humans they are defined as mycotoxins. Fungi can grow on crops pre-harvest and post-harvest, and have the potential to produce mycotoxins which occur regularly in animal feed and food for human consumption. In high doses, mycotoxins cause variety of problems that result in economic losses and extreme health issues. However, multiple mycotoxins will co-occur in nature and commonly occur in low doses. Thus chronic low daily doses of multiple mycotoxins overtime may result in wear and tear on the body, as well as disease development later in life (for both animals and humans). The goal of this thesis is to provide a broad review of current literature on mycotoxins ranging in topics from their biochemical synthesis and control to their toxicity in various organisms. To accomplish this, in this thesis we look at mycotoxin contamination from a wide perspective and from different disciplines in order to understand how our agricultural practices, trade and economics, diet and health, and regulatory policies both affect and are effected by mycotoxin contamination.

Chapter 1

Introduction and Brief History

Innumerable mysteries lie within the vast depths of the fungal kingdom. These lower eukaryotes have a ubiquitous presence and represent some of the greatest diversity known to man. The scale and range of phenotypic diversity of fungi (yeasts, molds, and mushrooms) further drives the curiosity to understand the genotypes behind them. A jack of all trades, they vary in size, shape, and habitat and fulfill various roles where they act as communication network links between plants, nutrient cyclers, decomposers of various chemical compounds, and producers of a wide variety of metabolites. Attempts to predict the number of species within this kingdom have been difficult to secure with numbers ranging from several hundred thousand up to 9.9 million [Hawksworth, 2001]. A conservative estimate of 1.5 million species was hypothesized by the British mycologist Dr. David L. Hawksworth in the early 1990s [Hawksworth, 1991]. However, recent research and scientific evidence from sequencing technologies and large scale environmental sampling suggest a number of around 5.1 million [Blackwell, 2011, O'Brien et al., 2005]. The number of fungi that have been identified is of a much smaller magnitude ($\approx 70,000$), of that number just about 3,000 fungal genomes are being or have been sequenced, and over 900 genome sequences are available for research [Aylward et al., 2017]. It stands to reason that these number estimates should continue to grow as should our understanding of fungal species diversity through the advancement and application of improved sequencing methods coupled with genomic research.

Whether we realize it or not, the natural world is connected through fungal interactions with virtually all living and dead things. Consider for example, mycorrhizal symbiosis, which is a quintessential relationship between soil fungi and over 90% of plant species [Allen et al., 2003, Voegelé and Mendgen, 2007]. They can exist in different kinds of medium (soil, water, and living organisms) and thrive in many dif-

ferent environments including those that are hostile and extreme [Blackwell, 2011]. The phylum Ascomycota encompasses a wide range of fungal extremists; such as species from genus *Aspergillum* and *Penicillium*, which have been found thriving in subzero whether conditions on the Antarctic mountain ranges and in highly radioactive environments on board the International Space Station [Dadachova and Casadevall, 2008]. One theory by Dr. Arturo Casadevall, a distinguished professor of molecular microbiology, immunology, and infectious disease, suggests that there may even be a connection between selection ability of pathogenic fungi and mammalian evolution. In a very broad sense, his theory suggests that the developmental evolution of mammals from the end of the cretaceous period to now was possible because a fungal filter selected for mammals against reptiles [Casadevall, 2005, Casadevall, 2012]. While this may be a hard stretch, mushrooms, molds, and yeasts have undoubtedly helped shape human existence, especially alongside agricultural development. From the worship of pagan gods of harvest to the development of antibiotics, the known or unknown impact of fungal effects permeates different aspects of civilization and society [Dugan, 2008].

The onset of agriculture was a game changer for humans but also a wide spread opportunity for fungus, specifically plant-pathogenic fungi such as those belonging to the genus *Claviceps*, *Fusarium*, *Aspergillum*, and *Penicillium*. These fungi grow on and infect cereal crops, nuts, and pulses in the field or on grains, seeds, and fruits in storage vessels after harvest, these opportunists can cause serious problems to food production and health around the world. One of the most studied and extraordinary examples of interaction between plant-pathogenic fungi, a staple crop, and human/animal disease is that of *Claviceps purpurea*, ergot alkaloids, and rye. Rye (*Secale cereale*) holds an undoubtedly sordid past throughout the ages as told through archeobotanical records and findings. Indigenous to Anatolia, rye had humble origins as a weed that migrated across Europe with other cereal (barley, oats,

and wheat) crops used for cultivation during Neolithic times [Hillman, 1978, Behre, 1992]. Its ability to persist in acidic soils with poor drainage and unfavorable frosty climate garnered the plant a competitive edge in some regions, however, evidence for its initial cultivation as a crop did not arise until after the introduction of new harvesting methods and technology in the early pre-Roman Iron Age; tools like sickles and the Gallo-Roman machine that harvested the stem near the ground and had less selectivity against weeds like rye [Behre, 1992]. Over the course of several centuries, rye production gradually expanded throughout Europe and Russia and became a highly cultivated, permanent crop thus establishing itself as a dietary and economic staple for the growing populations of the medieval period [Behre, 1992]. So what is the problem? Part of the problem is in ryes susceptibility to the fungus *Claviceps purpurea*. Unlike other self-pollinating (cleistogamy) crops such as wheat or barley, rye is a cross-pollinating (xenogamy) crop and thus their florets must be opened, which also allows for easy infection by ascospores of *Claviceps purpurea*. Cleistogamy is an effective avoidance mechanism which reduces the potential risk of ergot infection [Miedaner and Geiger, 2015]. The ergot (sclerotia) which form on florets after infection, can fall off during winter and remain dormant until conditions are optimal (hard frost) and germination begins again [Behre, 1992]. Additionally, *Claviceps purpurea* is biotrophic (needs to keep rye host alive), and can hi-jack the plants auxin/cytokinin homeostasis by directly secreting its own auxin/cytokinin like molecules that help it facilitate infection and go unnoticed by the plants immune response [Oeser et al., 2017]. *Claviceps spp.* can infect a wide range of hosts within the Poaceae grass family, specifically the entire subfamily of Pooideae (which includes all major cereals) and many true grass species within the subfamily Arundoideae [Pažoutová et al., 2000]. The climatic distribution of *Claviceps purpurea* favors temperate regions but can also be found from the Arctic to subtropical regions. They are not specialized for hosts but by climatic conditions [Pažoutová

et al., 2000, Miedaner and Geiger, 2015].

Historians and researchers have found various religious, medical, and cultural records (texts, chronicles, and painting) from western Europe that depict a rather unbelievable scene of dry gangrenous dismemberment, mass hysteria, formication (the sensation of insects crawling under your skin) and the *dance macabre* [Nemes, 2002, Nemes and Goerig, 2002]. The disease responsible, *ignis sacar* (holy fire, St. Anthonys Fire, or *Kriebelkrankheit*) is more commonly known as ergotism and is a result of consuming the ergot sclerotia on rye grain that has been infected by *Claviceps spp.* Outbreaks and epidemics occurred periodically throughout the middle ages and especially in times preceding poor harvests and famines, but it was not until the high middle ages and on that widespread devastation due to ergot poisoning was recorded [Nemes, 2002, Alm and Elvevåg, 2013]. Regions west of the Rhine (i.e. France and Spain) had more cases of gangrenous ergotism, while eastern regions (Germany) had convulsive ergotism [Nemes, 2002, Alm and Elvevåg, 2013]. People of all socioeconomic classes could be effected, especially the poor and pilgrims (peregrinos) on religious journeys to Santiago de Compostela [Nemes, 2002]. It was the affliction of the latter which spurred the development of the Antonite-Hospitals run by monks in the clerical order belonging to the lay fraternity of St. Anthony during the 11th and 12th centuries. These Antonite-Hospitals were solely dedicated to curing ergotism, and therefore they only admitted those with certain diagnosis of the disease so that they could miraculously cure it by administering foods free of ergot, herbal wines with potentially vasodilating and analgesic capacity, and Antonites-balsalm (a trans-dermal therapeutic system) [Nemes, 2002]. Over the next few centuries, the Antonites became one of the most impressive and extensive international medical welfare systems made up of hundreds of monasteries, hospitals, churches, and hostels/hotels located critical points (i.e. mountain passes and bridges) along the different paths leading to Santiago [Nemes, 2002, Alm and



Figure 1.1: Puente Románico de Puente la Reina. Medieval Bridge in Puente la Reina, Navarra, Spain along the Camino de Santiago with a modern day pilgrim. Photo credit Isobel Pribil.

Elvevåg, 2013]. Following the 16th century, the St. Anthony order began to gradually disintegrate out of existence in the late 1700s, some researchers suggest that they were integrated into the knights hospitallers while others suggest that it was in part due to the debauched behavior of the monks [Nemes, 2002, Alm and Elvevåg, 2013].

Ergotism became increasingly prevalent after the 16th and 17th centuries which coincided with significant global cooling and the onset of the Little Ice Age; and knowledge/information about the cause of holy fire became well known which was slightly remedied by preventative agricultural practices (switch to potatoes, field burning after harvest, cleaning grain) [Nemes, 2002, Miedaner and Geiger, 2015]. However, ergot poisoning still persisted, Alm & Elvevåg 2012 reviewed transcripts from Norwegian witchcraft trials in the late 1600s for possible incidences by comparing known symptoms with comments referring to witches cursing other peoples

bread, witches cursing peoples limbs to fall off, the feeling of ants crawling under skin, demonic convulsions, paralysis, and of course burning and freezing sensations. It has also been suggested to be the cause of witch epidemics in England and Salem, Massachusetts, however, not enough conclusive evidence or cursed bread has been uncovered yet [Alm and Elvevåg, 2013]. Several more outbreaks occurred across Western and Eastern Europe in the 1700s painting a rather perilous picture of morbid devastation and social revolutions [Alm and Elvevåg, 2013, Nemes, 2002]. In the early 1900s the ergotamine culprit was isolated and since over 50 alkaloids have been studied, these are broken up into three groups: i) clavines, ii) lysergic acid amides, and iii) peptides. Different kinds of alkaloids are produced by the endophytic *Epichloë spp.* and the parasitic *Aspergillus* and *Penicillium spp.* ([Klotz, 2015]). Ergot alkaloids are structurally similar to serotonin, norepinephrine, and dopamine (common tetracycline ergoline ring) so they have agonistic/antagonistic behavior or neurotransmitter receptors depending on the dose or the fungi that produces them, as well as the ability to bind to beta-adrenergic receptors; they can affect the central nervous system, the endocrine system and reproductive function, the immune systems, and have been shown to cause fat necrosis in the intestines of livestock [Klotz, 2015]. Its use in medicine has been seen for hundreds of years beginning with midwives for inducing childbirth and stopping postpartum hemorrhage around 350 AD, and is currently used today to treat neurological and endocrine disorders [Burn, 2000, Micale et al., 2006, Miedaner and Geiger, 2015, Alm and Elvevåg, 2013]. Despite strict regulations in place for food and feed; livestock are regularly effected. In the US alone, ergot alkaloid poisoning costs the livestock industry \$860 million a year [Coufal-Majewski et al., 2016].

Not to be overshadowed, *Aspergillus spp.* infect nuts, maize, other grains in the field as well as various commodities in storage, they are capable of producing aflatoxins (AF) A.7, sterigmatocystin (STE), cyclopiazonid Acid (CPA), ochratoxins

(OTA) A.6, and clavine alkaloids [CAST, 2003]. In 1998, aflatoxin contamination in the southern USA was estimated to have resulted in \$85 million to \$100 million in losses in Arkansas, Louisiana, Mississippi, and Texas [Abbas et al., 2012]. *Penicillium spp.* primarily grow in storage and can produce OTA, citrinin (CIT) A.3, patulin (PAT) A.5, CPA, and various other toxins that are found in special cheeses [Hymery et al., 2014]. *Fusarium spp.* of fungi produce fumonisins (FB1, FB2, and FB3) A.2, type a and b trichothecenes (i.e. DON and T-2) A.4, zearalenone (ZEA) A.8, and various emerging mycotoxins such as enniatins (ENN) and beauvericin (BEA) A.9. Some of these have their own diabolical histories and have been responsible for multiple outbreaks of disease and death throughout the 21st century in animals and humans [CAST, 2003]. Clearly, mycotoxins have always been around and our exposure to them is not necessarily new, however, there are so many things that we do that can exacerbate the problem without realizing it. For example, our selective breeding for various features in livestock and poultry (i.e. dairy cows that yield more milk or poultry with increased growth rate) to accommodate growing demands of consumers have resulted in serious metabolic and immune disorders, which in turn could make them more sensitive to mycotoxin exposure [Scheele, 2011, Rauw et al., 1998]. Also all the other chemicals and pharmaceutical compounds that we are exposed to could have interacting effect, Thus, those low background doses that seem insignificant, over time may actually be bigger problems in combination with other agriculture, diet, lifestyle, and genetic related factors.

For humans and animals, it would be beneficial to reduce the intake of and exposure to mycotoxins via dietary contamination. Though it is not possible to completely eliminate mycotoxin occurrence, there are methods for management, mitigation, and prevention that may provide an effective barrier against the negative effects on agricultural production and health. Consistent research and biomonitoring

Table 1.1: Major Mycotoxigenic *Fusarium spp.* in products and mycotoxins produced (adapted and modified from [CAST, 2003]).

	<i>verticilloides</i>	<i>fujikuroi</i>	<i>proliferatum</i>	<i>subglutinans</i>	<i>poae</i>	<i>sporotrichioides</i>	<i>acuminatum</i>	<i>graminearum</i>	<i>culmorum</i>	<i>crookwellense</i>	<i>avenaceum</i>	<i>equiseti</i>	<i>oxy-sporum</i>	<i>tricinctum</i>	<i>langue-hiae</i>
Fumonisin	+	+	+	+											
Trichothecenes					+	+	+	+	+	+	+	+	+		+
Zearalenone								+	+	+		+			
Fusaric Acid*	+	+	+	+								+	+		
Enniatins					+						+		+	+	
Beauvericin	+		+	+							+		+		
Moniliformin	+	+	+	+		+			+		+		+	+	
Pre-Harvest/Field	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Post Harvest/Storage									+						
MAIZE	+		+	+		+	+	+	+	+	+	+			
SMALL GRAINS	+				+	+	+	+	+	+	+	+		+	+
RICE	+		+					+							
SOYBEANS													+		
GRAPES		+	+									+	+		

is necessary to discern the effect of chronic low level mycotoxin exposure so that the public can be informed and educated as to help make consumer choices that drive market and regulatory changes. For the purpose of this paper, emphasis will be placed primarily on AFB1, OTA, FB1, ZEA, and DON.

1.1 Fungal genomics and host-plant interactions

To date, there are more than 400 mycotoxins that have been identified [Berthiller et al., 2007, Škrbić et al., 2012, Li et al., 2018, De Mets et al., 2018]. Primary metabolism and metabolites are essential for normal growth, development, reproduction of their producing organisms, and are the precursor molecules for secondary metabolism; therefore, secondary metabolites undergo production during limited/specific phases of the fungal life cycle [Keller et al., 2005, Bennett and Bentley, 1989]. Interestingly enough, Keller & Hohn 1997 have reported that genes required to for a fungus to express a fungal secondary metabolites are usually clustered together on a single chromosome [Keller and Hohn, 1997].

Because many different organisms (even those that are phylogenetically unre-

lated) share similar secondary metabolic capabilities, it is thought that these clusters can arise through Horizontal Gene Transfer (HGT), in which the DNA is transmitted from one genome to another. A study conducted by Wisecaver et al. 2014, investigated 208 different fungal genomes and 247, 202 enzyme encoded genes responsible for a variety of metabolic reactions to assess the involvement of gene innovation strategies such as gene duplication (GD) and HGT in the evolution of clustered and non-clustered metabolic pathways. Their results showed that, both HGT and GD give rise to clustered metabolic diversity, however GD was the more common process [Wisecaver et al., 2014]. This makes sense, especially in regards to bioactive compounds that imbue some kind of selective advantage. The physical linkage of gene and clustering of genes is an efficient system because i) it reduces the amount of regulatory steps in the biosynthetic machinery, ii) Chromatin based mechanisms, histone acetylases, deacetylases, methyltransferases control fungal SM biosynthesis by placing repressing chromatin domains on SM clusters during primary metabolism so that no SM production occurs with primary metabolism, then replacines will reprogram the chromatin landscape to for transcriptional activation [Gacek and Strauss, 2012]. Secondary metabolism produces a diverse array of complex chemical compounds through specialized biosynthetic pathways regulated by transcription factors (TFs) that are activated in response to some biotic or abiotic stimuli; these can be global regulators or pathway specific [Trienens and Rohlf, 2012]. Global TFs that

Table 1.2: Metabolic Gene Clusters for Mycotoxin in Select Plant-Pathogenic Fungi.

Fungus	Genome size (Mb)	Number of genes	Number of chromosomes	Metabolic gene clusters	Ref.
<i>A. flavus</i>	36.8	12,197	8	30 genes responsible for aflatoxin biosynthesis located in 75kb cluster on chromosome 3	(Payne et al., 2006, Amaike & Keller 2011)
<i>F. verticillioides</i>	41.7	14, 179	12	23 genes involved in fumonisin biosynthesis clustered in 80kb region on chromosome 1	(Ma et al., 2010)
<i>F. graminearum</i>	36.2	13,332	4	15 genes known for tricothecene biosynthesis over all 4 chromosomes with 12 genes clustered on one locus on chromosome 2	(Cuomo et al., 2007, Lee et al., 2008)

correspond to environmental signals are i) PacC that responds to pH, ii) CCAAT-binding complex CBC that responds to iron, iii) AreA that responds to nitrogen, iv) the Velvet complex which responds to light, and v) CreA that responds to carbon [Woloshuk and Shim, 2012]). Cluster specific TFs are sequence specific binding proteins like zinc fingers and leucine zippers [Woloshuk and Shim, 2012]. One of the most important things to realize about mycotoxin gene clusters is that they are co-regulated, thus a shared regulation method means that multiple types of mycotoxins can be expressed together, especially if they share the same pre-cursor molecule (i.e. Acetyl-CoA and the joint expression of OTA and CIT) [Geisen et al., 2017]. For the most part, mycotoxins are thought to help fungal strains adapt to their environment and thus by acting as i) virulence factors (i.e. DON), ii) antibiotic, iii) antioxidant, and iv) mineral storage [Geisen et al., 2017]. In addition to external factors, internal host factors may also play a large role in mycotoxin accumulation. Metabolites produced by the host or other organisms within the host could potentially alter the mycotoxin biosynthesis pathway. Kumar et al., 2017 describe two possible host mechanisms that could modulate fungal mycotoxin cluster activation and accumulation: i) pathogen-modified nutritional factors present in the host that become precursors for activation of the mechanism of mycotoxin production and ii) natural chemical factors present in the host that directly modulate the transcription factors that induce mycotoxin production” [Kumar et al., 2017]. Plants are able to both metabolize (with Phase I and II reactions) and compartmentalize (Phase III) mycotoxins. Phase I reactions may convert mycotoxins into phytotoxic compounds, while phase II conjugation reactions reduce its toxicity, then phase III will sequester the the glucose or GSH conjugated mycotoxins into vacuoles or bind them irreversible to the cell wall [Berthiller et al., 2013]. These are effectively known as ‘masked’ mycotoxins, and they they may no longer be toxic to the plant, but upon ingestion in animals and humans can be transformed back into the parent compound

and once again be toxic [Berthiller et al., 2013].

Chapter 2

Control, Prevention, and Detection

Mycotoxins circulate through the environment and contaminate various resources. Secondary metabolites are allelopathic, meaning they have a direct or indirect, harmful or even beneficial effect on another organisms from the biochemicals they produce [Keller et al., 2005]. It is unrealistic if not impossible to eliminate mycotoxins, therefore, more research about a mycotoxin role in different ecological and agricultural settings, occurrence under different conditions, and its interactions with other organisms is necessary. The effect of mycotoxins on some of these ecosystems remains largely unknown, however, it is important to understand how, why, and in what forms are they getting there in order to mitigate undesirable outcomes to the best of our ability. With this knowledge we can help prevent, control, and decontaminate mycotoxins in our environment. Figure 2.1 represents of flow diagram of how mycotoxin move through the environment, from this we can see potential areas where mycotoxins can be monitored, identified, and perhaps prevented to some extent.

2.1 Pre-harvest Control and Prevention

2.1.1 Soil Tillage and Crop Rotation

There are many kinds of soil tillage management practices: conservation tilling (no-till, reduced-tilling) and conventional tilling [Conservation Technology Information Center, 2008, Towery and Werblow, 2010]. Tillage systems effectively alter the chemical, biological, and physical property of a soil. The degree of physical mixing of a soil can alter the distribution of nutrients, microbial communities, and complexity of a substrate [Towery and Werblow, 2010]. In conservation tilling systems, 30% or more of crop residue is left on the surface after planting, this allows for high accumulation of organic matter and can influence the type of microbial organisms that dominate soil. It also helps reduce nutrient loss, prevent erosion, and help with water reten-

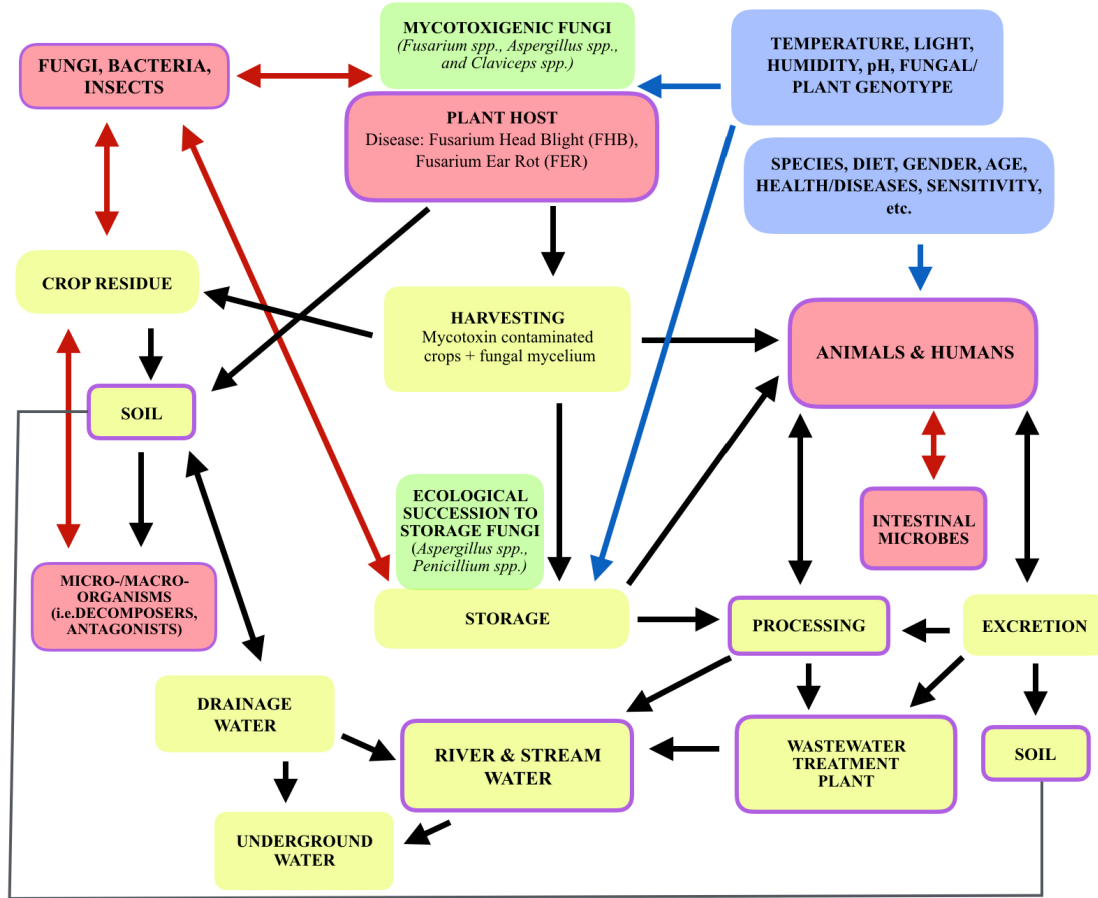


Figure 2.1: The green boxes are mycotoxin producing fungi, the blue boxes represents influencing environmental factors, pink boxes are other interacting organisms, yellow boxes are steps or areas, boxes with purple outline indicate areas or organisms that can potentially alter the mycotoxin compound (i.e. conjugation, degradation, or detoxification). The blue single arrows show factors can influence fungi, the red double arrows show competition between organisms, and the black single end or double end arrows represent the flow of mycotoxigenic fungi and mycotoxins (modified from [Abid, 2012]).

tion in the soil [Towery and Werblow, 2010]. In conventional tilling (deep tilling, sub-soiling) systems, less than 30% of the plant residue is left on the surface and is instead plowed/buried into the soil [Conservation Technology Information Center, 2008, Towery and Werblow, 2010]. Some research studies suggest that burying contaminated detrital matter in order to reduce the spread of plant pathogenic fungi to the next crop may decrease the spread and subsequent mycotoxin contamination,

however, there has yet to be any significant differences between the incidence of certain mycotoxins and the use of conventional versus no till management [Marocco et al., 2008, Ariño et al., 2009].

The high accumulation of organic matter and debris associated with no-till can influence the microbial community ecology by favoring fungal decomposers [Widstrom et al., 2003, Angle, 1987a, Angle, 1987b]. A study conducted by Sipilä et al., 2012, looked at cross-site microbial communities under different tillage regimes (till and no-till) and the effect on *Fusarium* fungistasis in soil and the extent at which soil conditions can inhibit fungal germination and growth. They looked at bacterial and actinobacterial markers (16rRNA) in terminal restriction fragment length polymorphism (T-RFLP), phospholipid fatty acid (PLFA) analyses, and conducted fungistasis biotests on the model organism *Fusarium culmorum* to elucidate predictions based on long-term tillage practices and link microbial markers (16S rRNA T-RFLP, Actinobacteria-specific T-RFLP, and PLFA) with soil fungistasis. It was found that, microbial biomass, fungal biomass, and C/N ratio all correlated with the strength of fungistasis. In general, it is no specific management strategy or bacterial strain but the higher activity in the soil that had had greater impacts on plant pathogen suppressiveness due to the high microbial biomass, increased interaction and competition, and greater amount of resources to support these interactions [Sipilä et al., 2012, Weller et al., 2002].

The FAO/CAC recommend no-till management practice for reducing mycotoxin contamination in crops [Widstrom et al., 2003, Codex Alimentarius Commission, 2003]. Though it may seem counter intuitive, because plant residues can act as a reservoir for mycotoxigenic fungi, it may have positive effect on reducing mycotoxin concentration by i) limiting water and nutrient loss from soil which helps alleviate plant stress and subsequent fungal infection and mycotoxin production, ii) encouraging higher microbial (bacteria and fungi) soil activity at the surface layer

that strengthen soil fungistasis [Widstrom et al., 2003, Sipilä et al., 2012, Angle, 1987a, Angle, 1987b].

Crop rotation is an advantageous agricultural practice because it has the potential to limit pathogen inoculation, reduce soil erosion, diversify the nutrient use so there is time for them to be replenished. GAP recommend that highly susceptible crops should not be planted consecutively nor should they be rotated with another susceptible crop [Codex Alimentarius Commission, 2003]. Peanuts, maize, sorghum, and small grains (wheat, oats, rye) are amongst the more susceptible crop types. A better rotation crop would be one that is significantly less susceptible like soybeans or forage crops [Codex Alimentarius Commission, 2003].

Abbas et al., 2012 studied the implications of mycotoxin (aflatoxin and fumonisin) production by soil *Aspergillus flavus* in different corn-soybean rotation systems in the Mississippi Delta region [Abbas et al., 2012]. Over four years (2005-2008), they planted and studied 8 different corn and soybean rotations that were under conventional tillage (common practice for sandy soils of southeastern coastal plains) and the resulting *A. flavus* soil populations and contamination [Widstrom et al., 2003]. Soybean is a poor substrate for aflatoxin production, possibly due to low C/N ratio or the greater amount of nitrogen than carbon [Shotwell et al., 1978, Abbas et al., 2012]. The results of their study and other similar studies indicated that i) soybeans had greater colonization by non-aflatoxigenic isolates compared to corn which had a greater specificity for aflatoxigenic strains, ii) rotating soybean and corn helped to reduce *A. flavus* propagules in soil thus reducing potential for corn inoculum and aflatoxin production, iii) rotating with soybeans also helps reduce the production and contamination of fumonisins in corn [Abbas et al., 2012, Abbas and Bosch, 1990].

2.1.2 Cultivar Selection and Breeding

Selecting the right cultivars will help to reduce mycotoxin accumulation through various mechanisms that allow the plants to either resist infection and growth from a plant pathogenic fungi or suppress fungal production of a mycotoxin after infection [Brown et al., 1999, Warburton and Williams, 2014]. There are several ongoing breeding programs for many crops such as the USDA-ARS Genetic Enhancement of Maize (GEM) project which partners and cooperates with many public and private industries and researchers from around the world [USDA-ARS et al., 2018].

Maize and wheat are some of the most intensely studied and developed genetically modified crops and as a result have commercially available cultivars [Warburton and Williams, 2014]. Conventional approaches revolve around selection for breeding based on observed variation and controlled mating, while modern/advanced techniques implement various gene modification and recombination technologies [Podevin et al., 2012, Onaga and Wydra, 2016]. Screening for desirable traits of resistance can be done through inducing infection epidemics on different cultivars, where the superior phenotypic trait can be assessed visually (assisted by digital imaging analysis) or with the help of molecular markers and genetic sequencing [Steiner et al., 2017]. There is no singular trait that controls host resistance to fungal infection and mycotoxin accumulation [Steiner et al., 2017, Milgroom et al., 2014]. The traits are complex, quantitative, and have a high degree of Gene x Environment interaction [Milgroom et al., 2014].

2.1.3 Planting/Harvesting, Irrigation, Fertilization, and Plant Density

The dates at which seeds are planted and harvested vary by region, crop type, favorable weather conditions to plant/harvest, and a variety of other human factors (see Table 2.1). It is generally recommended to adhere to timely planting and harvesting as to avoid unfavorable disease development at susceptible times (i.e anthesis) or prolonged disease exposure by late harvesting, which has been found to

Table 2.1: Planting and harvesting dates for different crops in the US. (data from [USDA-NASS, 2010]).

	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Corn				PLANT			SILK			HARVEST		
Soybean					PLANT		BLOOM			HARV.		
Peanut					PLT.		PEG			HARV.		
Rice				PLANT			HEAD		HARV.			
W. Wheat					HEAD		HARV.			PLANT		
S. Wheat				PLANT			HEAD		HARV.			
D. Wheat				PLANT			HE.		HARV.			
Barley				PLANT			HEAD		HARV.			
Oats				PLANT			HEAD		HARV.			
Sorghum				PLT.			HEAD		HARV.			
Sugarcane		HARV.							PLT.		HARV.	
Sugarbeets				PLANT						HARV.		

severely increase mycotoxin contamination levels and other negative impact on the crop.

Parsons & Munkvold, 2012 conducted a two year field study on *Fusarium* ear rot (FER) maize caused by *Fusarium verticillioides* in 6 different locations in the US (Garden City, KS; Johnstn IA; Tifton, GA; Union City, TN; Waimea, HI; and Woodland, CA) and the subsequent contamination of fumonisins [Parsons and Munkvold, 2012]. In order to determine what factors play a role in disease severity and mycotoxin contamination, they evaluated cultural practices such as planting date and hybrid type as well as location and environmental factors such as rainfall, temperature, insect populations, and symptom specific contamination. The results of their studies illustrated that when planted early (relative to the date instructions indicated for the hybrid plant types for each region) there was a lower disease severity, less FB1 levels, and less insect damage. Additionally, locations with dry conditions and increased insect population levels (thrips) were at greater risk for fumonisin contamination.

In comparison to corn grown in the Midwest, corn from the southeastern U.S. has been reported to have higher contamination levels of of aflatoxins from *Aspergillus*

flavus infection [Tubajika et al., 2000]. The higher temperatures and greater humidity in some southern states, such as Louisiana, could consequently have increased AF levels. A study by Tubajika et al., 2000, experimented with three different planting dates (March, April, and May) and four different harvest moisture (15, 20, 25, and 30%) for a corn hybrid (Pioneer Brand 3167) [Tubajika et al., 2000]. Ultimately, their results indicated that an early March planting date followed by harvesting at a moisture percentage below 20% would be a good plan of action for reducing AF contamination.

Competition for resources permeates every aspect of life, and especially for plants. Each plant is competing for space, nutrients, water, and light. When these resources become limited, stress conditions in the plants can occur and have negative impacts. Ways in which these stressors can be amended are through proper seeding ratios for plant density, weed control, irrigation, and fertilization [Ariño et al., 2009, Parsons and Munkvold, 2012]. Crop production must find a balance for plant density that allows them to obtain high yields without compromising the quality or inducing stress conditions that can lead to fungal infection and mycotoxin production. Plant density is affected by weeds that are present in the field, therefore effective removal and control strategies should be considered. Environmental conditions of the area (i.e. drought) will also determine the acceptable plant density. Even hybrids that are high-density tolerant can experience yield reductions and mycotoxin accumulation under severely stressed conditions [Tokatlidis et al., 2010]. How this in turn affects fungal growth and mycotoxin production is dependent on many things ranging from host-species type to temperature. However, the general idea is that greater stress conditions lead to plant disease susceptibility, especially those that favor fungal growth and mycotoxin production.

With regards to irrigation in plants, it is important to adhere to proper timing and the methods used for irrigation. There is much debate as to whether there is any

significant effect of irrigation on fungal growth and mycotoxin production, but some research has shown that irrigation can help improve abiotic stress conditions [Codex Alimentarius Commission, 2003]. When drought and heat stress occurs irrigating can help relieve plant stress, however, different host-species and fungi will have different responses to the amount and timing of the water application. The use of drought tolerant plants such as pearl millet instead of corn in extremely dry land environments is also advised for reducing water stress in plants [Wilson et al., 2006].

For some fungi, dry weather conditions can cause sporulation in crop debris, and when followed by rain the conidia can be spread by splashing. Sprinkle irrigation can have the same effect and can also cause splash dispersal [Aldred and Magan, 2004a, Ariño et al., 2009]. Studies have shown that height of host plant is an indicator for their susceptibility to fungal infection and disease severity; short plants are more likely to be inoculated by splash dispersal during flowering or grain filling stages [Ariño et al., 2009]. Therefore, the the type of irrigation used during anthesis should be one that minimizes potential conidia dispersal. For maize, water stress mitigation has been shown to reduce fumonisin contamination and infection from *F. verticilloides* and aflatoxins from *A. flavus*, however, some suggest that the reduction of temperature in the crop canopy from irrigation, may have a considerable affect in limiting the production of aflatoxin [Torelli et al., 2012, Widstrom et al., 2003, Fortnum, 1987].

Nitrogen fertilization also has mixed results with regards to its ability to suppress, hinder, or encourage fungal infection and mycotoxin production [Codex Alimentarius Commission, 2003]. Nitrogen fertilization can affect crop canopy by increasing canopy density that then can lower temperature and indirectly preserve humidity by reducing light penetration and wind speed [Hofer et al., 2016]. Hofer et al., 2016 conducted a study on nitrogen fertilization and its effect on FHB in spring barley. Both field and green house experiment plots were subjected to soil-

inoculation treatments with *F. culmorum* and *F. avenaceum* as a way to simulate increased pathogen pressure; fungal genomic DNA was isolated and then quantified with quantitative realtime polymerase chain reaction (qPCR) while mycotoxins (DON and ENN) were quantified using liquid chromatography tandem mass spectrometry LC-MS/MS analysis [Hofer et al., 2016]. In unfertilized control plots, they found higher amounts of DNA and mycotoxins in the barley compared to those that were fertilized. Other studies have found increased FHB and mycotoxin production in wheat from increased nitrogen input [Lemmens et al., 2004]. The difference is nitrogen effects could be the result of many factors involving the type of nitrogen used, environmental conditions, host species, and much more.

2.1.4 Chemical and Biological Control (Weeds, Insects, and Fungi)

Weed control is necessary for many reasons, they can act as an inoculum source for fungi and can spread diseases to crops [Widstrom et al., 2003, Mourellos et al., 2014]. They also compete with the crop plants for resources which can create stressful conditions. How farmers choose to deal with weeds has a variety of impacts and a farmer must consider the best method for their region, crop type, financial means, etc. There are several different weed management strategies for weed control with different crop types, each have pros and cons; i) mechanical removal of weeds is labor intensive and disturbs soil possibly leading to erosion and loss of water; ii) flooding fields can be effective for suppressing weeds in rice paddy fields but may also impact root branching which in turn might affect symbiosis with arbuscular mycorrhizal fungi (AMF); iii) chemical treatment with herbicides such as glyphosate are the most wide-spread and most used weed control strategies but can impair plant defense mechanism while increasing the virulence of pathogen populations [Widstrom et al., 2003, Johal and Huber, 2009].

Insect management is critical during pre-harvest, post-harvest, and storage/transportation. Fungal infection can be exacerbated by physical damage from feed-

ing invasion and act as a vector for infection. The severity of *Fusarium* ear rot (FER) in maize and *Fusarium* head blight (FHB) in wheat, has been linked to the severity of insects/pests such as corn borers, cutworms, ear worms, and thrips [Dmello et al., 1999, CAST, 2003]. The benefit of insecticides is the indirect reduction of mycotoxins by reducing the insect population and thus eliminating any potential damage they could inflict on the crop. As a bonus, however, some insecticides (i.e. naled, an organophosphate) have been shown to directly reduce mycotoxin production by reducing fungal growth as well [Dmello et al., 1999].

In order to curtail fungal infection in crops pre-harvest, fungicides can also be helpful. However, they can also cause many other issues. Therefore, the use of fungicide as a management tool requires careful consideration and should only be considered as part of an integrated management plan and never as a sole control solution. Following manufacturer recommendations and literature about appropriate application and timing will help with obtaining the best results and reducing disease severity and mycotoxin incidence. Application of fungicides is recommended at anthesis (flowering) when a plant is most susceptible to infection [Codex Alimentarius Commission, 2003].

Factors that can reduce a fungicides efficacy or increase undesirable results are i) selection for resistance to fungicide, ii) stimulation of mycotoxin production, iii) differential reactions of crops/hybrids [CAST, 2003, Schmidt-Heydt et al., 2012]. The Fungicide Resistance Action Committee (FRAC) is dedicated to coordinating with/between different companies that market fungicides in the hopes to implement appropriate strategies and solutions for minimizing the risk of fungicide resistance and disseminating vital information on the chemistry, resistance, mechanisms, and management of fungicides [FRAC, 2007]. Selection for fungicide resistance genotypes depends on many factors and can be managed by adjusting the amount and frequency of application or through the use of fungicide mixtures (alternating with

different types) [Milgroom et al., 2014].

Fungicides that are recommended for use with pre-harvest plant pathogens are azole chemicals. They have been studied extensively under both *in vitro* and field conditions for *Fusarium spp.* and *Aspergillus spp.* [Magan et al., 2011, FRAC, 2007]. Azole fungicides (FRAC CODE 3) are demethylation inhibitors (DMI), their mechanism of action is to inhibit sterol biosynthesis in membranes and are considered a medium risk for fungicide resistance [FRAC, 2007]. Mateo et al., 2017 assessed the ability of azole fungicides (prochloraz, tebuconazole, and prochloraz+tebuconazole) to control *A. flavus* growth and AF production in maize and on YES (yeast-extract-sucrose) solid medium under different temperature (25 and 37°C) and a_w (0.95 and 0.99) conditions [Mateo et al., 2017]. Prochloraz was the most efficient at reducing *A. flavus* growth and had the lowest AF production, and all fungicides were more successful at higher water activity (0.99). It was also shown that at a certain temperature (25°C), a_w (0.99), and the fungicide mixture prochloraz + tebuconazole (0.01 ± 0.005 mg/L) at low doses enhanced AF production [Mateo et al., 2017]. Reconfirming that sub-inhibitory levels fungicides may actually promote mycotoxin production because of any stress put on the species by the fungicide [Dmello et al., 1999, Edwards and Godley, 2010, Mateo et al., 2017]. The results of their study exhibited information on the interaction between azole fungicide treatment and environmental factors. This is especially important to understand given the changing climate conditions because increasing temperatures can cause additional environmental stress that can result in mycotoxin production as well as drive an ecological shift towards more xenotolerant species [Magan et al., 2011, Mateo et al., 2017].

Fungicides such as quinone outside inhibitors (QoI) are not recommended for treatment on *Fusarium spp.* as they tend to increase DON production in wheat [Ellner, 2005]. QoI or strobilurins (FRAC CODE 11) have multi-site actions, inhibit mitochondrial respiration and disrupt the electron transport chain and ATP pro-

duction in fungi [FRAC, 2007]. They are high risks for fungicide resistance [FRAC, 2007]. Additionally, they have a greening-effect on plants where they delay senescence until later on in the season [Dmello et al., 1999, Fromme et al., 2017, Ellner, 2005]. Ellner et al., 2005 found that applications of QoI fungicides on field tests before blossom resulted in increased DON contamination compared to untreated controls [Ellner, 2005].

Makers of certain fungicides have been shown to advertise their product as a prophylactic treatment, even in the face of low pathogen pressure for disease, because it might help increase water retention and nitrogen use efficiency and in turn produce a higher yield; however, Fromme et al., 2017 tested this on sorghum in the absence of pathogen pressure and found that there was no effect on overall plant health or yield [Fromme et al., 2017]. In an effort to reduce the use of chemical pesticides several biological control methods have been developed, which includes resistant cultivars of plants. Ideally, these microorganisms, bioactive metabolites (i.e. phenolic compounds and antioxidants), or essential oils can help to control infection and reduce mycotoxins by several means including competition for nutrients, detoxification of mycotoxins, and much more [Tsitsigiannis et al., 2012]. Microorganisms that have antagonistic ability towards toxigenic fungal species have been evaluated based on their ability to reduce mycotoxin contamination [Köhl et al., 2011]. Aflatoxin and ochratoxin production by *Aspergillus spp.* can be controlled by bacteria (lactic acid bacteria and *Bacillus spp.*), nontoxigenic *Aspergillus* strains or strains that can degrade mycotoxins, and yeasts [Tsitsigiannis et al., 2012, Varga et al., 2010]. Mycotoxins (trichothecenes, fumonisins, etc.) produced by *Fusarium spp.* can be controlled by bacteria (*Bacillus*, *Paenibacillus*, *Pseudomonas*, and *Streptomyces spp.*), non-toxigenic *Fusarium spp.*, and yeast [Tsitsigiannis et al., 2012].

Table 2.2: Storage fungi and temperature and a_w parameters that promote their growth and mycotoxin production (adapted from [Paster et al., 1995, Aldred and Magan, 2004b, Manna and Kim, 2016, Zong et al., 2015, Fleurat-Lessard, 2017]).

Fungi	Mycotoxin	Temp. for growth /mycotoxin prod.	a_w growth/mycotoxin prod.
<i>Fusarium graminearum</i>	ZEA	-/25-30	/0.98
<i>Fusarium spp.</i>	FB1	30/15-30	0.88/0.93
<i>Fusarium spp.</i>	DON	20-25/29-30	0.90/0.93
<i>Aspergillus flavus</i>	AFB1	35/28	0.78 - 0.85/0.84-0.99
<i>Aspergillus parasiticus</i>	AFB1	35/33	0.84-0.95/0.99
<i>Aspergillus ochraceus</i>	OTA	-/30-37	0.77/0.85
<i>Penicillium verrucosum</i>	OTA	-/25	0.80 - 0.95/0.83-0.95
<i>Penicillium expansum</i>	PAT	-/23-25	0.82 -0.84/0.99

2.2 Post-harvest and storage control/prevention

Good Hygiene Practices (GHP) begin with proper harvest protocol and follow through with good storage and good manufacturing practices (GSP and GMP respectively) [Codex Alimentarius Commission, 2003, CAST, 2003]. During the post-harvest and storage stage the predominant mycotoxigenic fungi shift to *Aspergillus spp.* and *Penicillium spp.*; this ecological succession can be attributed to dramatic difference in storage environment conditions [Lacey et al., 1980, Aldred and Magan, 2004b, Manna and Kim, 2016]. Lacey et al., 1980 classified the successional traits into four eco-physiological groups i) hygrophillic (i.e. *Fusarium spp.* and *Claviceps spp.*), ii) cryotolerant (i.e. *Penicillium spp.*), iii) thermotolerant (i.e. *Aspergillus flavus* and *A. nidulans*), and iv) xerotolerant (i.e. *Aspergillus spp.*) [Lacey et al., 1980]. Some of the factors that determine the shift in species depend on tolerance to low oxygen, low a_w , and low temperatures (see Table 2.2) [Manna and Kim, 2016].

In addition to the production of mycotoxins, fungal activity in stored grains can cause discoloration, dry matter losses (carbohydrates used as energy source), heating, lipid and protein degradation, emission of volatile metabolites and odors, decrease quality and germination viability [Aldred and Magan, 2004b]. Some of

these can act as indicators and can be easily detected in storage fungi.

2.2.1 Harvesting and Storage

Leading up to harvest time, simple steps can be taken to reduce excessive crop contamination by i) equipment care and maintenance, ii) pre-harvest field monitoring, and iii) proper drying/cleaning procedures [CAST, 2003]. All field equipment that has any contact with seeds, plants, or soil should be regularly cleaned to reduce spreading fungal contaminants and maintained to ensure proper function to avoid delayed harvests or damage to crop. Delayed harvest times, damaged kernels, and contact with soil are associated with increased risk of fungal growth or mycotoxin production. Pre-harvest field monitoring, sampling, and testing to locate pocket/areas that are infected (several methods will be discussed in the next section) can help reduce the spread of fungal infection and mycotoxin production because they can be harvested separately. Additionally, monitoring for water content, humidity, and temperature in the environment or within the crop canopy can have huge effects on mycotoxin production [Fleurat-Lessard, 2017]. After harvesting at a low moisture content, grains should be cleaned, separated based on different characteristics (i.e. market quality, moisture content, crop type, or protein content), and all grain should be dried properly to ensure moisture content levels are below that which is favored for fungal growth and mycotoxin production [Fleurat-Lessard, 2017].

In accordance with good hygiene practices, all containment devices need to be cleaned before and after use and all storage or transportation facility for food commodities should be well maintained to prevent for leaks, pests, and fungal growth [CAST, 2003]. Good storage practices suggest that conditions are cool, dry, and well ventilated, and the moisture content should not exceed 14.5% [Mannaa and Kim, 2016]. A large part of best (good) practices is having proper documentation, which can help officials, producers, and consumers keep track of potentially

hazardous products and to allow for interventions, if hazardous products are found. Proper documentation can also ensure that the crop meets any regulation compliance necessary for different areas [Codex Alimentarius Commission, 2003].

2.2.2 Detection Methods and Sampling

In order to monitor fungal activity and mycotoxin production, appropriate sampling methods and analytical testing methods must be used. For on-field monitoring methods and sampling/testing at pre-harvest stages, digital imaging and analysis can be used to identify diseases or screen for resistant phenotypes [Li et al., 2014]. Different digital imaging, sensing, processing, and analysis techniques from the past three decades has been reviewed by Berbedo 2013. In the past, imaging analysis were limited by the need for apparent and visible symptoms or patterns to distinguish healthy plants from infected plants (Berbedo 2013). Current optical sensor techniques that have promising potential for use and application in the field are thermography, chlorophyll fluorescence imaging, and hyperspectral techniques [Fang and Ramasamy, 2015, Bauriegel and Herppich, 2014]. In addition, spore traps can help track and control plant disease by fungal airborne inoculum and monitoring of pathogen populations [West and Kimber, 2015, West et al., 2017]. These kinds of sensors can be hand held portable devices or mounted on different platforms such as unmanned aerial vehicles (UAV), tractors/trucks, stationary posts, or using satellite technology [West et al., 2017].

Fungal pathogens can cause a huge range of plant responses in physical appearance and biochemical processes; for example: leaf color, canopy morphology, canopy density, and disturbances in photosynthesis, transpiration, and respiration [Fang and Ramasamy, 2015, West et al., 2017]. These responses can be measured across the electromagnetic spectrum. Imaging methods can help reduce infection in the field by prompting fungicide or pesticide application. However, in the event that the infection is detected too late, harvest control applications can be taken to harvest

areas with high infection and damage separately from areas that are not [West et al., 2017, Bauriegel and Herppich, 2014]. It is often helpful to use vegetation indices such as normalized difference vegetation index [Li et al., 2014]. While these sensors are helpful, they are limited by the statistical algorithms used for data analysis, field of view of the sensor, sub-optimal environmental conditions, and more [West et al., 2017]. Sensors for monitoring and early detection of storage fungi are the electronic nose (EN) and CO₂ micro sensors are simple and fast qualitative detection methods [Fromme and O’connor, 2012, Fleurat-Lessard, 2017]. For more information on classical methods of detection as well as sequencing methods for used for novel fungal identification with research driven understanding of fungal genomics see 2.3, 2.4, and 2.5.

Many regulatory sampling and detection methods are conducted by government agencies or by private industries, after harvest, before transfer/transportation, upon receiving shipments, or at various identified critical control points [FAO, 2001]. Under the United States Grain Standards Act (USGSA), the American Market Act (AMA), and the Grain Quality International Act (GQIA), USDA/GIPSA’s Federal Grain Inspection Service (FGIS) is authorized to test for AFs in corn, wheat, sorghum, and soybeans and DON in barley, corn, oats, and wheat to ensure they are below the maximum levels. There are several testing labs across the united states that offer testing services and produce/sell validated test kits (i.e Romer Labs and EnviroLogix). The kinds of well validated tests that are approved by GIPSA are: enzyme linked immunosorbent assay (ELISA), monoclonal antibody affinity chromatography, lateral flow strip, fluorescence technology, and on specific request or case-by-case basis high performance liquid chromatography (HPLC) [USDA and FIGS, 2018].

In the US, it is prohibited to use unauthorized test kits or sampling methods. These analytical methods require sample preparation with solvent extraction (spe-

cific to mycotoxin chemical properties), filtration, cleanup, dilution, and reference method (i.e. LCMS) with reference material (use of commercial standards) [CAST, 2003]. Sampling procedures have been extensively reviewed, and protocols are discussed in the GIPSA/USDA handbook and EU sampling handbook [USDA and FIGS, 2018]. Adherence to proper sampling plans for specific toxins and matrices (products) is necessary in order to obtain the most accurate reflection of mycotoxin contamination, because the sampling step is the largest source of variation and uncertainty [CAST, 2003]. Currently there are sampling plans for aflatoxins, fumonisins, and deoxynivalenol but none for OTA, T-2, and other emerging mycotoxins [CAST, 2003]. Sampling protocols can be specific to: i) lot weight, ii) analytical method, iii) product being sampled, and iv) mycotoxin being tested [CAST, 2003]. From the representative lot, incremental samples (number of incremental samples reflect the distribution of the mycotoxin) are collected then homogenized/aggregated and sub-samples are further prepared and divided for replicate testing [CAST, 2003]. This process, however, loses information on the actual spatial distribution in the bulk/lot sample. A study by Rivas-Casado et al., 2009 on DON in bulk storage samples proposed that sampling plans for mycotoxins with known spatial structure should also consider the incremental sample point location to improve estimates [Rivas Casado et al., 2009]. However, as the product moves through various stages of the food chain (transporting, milling, processing, etc.), a mycotoxins heterogeneous distribution will decrease thus reducing its variability [Rivas Casado et al., 2009]. Therefore the sample point location would not be as telling later in the grains processing journey as it is at the beginning.

2.2.3 Decontamination

Decontamination methods should meet certain standards and require considerations. First, processing methods should reduce mycotoxin level; mycotoxins can be physically removed, transformed/detoxified by chemical or biological means, or can be

adsorbed to solid surfaces. Specific structural groups give mycotoxins their toxicity (i.e. lactone rings, epoxy group), and when modified they may become less toxic (see Figure 2.2) [Karlovsky et al., 2016]. Not all treatments will yield a metabolite with reduced toxicity, some masked metabolites might be released and therefore have increased bioavailability and toxic potential [Karlovsky et al., 2016]. Second, the cost is effective and the method readily usable; the method should be economically feasible for consistent use and technologically appropriate for a wide demographic range. Also its process should be effective enough to remove mostly contaminated grains without high losses of uncontaminated grains. Third, the quality and safety of product should not be compromised; regardless of method used, there should

Device	Use	Method	Advantages	Disadvantages
FIELD/PRE-HARVEST				
Thermal Imaging	<ul style="list-style-type: none"> - On-field after infection in pre-harvest - Storage detection 	Thermal cameras (8000-14000 nm) and near infrared cameras (700-1200 nm) capture images and colors that are then analyzed for canopy of leaf temperatures or fungal infection. Reduced transpiration from infections can increase leaf temperature by closing stomata at early stage of infection. Water content can also be detected and can appear cooler.	<ul style="list-style-type: none"> - Can detect plant disease and monitor through water transpiration - Monitor heterogeneity of soil borne pathogens - Costs have been decreased in the past years 	<ul style="list-style-type: none"> - Not specific for diseases, does not i.d. infection type - Dependent on statistical method application - Does not distinguish between diseases with similar patterns - Sensitive to fluctuations in environment during measurements
Chlorophyll Fluorescence Imaging (CFI)	<ul style="list-style-type: none"> - On-field during early stages of infection 	Measured on leaves as function of incident light and change in fluorescence patterns used to analyze pathogen infections, which can be from alterations in photosynthetic electron transport reactions. Use of charge-coupled device (CCD) cameras with light application (UV or LED).	<ul style="list-style-type: none"> - Sensitive detection before visible symptoms appear 	<ul style="list-style-type: none"> - Dependent on statistical method application - Requires subsequent diagnostic method for isolate i.d.
Hyperspectral Imaging Across the Electromagnetic spectrum	<ul style="list-style-type: none"> - On-field after infection in pre-harvest 	Measures the change in reflectance that occur from changes in biophysical and biochemical characteristics after infection. Measures contiguous spectral bands with use of NIR instruments, hyper spectral cameras, and thermal cameras.	<ul style="list-style-type: none"> - Used for plant phenotyping and crop disease identification - Robust and fast analysis - Cameras have data collection in 3 dimensions 	<ul style="list-style-type: none"> - Dependent on statistical method application - Requires subsequent diagnostic method for isolate i.d.
Spore Traps	<ul style="list-style-type: none"> - On-field during early stages of infection 	Device captures air particulate samples (dust, spores, pollen, plant, microscopic materials) onto a sticky surface or with airflow manipulation to collect samples in a tube. When sample collections are at threshold levels can indicate disease infection on plants.	<ul style="list-style-type: none"> - Monitor changes in pathogen populations - Can help target crucial times when crop protection agents need to be applied 	<ul style="list-style-type: none"> - ID possible for large, visually characteristic spores - Does not identify to species level without uncertainty; requires subsequent diagnostic methods - Weather interferes with inoculum detection; needs forecasting models - Spatial variability in spore concentration related to disease risk is uncertain

Table 2.3: Detection methods, their use, and advantages/disadvantages for the pre-harvest period [Fang and Ramasamy, 2015, West and Kimber, 2015, West et al., 2017, Bauriegel and Herppich, 2014, Li et al., 2014, Fromme and O’connor, 2012, Niessen, 2007, Xu et al., 2016, Cellini et al., 2017, Lezar and Barros, 2010, Olarte et al., 2015, Emri et al., 2017, Riccombeni and Butler, 2012, Fleurat-Lessard, 2017].

Table 2.4: Detection methods, their use, and advantages/disadvantages in the post-harvest period [Fang and Ramasamy, 2015, West and Kimber, 2015, West et al., 2017, Bauriegel and Herppich, 2014, Li et al., 2014, Fromme and O’connor, 2012, Niessen, 2007, Xu et al., 2016, Cellini et al., 2017, Lezar and Barros, 2010, Olarte et al., 2015, Emri et al., 2017, Riccombeni and Butler, 2012, Fleurat-Lessard, 2017].

Device	Use	Method	Advantages	Disadvantages
POST-HARVEST/STORAGE				
Electronic Nose	<ul style="list-style-type: none"> - Early storage detection - Potential for use in field 	Device samples air from head space above the grain bulk sample. It has an array of sensors (metal-oxide semiconductors) that can detect molecules from several chemical classes or with diverse functional groups (VOC, odor) and interpret the data with pattern recognition strategies (neural networks).	<ul style="list-style-type: none"> - Non - destructive - Recognizes signatures of targeted fungal metabolites - Portable devices for <i>in situ</i> measurement of grain bulk - Semi-quantitative, detects above or below legal limits 	<ul style="list-style-type: none"> - Limited by high variability of spoilage level - Does not quantify - Shows highly variable results
CO ₂ Micro Sensor	<ul style="list-style-type: none"> - Early storage detection 	Production of CO ₂ is associated with moisture content above threshold levels. Monitoring for CO ₂ in grain bulk can help identify places where fungi is growing , safe grain storage has CO ₂ levels between 400-500 ppm, levels of 3000 ppm or more indicate fungal spoilage.	<ul style="list-style-type: none"> - Screening for spoilage that is occurring allows for intervention and control - Fast results 	<ul style="list-style-type: none"> - Does not determine type or location of spoilage - Does not quantify level of contamination
Immunochromatographic membrane-based test strip (ICS) or Lateral Flow Assay (LFA)	<ul style="list-style-type: none"> - Storage detection from sample 	Qualitative/semi-quantitative fast visual test can detect presence of a target analyte (mycotoxin) in the sample matrix. A sample is placed on the sample pad and then travels to the conjugate pad where the target molecule will react with its antibody conjugated Tag. Then the mix moves toward a third 'capture' molecule in the test zone to immobilize the complex and its concentration there forms a visible result.	<ul style="list-style-type: none"> - Fast and commercially available and easy to use - Approved/used by GIPSA 	<ul style="list-style-type: none"> - Sample preparation and pre-treatment required (toxin extraction and clean up, solvent extraction, filtration, and clean up) - Reproducibility varies - Only semi-quantitative
Enzyme Linked Immunosorbent Assay (ELISA)	<ul style="list-style-type: none"> - Storage detection - Can be used at any point along food chain 	Assay uses antibodies specific to a particular antigen to immobilize and then identify/detect the substance by producing a visible signal. This can also be used for plant pathology and biomarkers from mycotoxin interaction. There are several types of ELISA: Direct, Sandwich, and competitive.	<ul style="list-style-type: none"> - Semi-quantitative fast visual test - Approved/used by GIPSA 	<ul style="list-style-type: none"> - Sample preparation and pre-treatment required (toxin extraction and clean up, solvent extraction, filtration, and clean up)
Fluorescence Sensing Assay	<ul style="list-style-type: none"> - Storage detection - Can be used at any point along food chain 	Quantitative method based on fluorescence resonance energy transfer (FRET).	<ul style="list-style-type: none"> - Approved/used by GIPSA (ex. FluoroQuant®) - Can be set up for single or multiple mycotoxins (CITE ROMER LAB PRODUCT) 	<ul style="list-style-type: none"> - Preparation of sample can be destructive - Needs alternative reference method with HPLC or ELISA
Near Infrared Spectroscopy	<ul style="list-style-type: none"> - Storage detection - On-Field detection in pre-harvest 	(800-2500 nm) Detects wavelength of functional groups.	<ul style="list-style-type: none"> - Fast, non-destructive - Environmentally friendly (no extraction/cleanup chemicals) 	<ul style="list-style-type: none"> - Not good for low regulatory level threshold - Dependent on statistical method application
Thin Layer Chromatography (TLC)	<ul style="list-style-type: none"> - Storage detection - Can be used to quantify at any point 	See 4.2.1	<ul style="list-style-type: none"> - Standard reference methodology for some mycotoxins (AF) 	<ul style="list-style-type: none"> - Sample preparation can be destructive - Outdated technology
HPLC, LC-MS/MS	<ul style="list-style-type: none"> - Storage detection - Can be used to quantify at any point 	See 4.2.1	<ul style="list-style-type: none"> - Standard reference methodology (reliable and repeatable) for some mycotoxins (AF) - Simultaneous analysis on mycotoxins - Can be used for biomarker analysis in organisms 	<ul style="list-style-type: none"> - Costly equipment and special training required - Solvent extraction and preparation could destroy sample - Matrix related issues

be no undesirable alterations to the nutritional quality or sensory properties of the product, nor should there be residual chemicals that could be harmful to consumer

separately or in conjunction with any mycotoxins present [Kabak et al., 2006, Varga et al., 2010]. This can be accomplished through physical, chemical, and microbial methods. In addition to treatments that we use specifically to manage both fungal presences and mycotoxin levels. Additionally, food additives (binders, prebiotics, probiotics, and enzymes) can be added to feed or food as a way to further reduce toxicity of mycotoxins by reducing absorption or promoting excretion [CAST, 2003].

Table 2.5: Sequencing methods, their use, and advantages/disadvantages [Fang and Ramasamy, 2015, West and Kimber, 2015, West et al., 2017, Bauriegel and Herppich, 2014, Li et al., 2014, Fromme and O’connor, 2012, Niessen, 2007, Xu et al., 2016, Cellini et al., 2017, Lezar and Barros, 2010, Olarte et al., 2015, Emri et al., 2017, Riccombeni and Butler, 2012, Fleurat-Lessard, 2017].

Device	Use	Method	Advantages	Disadvantages
Sequencing				
Polymerase Chain Reaction (PCR) - based methods: Multiplex-PCR (m-PCR), Real-time PCR (q-PCR), and Digital PCR (d-PCR)	<ul style="list-style-type: none"> - Storage detection - Can be used to quantify at any point along food chain - Can be used to evaluate biological markers in animals and humans 	Genomic markers allow for the detection of targeted mycotoxigenic species by adding the following components to a solution with the target sequence: i) a pair of primers that hybridize with the flanking sequences of the target, ii) all four deoxyribonucleoside triphosphates (dNTPs), and iii) a heat-stable DNA polymerase. One cycle of PCR requires strand separation, then hybridization of primers, followed by DNA synthesis. This can be carried out for n cycles with 2^n -fold amplification. Quantification of fungal biomass production (can be correlated with mycotoxin content). Depending on the gene within the fungal genome being targeted, different PCR methods will be used.	<ul style="list-style-type: none"> - High specificity and sensitivity, can detect and quantify - Results within 24 hrs - Can be used to genotype resistant plant cultivars or fungi 	<ul style="list-style-type: none"> - Requires complicated set up and training for use - Requires DNA/RNA extraction - Needs alternative methods for reference (i.e. LC-MS/MS) - Can only i.d. one isolate at a time
DNA chip/ Microarray	<ul style="list-style-type: none"> - Storage detection - Can be used to detect at any point along food chain - Can be used to evaluate biological markers in animals and humans 	Hybridization-based transcriptomics technique. Microarray slide made up of species- or genus-specific oligonucleotide probes from rRNA internal transcribed spacers (ITS) or elongation factor1-a (EF-1a) to identify polymorphisms, fluorescent labeling is added during PCR step. Target sequence samples are amplified, purified, and hybridized. Images of the probe intensities can be read and then processed and analyzed with software programs. For more information please see Lezar & Barros 2010, Olarte et al. 2015, and Emri et al. 2017.	<ul style="list-style-type: none"> - Maps global transcriptional change and predict mycotoxin producing fungi by detecting secondary metabolite genes - Multiple organism i.d. at same time - Growing fungal sequence collections available in online database - Low cost relative to RNA-seq 	<ul style="list-style-type: none"> - Requires extensive training and set up for use (need prior sequence library for comparison (biased), preparation of the DNA chip with , extensive preparation of target DNA with PCR) - Dependent on statistical method application; require software to store and process data and eliminate background noise - Cross-Hybridization artifacts - Can not detect isoforms or structural variation and does not provide sequence data - Lengthy process (multiple days)
RNA-sequencing	<ul style="list-style-type: none"> - Storage detection - Can be used to detect at any point along food chain - Can be used to evaluate biological markers in animals and humans 	Next generation sequencing (NGS) technology; has variety of methods. The input RNA is isolated then reverse transcribed to cDNA, amplified with PCR and then sequenced. Then the sequences can then be processed, analyzed, and compared using software programs. For more information please see Riccombeni & Butler 2012.	<ul style="list-style-type: none"> - Not dependent on prior sequence information, can i.d. novel genes and transcripts without reference genome (<i>de novo</i>) - Very sensitive, detects low levels of expression, isoforms, and structural forms 	<ul style="list-style-type: none"> - Requires extensive training and set up for use - Expensive - Dependent on statistical method application; biases not well understood - Background noise must be eliminated

Physical

Physical processes/methods can reduce mycotoxin contamination by removing portions of contaminated grain or contaminated parts. The process of sorting grains (removing contaminated from non-contaminated) can be accomplished through basic hand sorting, flotation and density segregation and more advanced high-speed techniques, such as optical color sorting. Generally, sorting of grains and products has been shown to reduce all mycotoxin levels in maize, cereals, nuts, apples, grapes, and beans (cocoa and coffee) [Varga et al., 2010, Karlovsky et al., 2016, CAST, 2003]. Optical color sorting is especially important for removing ergot sclerotia rye (purple-black color) from unaffected rye due to the high correlation between the presence of ergot sclerotia and the contamination of ergot alkaloids [Tittlemier et al., 2015]. It is important to note, however, that grain infected by some fungi may not always show visible symptoms, thus optical sensing methods may not reject the afflicted kernels, this can be overcome by using a series of sorting, washing/treating, and then re-sorting. This can be seen in peanut processing where electronic color sorting (ECS) is able to remove a high percentage (70%) of discolored peanuts (indicated by aflatoxigenic fungi colonization), however, when followed up by blanching (removal of seed coat from kernels) and subjected to ECS again a reduction of 91% for mean aflatoxin content has been shown [Dorner, 2008, Cole et al., 1995].

Washing grains or sieve cleaning (with various filter sizes) grains can help remove dust, broken kernels, and particulate matter which may be harboring fungal spores or mycotoxins. Washing with water has been shown to be somewhat effective for water-soluble mycotoxins such as DON, however, mycotoxins that are not water-soluble such as ZEA could be washed with the addition of sodium carbonate solutions [Karlovsky et al., 2016]. Therefore knowledge about a mycotoxins solubility and partition coefficient is necessary (See Figure 2.6). Dehulling (to remove the husk/hull) and pearling (to remove the bran of a grain) of grains are necessary pre-

requisites to milling/grinding. They can also effectively reduce mycotoxin content because fungi are relegated to the surface of the grain and mycotoxins are known to accumulate in the bran fraction [Karlovsky et al., 2016]. The removed husk and bran can be used in animal feed ingredients (if within accept limits for mycotoxin contamination) or in biofuels [CAST, 2003].

Thermal inactivation can occur during raw product processing, industrial commodity processing (i.e. biscuit, pasta, bread making), and at home in the kitchen when items are prepared. Theoretically, by the time any product (be it flour, uncooked pasta, rice, or bread) has been purchased by the consumer, it has undergone significant treatment and testing and levels are well below tolerable or suggested daily intakes, but even if not, the way people cook things may have some effect

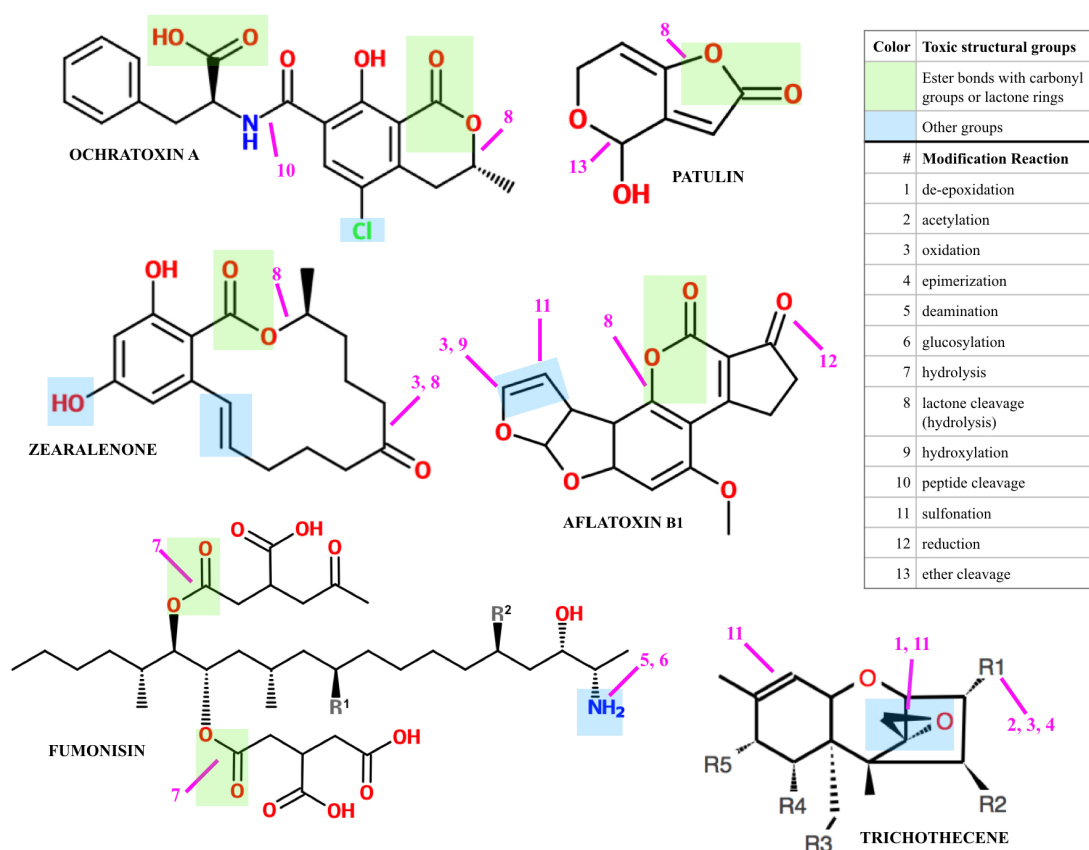


Figure 2.2: Toxic structural groups and possible modification reactions (adapted from [Karlovsky et al., 2016]).

on mycotoxin degradation. Thermal treatments, do have the potential to cause unwanted nutritional and quality effects and require a great deal of energy to be effective. There are many kinds of thermal treatments/cooking such as extrusion cooking (forces product through a mold and heats to a high degree), roasting, frying, baking, pasteurization, evaporation, etc. which all have varying degrees of effect depending on the type product being treated (raw or in a formula for something else), the duration of the method, the mycotoxin (ZEA and FB1 are more resistant to heat treatments), and other processing factors (i.e. pH of formula, other added ingredients) [Varga et al., 2010, CAST, 2003].

Irradiation can destroy mycotoxigenic fungi and mycotoxins with non-ionizing (photon energy below 10eV; solar, UV, microwave) or ionizing (photon energy greater than 10 eV, Co60, high energy electron beams) processes [Karlovsy et al., 2016, Freita-Silva et al., 2015, Calado et al., 2014]. These can be done as an industrial processing treatments and can occur naturally in the environment from solar radiation, for example the phototransformation of ZEA in natural waters [Emídio et al., 2017]. Radiation works by causing either direct damage to DNA or indirectly through generation of oxidative radicals through radiolysis/photolysis [Le Caër, 2011, Farkas, 2006, Emídio et al., 2017]. When water molecules are ionized and excited, they split into the positively charged radicals (H_2O^+) and the negatively charged free solvated electrons (e^-), these products (e^- , H^\bullet , HO^\bullet , HO_2^\bullet , OH^- , H_3O^+ , H_2 , and H_2O_2) are reactive with any substrate and can break down organic molecules [Le Caër, 2011, Emídio et al., 2017, Calado et al., 2014].

A review article by Calado et al., 2014 discusses several aspects of using irradiation for food processing and notes that many factors can influence its effectiveness for controlling fungi within a food matrix such as, “ i) the fungal strain (inherent ability to overcome the DNA damage) and load, ii) fungal form (spore are incredibly resistant to radiation) and age, iii) moisture of spores/ food matrix, iv) temperature

before and after treatment.” Adversely, for destroying mycotoxins they discuss, “i) type and concentration of mycotoxin, ii) presence of other chemical compounds, iii) formation of toxic degradation products,” [Calado et al., 2014].

Doses of irradiation in food should not exceed 10 kGy, which is sufficient for controlling fungal growth, however, in order to reduce a significant amount of mycotoxins amounts >10 kGy are used [Freita-Silva et al., 2015, Calado et al., 2014]. Part of the problem with using irradiation is that some units are very costly and consumers have a negative association with it, however, switching to alternatives like electron beams over gamma rays can reduce energy costs has a better reception [Freita-Silva et al., 2015, Calado et al., 2014]. Further more, any use of theses energy or radiation sources can be used to ionize gas and generate plasma under different temperatures and pressures. These plasma species (O, HO•, O₃, H₂O₂, and peroxyxynitrate) have high oxidative potential, thus Cold Plasma (CP) technology has the capacity to be an even greater alternative to radiation technology for fungal and mycotoxin control but requires more research and attention at this time [Hojnik et al., 2017, Pankaj et al., 2018].

Chemical

The use of chemical treatments such as acids, bases, and oxidizing/reducing agents is widely used and applied in conjunction with other physical treatments (thermal, irradiation, etc.), however, chemical residues may persist in the product and the environment. Acidic treatments (or additives) with a pH (< 3) that is less than the optimum for fungal growth (range 3 - 5) can be used as anti-fungal, antimicrobial, and preservation for food processing but very few have mycotoxin degradation abilities [CAST, 2003, Ray and Liewen, 2004]. Basic treatments with alkaline conditions pH (>10) such as ammoniation and nixtamalization (soaking/cooking in alkaline solution and hydrolyzes ester bonds), have been effective for some mycotoxins (AFB1 and FB1). Ammoniation is not recommended for detoxification of

OTA contaminated food/feed but it is allowed for aflatoxin contaminated products in several states such as Arizona, California, Texas, Alabama, and Georgia [Varga et al., 2010]. Alkaline conditions are very unstable for mycotoxins and can be the first step of degrading mycotoxins with lactone rings [Karlovsky et al., 2016, CAST, 2003]. The use of oxidizing agents such as ozone gas (O₃) or reducing agents like sodium bisulfite have also been shown to detoxify and degrade multiple mycotoxins [Karlovsky et al., 2016, CAST, 2003]. Most of these treatments are dependent upon the pH conditions, duration of the treatment, temperature, atmospheric conditions, and subsequent treatment/ processing conditions.

In addition to the use of herbs, spices and essential oils for their pleasing taste or smell, their phenolic antioxidants impart some antimicrobial/anti-fungal activity which helps with food preservation and acts akin to the natural defense response for the plant itself can have [Ray and Liewen, 2004]. Studies have shown that in excessive quantities, they can inhibit mold growth and mycotoxin production [Ray

Table 2.6: Physical and Chemical Decontamination: Mycotoxin levels reduced and experimentally demonstrated (green), mycotoxin level reduced partially/or effective at inhibiting fungi from producing mycotoxins (yellow), No effect on mycotoxins/Not compatible (red), not enough information (white) (adapted from [Karlovsky et al., 2016, CAST, 2003, Calado et al., 2014, Ray and Liewen, 2004]).

Treatment Method	Food Commodity	Aflatoxins	Ochratoxins	Fumonisin	Zearalenone	Trichothecenes	Ergot Alkaloids
Sorting	cereal, maize, nuts, fruit, cocoa, coffee	Green	Yellow	Green	Yellow	Green	Green
Sieve cleaning	cereal, maize	Yellow	Yellow	Yellow	Yellow	Green	Green
Washing	cereal, maize	Yellow	White	White	Red	Yellow	Yellow
Dehulling/pearling	cereals, maize, cocoa, coffee	Green	Yellow	White	Yellow	Green	Yellow
Steeping	cereal, maize	Yellow	Yellow	White	White	Green	Yellow
Milling	cereal, maize	Green	Green	Green	Green	Green	Yellow
Heat treatment	cereal, maize, nuts, fruit, cocoa, coffee	Green	Green	Green	Yellow	Yellow	Yellow
Irradiation	cereal, maize, nuts, fruit, cocoa, coffee	Green	Yellow	Yellow	Yellow	Yellow	Yellow
Cold plasma	cereal, maize, nuts, fruit, cocoa, coffee	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow
Acid treatment: Organic acid	cereals, maize, fruit	Yellow	Yellow	Yellow	Yellow	Yellow	White
Base treatment: Nixtamalization	cereals, maize, nuts, cocoa, coffee	Green	Yellow	Yellow	Green	Green	Yellow
Ammoniation	cereals, maize, nuts, cocoa, coffee	Green	Green	Red	Green	Yellow	White
Oxidizing Agent	cereals, maize, fruit, nuts, cocoa, coffee	Yellow	Red	Yellow	Yellow	Yellow	Yellow
Reducing Agent	cereals, maize, nuts, cocoa, coffee	Green	Red	Red	Red	Yellow	White

and Liewen, 2004]. El Khoury et al., 2017 investigated the ability of phenolic acid extracts and essential oils from several herbs and plant (bay leaves, cumin, fenugreek, melissa, mint, sage, anise, chamomile, fennel, rosemary, and thyme) to control *A. carbonarius* S402 growth and prevention of OTA production on synthetic grape medium (SGM) at 28°C for the duration of 4 days. None affected the growth, however, results indicated that essential oils were more potent than phenolic extracts on reducing the production of OTA [El Khoury et al., 2017].

Biological Transformation

Fermentation (could be considered chemical) using microorganisms have mechanisms that can reduce the level of mycotoxins in contaminated products. The use of yeast and lactic acid bacterial strains (LABs) has been used for thousands of years for the brewing of beer and wine or the production of yogurts and cheese, as well as ensilage for hay [CAST, 2003]. The fate of various mycotoxins during the brewing process has been reviewed by Inoue et al., 2013, showing that most mycotoxin levels are reduced from adsorption to yeast and then removal with the spent grain [Inoue et al., 2013]. The LABs, *Oenococcus oeni*, which is found on grapes and is involved in the malolactic fermentation for wine, is able to cellularly bind to OTA [Varga et al., 2010, Del Prete et al., 2007]. Processing of milk with AFM1 has various degrees of success, however, a study by Shigute & Washe 2018, investigated the effect of LAB species (from genre *Lactobacillus*, *Pediococcus*, *Enterococcus*, *Streptococcus*, and *Lactococcus*) on the reduction of AFM1 in the traditional Ethiopian production of the fermented milk, Ergo. These LABs are commonly isolated from the traditional smoked clay pot vessels used in this milk fermentation process. Their results demonstrated that LABs strains were able to reduce up to 57.33% of AFM1 [Shigute and Washe, 2018].

As mentioned earlier, there are several fungi and bacterium that can biodegrade mycotoxins as well as several other hazardous chemical compounds [Chanda

et al., 2016]. Before a mycotoxin biotransforming organism can be used commercially it must be found, characterized, and assessed. A recent review by Zhu et al., 2017, explained the necessary methodologies for development: i) identify environmental source/screen from identified groups, ii) identify the detoxification activity/biotransformation product, iii) safety assessment for functional strain and biotransformation product, iv) isolate, identify, clone/express enzymes responsible, and v) validate process and feasibility. Microbial organisms from fish, rumen, and chicken intestines as well as soil bacterium have been isolated and studied for their ability to detoxify or degrade certain mycotoxins [Zhu et al., 2016, He et al., 2016]. He et al., 2016 used a soil enrichment scheme to screen for aerobic bacteria capable of detoxifying DON and NIV. They found a *Desulfitobacterium* species that was capable of aerobic de-epoxidation of DON and NIV into dE-DON and dE-NIV, both compounds were assayed/ tested for toxicity and were found to be less toxic than their precursors [Pierron et al., 2016, Eriksen et al., 2004, He et al., 2016]. Ferenczi et al., 2014 screened *Cupriavidus basilensis* R16 through a genome project and found that it was able to cleave OTAs peptide bond and transform it into OT α and phenylalanine [Ferenczi et al., 2014]. They then conducted an *in vivo* analysis based on morphological alterations in kidney and spleen weight and changes in expression of OTA-affected genes in kidney tissue [Ferenczi et al., 2014]. For more information about mycotoxin degrading microorganisms please see additional review materials [Young et al., 2007, Zhu et al., 2017].

Feed additives: chemo-protection, binding agents/adsorbents, and Enzymes

Microorganisms also offer a potential mechanism for mycotoxin binding instead of degradation. The polysaccharides, proteins, and lipids that comprise cell walls have many functional groups and hydrophobic adsorption sites which may bind a wide variety of mycotoxins [Campagnollo et al., 2015]. This chemoprotection allows bi-

ological effects of mycotoxins to be modified through dietary components (i.e. the addition of folate to wheat), use of phytochemicals in the diet/ nutrient as mediated histone modifiers, and probiotics/pre-biotics [Gao and Tollefsbol, 2015, CAST, 2003].

Campagnollo et al., 2015 reported that beer fermentation residue (BFR) with *Saccharomyces cerevisiae* obtained from brewers spent grains (brewing byproduct) has been suggested for use to bind to mycotoxins in poultry and cattle feed. There was low binding ability to AFB1, OTA, or DON at pH 3.0 and 6.5, but had highest binding capacity for ZEA and was able to reduce bioavailability of ZEA in contaminated feedstuffs by more than 70% [Campagnollo et al., 2015]. Calcium montmorillonite clay and activated carbon have been shown to have success in binding to AFB1 and DON respectively and preventing them from being absorbed in the intestinal tract in several model animals studies [Devreese et al., 2014, Wang et al., 2008]. Enzymes that have been successfully isolated and identified from microorganisms can be purified and added to feed like the fumonisin carboxyl esterase FumD (FumDIFUMzyme) which degrades FB1 to HFB1 and has been shown in swine and Turkey [Masching et al., 2016]. The addition of *Bacillus subtilis* to laying hen feed is capable of counteracting adverse effects of mycotoxins on laying performance, im-

Table 2.7: Food/feed additives for potential animal species and their effect at protecting against mycotoxins.

Additive	Species	Mycotoxin	Mechanism/effect	Ref.
Activated Carbon	Pigs	DON	- Activated carbon prevented absorption of DON in intestinal tract	(Devreese et al., 2014)
Calcium montmorillonite clay (NovaSil)	Rats, Humans	AFB1	- Decreases uptake of AF in GI tract by binding to AF - Reduce bioavailability to blood, liver, etc.	(Wang et al., 2008)
Fumonisin carboxyl esterase FumD (FumDIFUMzyme)	Turkey, Swine	FB1	- Enzyme catalyzes hydrolysis of tricarballic acid side chains: FB1 degraded to HFB1	(Masching et al., 2016)
Beer Fermentation Residue (BFR) with <i>Saccharomyces cerevisiae</i>	Poultry, Swine	AFB1, ZEA, OTA, DON	- Bind to toxins; BFR had highest binding capacity for ZEA	(Campagnollo et al., 2015)
<i>Bacillus subtilis</i>	Laying Hens	AF and ZEA	- Spores of <i>B. subtilis</i> germinate in animals intestinal tract, and secrete enzymes which degrade toxin (prevent intestinal injury)	(Jia et al., 2016)
Commercial feed additive (CFA)	Rainbow trout	DON	- No significant effect detected in alleviating DON effects on growth or nutrient utilization	(Hooft et al., 2017)

prove egg quality, and reduce accumulation of AF residues in egg because spores of *B. subtilis* germinate in animals intestinal tract and secrete helpful enzymes that can degrade AF and ZEA [Jia et al., 2016]. This approach of using the organisms means you don't have to produce the enzyme. Although some feed additives have shown promising results in many birds and mammals, the feed additives designed/intended for these animal models may not be efficient in fish species. This is demonstrated by Hooft et al., 2017, where a commercial feed additive had no effect on alleviating the effects of DON on the growth of nutrient utilization in rainbow trout (*Oncorhynchus mykiss*) [Hooft and Bureau, 2017]. Additionally, mycotoxin binders and detoxifiers may interact with other nutritional components in food, thus limiting their efficiency and potentially causing other complications.

2.3 Summary

Farmers can protect their crops and financial future as well as ensure safe food/feed by following GAP, GSP, GHP, and GMP recombinations before, during, and after harvest. During the pre-harvest stage: i) conservation tillage systems for increased water and soil retention and diverse microbial activity, ii) crop rotation with non-/less-susceptible hosts to reduce continued transmission, iii) cultivar selection with breeding programs for resistant host phenotypes, iv) avoiding mono-culture crops by planting a variety, v) planting and harvesting on dates which adhere to recommendations to avoid unfavorable weather conditions during critical plant development times, vi) plan for optimal plant density which reduces plant stress from competition for resources over high yields, vii) appropriate timing for irrigation and fertilization (also the method and type), viii) chemical treatment for weeds, insects, and fungi with the correct type and quantity as prescribed by manufacturer and avoid prolonged exposure, ix) have an integrated plan that utilizes different methods from each part. During harvest and pre-harvest stages, good hygiene, storage, and manufacturing practices should be followed to prevent storage fungi from contaminating

products. Storage containers should be cleaned, maintained, and monitored. Different processing methods have a variety of results on mycotoxin control either by inhibiting fungal growth or production of toxins or by altering the chemical structure of the compound to reduce its toxicity.

There are many uncertainties with mycotoxin binders and detoxifiers at this time, and the adverse affects of there interactions with over feed components and nutrients is still under investigation. More evaluations on the efficiency of mycotoxin binders in ruminant, pig, and poultry and especially aquatic species is necessary [Hooft and Bureau, 2017].

More information is needed for use biological control agents (i.e resistant plant cultivars and non-toxigenic strain), specifically on the introduction of non-toxigenic strains to control toxin producing strains. Some research has suggested that under laboratory conditions, mating of two different species of *Aspergillus* resulted in hybrid progeny with a different mycotoxin profile [Olarde et al., 2015]. A deeper understanding of the genes and mechanism that produce mycotoxins can be achieved through transcriptomic analysis like DNA microarray and RNA-seq. Thus more transcriptionally active mycotoxin production pathways or detoxification pathways can be discovered and applied for prevention and control of mycotoxin contamination.

Chapter 3

Toxicity in Animals and Humans

Over the past several decades, there have been many *in vivo* and *in vitro* toxicity studies, as well as metabolic and kinetic studies, on several model species. This section will discuss the absorption, distribution, metabolism, and excretion processes of a few different mycotoxins briefly, as well as some of their well known effects in livestock animals, aquatic species, and humans. Additional focus will be placed on mycotoxin implications on intestinal health, immune response, and reproductive system dysfunction.

3.1 Absorption, Distribution, Metabolism, and Excretion

During absorption, a mycotoxin (or other contaminant) is transferred from the administration site into the circulation of the organism [World Health Organization, 2009]. Most human and animal exposure to mycotoxins occurs via food ingestion, however, animals and humans that come into close contact with grain dust may also be at risk of inhalation and dermal contact [Niculita-Hirzel et al., 2016]. Factors that will determine a mycotoxins absorption characteristics can be its ionization, molecular weight, and solubility. Mycotoxins and their metabolites can be partitioned into different body tissues depending on their chemical nature. Entry into specific tissues can be limited to either lipid soluble chemicals due to barriers with tight junctions between endothelial cells that prevent water soluble molecules from leaving the lumen of blood vessels, or by the selectivity of transporter proteins [Anzai et al., 2010, Pfohl-Leszkowicz and Manderville, 2007]. ABC transporters in the luminal membrane of intestines help to counteract absorption and increase excretion of xenobiotics and drugs [Anzai et al., 2010]. Biotransformation process structurally alters any administered mycotoxin so that it may be excreted and eliminated from the organism. Phase I metabolism systems can be microsomal (i.e cytochrome P450s, cyclooxygenases, epoxide hydrolase, etc.) or non-microsomal (i.e. alcohol/aldehyde

dehydrogenases, monoamine oxidases, and esterases) and can cause oxidation, reduction, hydroxylation, hydrolysis, and epoxidation reactions. This process acts to convert the toxin into more polar metabolite by either adding a functional group (-OH, -NH₂, -COOH) or by un-masking them; mycotoxins may be inactivated but some are activated (i.e. AFB1 and OTA). Many mycotoxins have been shown to occur in food and feed in their metabolized fungal and plant derivatives, these can then be activated by the consuming organisms system [Berthiller et al., 2013, Maresca, 2013]. Phase II metabolism acts to further increase a mycotoxins solubility, through conjugation reactions and covalent binding of glucuronide, glucoside, sulfate, glutathione, and amino acid, or with acetylation and methylation reactions. If ingested, the mycotoxins undergo metabolism by enzymes and microflora in the intestine first, however, the liver is the major site for metabolism [Sousa et al., 2008]. Biotransformation can also occur in the cells of the kidneys, lungs, plasma, testes, placenta, adrenal glands, and even the brain. Mycotoxins can be excreted via renal (urine) and biliary (fecal) secretions and through milk from lactational transfer. Compounds can also be passively reabsorbed in the distal and renal tubules leading to enterohepatic circulation. This section will be broadly discussed to give an idea about how mycotoxins are absorbed, distributed, transformed, and excreted. Should you wish to read about these processes with more specific criteria about clearance, elimination, and excretion rates please refer to reviews and articles cited according to the mycotoxin.

3.1.1 AFB1

Aflatoxins are rapidly absorbed from the small intestines by passive diffusion (duodenum is major site of absorption); AB1 and AFB2 are more rapidly absorbed than AFG1 and AFG2 [Kensler et al., 2010, CAST, 2003, Wen et al., 2016]. After AFB1 is absorbed, it is non covalently bound to albumin and can be transported throughout the body to other tissues. AFB1 can be hydroxylated by CYP enzymes into

Table 3.1: ADME for various mycotoxins.

	AFB1	OTA	FB1	ZEA	DON
Bioavailable (%)	chicken: >80 pig: >80	fish: 1.6 chicken: 40 pig: 66 human: 93	chicken: 1 pigs: 3 - 6 rats: 3.5	pig: 80 - 85 rat: 1.1 - 2.7	chicken: 20 pigs: 55 - 80 rats: 25 ruminants: 7
Absorption Mechanism	passive transport	passive transport by diffusion	interaction with bile salts	passive transport by diffusion	passive transport (paracellular pathway)
Renal Reabsorption/ Enterohepatic Circulation	-/Y	Y/Y	-/Y	-/Y	-/Y
Comparative Toxicity	AFB1>(AFM1,AFG1)>(AFB2,AFG2)	OTA>OTC>OTB>OT α	FB1>FB2>FB3	α -ZEL > α -ZAL > ZEA = ZAN = β -ZAL > β -ZEL	DON=3ADON>15ADON
LD ₅₀ range (mg/kg bw)	trout: 3.0 poultry: 3.3 pig: 1.0 rat: 20 - 30 mouse: 48 - 58 sheep: 2.0 dog: 0.2	carp: 0.08 trout: 0.5 - 1.0 poultry: 0.025 - 2.0 pig: 0.6 rat: 1.0 - 7.4 mouse: 9.0 dog: 0.5	nile tilapia: 0.6 horse: 0.15 - 0.22	rat: 4,000 - 10,000 mouse: 2,000 - 20,000	rat: 7.3 mouse: 46 - 48

AFM1, AFQ1, AFP1 and then excreted into urine or milk [CAST, 2003]. Alternatively, various liver CYP450 can transform AFB1 into AFB1-8,9-epoxide which can then proceed to i) react with DNA or RNA to form AFB1-adducts (i.e. AFB1-N7-Guanine) that can either be repaired or will undergo G-T transversion that will become a fixed mutation after two rounds of DNA synthesis leading to mutagenicity and carcinogenicity (this has been shown in codon 249 of the p53 tumor suppressor in human liver), ii) undergo hydrolysis by microsomal epoxide hydrolase enzymes to AFB1-8,9-dihydrodiol then bind to albumin to form AFB1-albumin products, or iv) conjugated by phase II glutathione-s-transferase (GST) and excreted in urine [McGlynn et al., 1995, Wen et al., 2016]. Additionally, Guindon-Kezis et al., 2014 showed

Table 3.2: Adverse effects on cells. Mycotoxins can cause effect (green), unknown (white). Adapted from [Wen et al., 2016].

	AFB1	OTA	FB1	ZEA	DON	T-2	CIT	PAT
Oxidative Stress								
Immune Suppression								
Apoptosis								
Cell death/Necrosis								
Lipid peroxidation								
DNA damage								
Cell cycle arrest								

Table 3.3: IARC classified groups of carcinogens.

Mycotoxin	Group	Classification	Ref.
AFB1	1	Carcinogenic to humans	(IARC 1993)
AFM1	2B	Possibly carcinogenic to humans	(IARC 2002)
OTA	2B	Possibly carcinogenic to humans	(IARC 1993)
FB1	2B	Possibly carcinogenic to humans	(IARC 2002)
ZEA	3	Not classifiable as to its carcinogenicity to humans	(IARC 1993)
DON, T-2	3	Not classifiable as to its carcinogenicity to humans	(IARC 1993)
CIT	3	Not classifiable as to its carcinogenicity to humans	(IARC 1987)
PAT	3	Not classifiable as to its carcinogenicity to humans	(IARC 1987)

that treatment with AFB1 on female A/J mice of 50 mg/kg AFB1 (i.p.) resulted in oxidative stress in the liver and lungs [Guindon-Kezis et al., 2014]. The increased levels of DNA oxidation products (8-hydroxy-2-deoxyguanosine (8-OHdG) and 5-hydroxy-2'-deoxycytidine (5-OHdC)), increased immunoreactive protein 8-oxoguanine glycosylase (OGG1), and increased base excision repair. Thus the oxidative DNA damage might lead to carcinogenicity [Guindon-Kezis et al., 2014]. Aflatoxins have the ability to decrease protein synthesis, disrupt lipid metabolism, and interfere with mitochondrial respiration [Wen et al., 2016]. AFB1 is a group 1 carcinogen and its hydroxylated metabolite AFM1 is a group 2B carcinogen [Kensler et al., 2010, CAST, 2003, Wen et al., 2016]. Anorexia, malaise, vomiting, and jaundice are a few of the early symptoms associated with aflatoxicosis and hepatotoxicity [CAST, 2003, Ngindu et al., 1982, Azziz-Baumgartner et al., 2005]. There have been many outbreaks of aflatoxicosis, the most recent was in 2004 [Onsongo, 2004]. In the wake of a food shortage, eastern and central provinces of Kenya experienced a severe aflatoxicosis outbreak, in which 317 cases of acute hepatic failure and 125 deaths during a time period in which maize samples reached AFB1 levels of 4,400 - 8,000 ppb [Onsongo, 2004, Azziz-Baumgartner et al., 2005]. A case-study conducted in the area by Azziz-Baumgartner et al., 2005 collected home grown maize samples for AFB1 concentration as well serum albumin from participants for AFB1-lysine

adducts and positive hepatitis B surface antigen (HBsAg-positive). Serum albumin with AFB1-lysine adducts at or above 0.25 ng/mg was and HBsAg-positive is considered a risk factor for aflatoxicosis and hepatocellular carcinoma [CAST, 2003, Wen et al., 2016, Kensler et al., 2010]. Chronic exposure to lower levels of AFB1 cause reduced weight gain, low feed conversion, immunosuppression, carcinogenesis (liver cancer), and encephalopathy [Kensler et al., 2010, CAST, 2003, Wen et al., 2016].

OTA

The amount of OTA that is absorbed depends on the species and ranges from 1.6% in fish, 40% in chickens to 66% in pigs and 93% in humans [Ringot et al., 2006, Kőszegi and Poór, 2016, Bernhoft et al., 2017]. The non-ionized (OTA^0) and monoanion (OTA^-) forms of OTA are passively absorbed in the stomach and proximal jejunum [Ringot et al., 2006]. No specific transport mechanisms have not been implicated in the transport of OTA across intestinal epithelial cells, therefore, it has been suggested to be a result of simple diffusion [Berger et al., 2003, Ringot et al., 2006]. OTA absorption is more efficient at a pH closest to 6 (apical) than a pH 7.4 (basolateral), showing that the apical to basolateral passage of OTA is more common [Maresca et al., 2001, Berger et al., 2003]. It has been suggested that intestinal absorption may be limited by the efflux of the toxin by transporter proteins which counteracts absorption, adds a protective tissue barrier, and acts in the elimination [Pfohl-Leszkowicz and Manderville, 2007]. The multi drug resistance-associated protein ATP-Binding Cassette C2 and breast cancer resistance-associated protein (BCRP) have been implicated in this process [Berger et al., 2003]. The intestinal microflora in ruminants and rodents has been shown to hydrolyze OTA into $\text{OT}\alpha$ (less toxic metabolite), which has been found in urine [Heussner and Bingle, 2015]. OTA is distributed to other tissues and organs through the portal system. OTA binds to serum proteins (albumin) in the blood stream; 99.8% of OTA found is albumin bound [Pfohl-Leszkowicz and Manderville, 2007]. The dianionic

form of OTA is bound to human serum albumin and does not readily pass through membranes [Pfohl-Leszkowicz and Manderville, 2007]. Various CYP450 enzymes or peroxidase can remove the chlorine ion from OTA and transform it into OTB or aryl radicals, phenoxyl, and benzoquinone intermediate that can then go on to form DNA adducts [Kőszegi and Poór, 2016]. Organs that have been found to accumulate OTA include kidneys, liver, skeletal muscle, fat tissue, brain, and testes [Kőszegi and Poór, 2016]. An *in vivo* study conducted by Hallén et al., 1998 was able to show placental and lactational transfer of OTA in rats [Hallén et al., 1998]. OTA and metabolites can be eliminated via renal excretion route, however, glomerular filtration is limited due to the high binding affinity of OTA to plasma proteins, which increases its ionization and decreases its lipid solubility [Ringot et al., 2006]. Instead, carrier-mediated transport across tubular membranes by transporter proteins (i.e. organic anion transporters OAT) eliminate OTA into urine [Pfohl-Leszkowicz and Manderville, 2007]. However, OATs have a dual nature, in which they mediate the efflux of OTA as well as its reabsorption [Anzai et al., 2010, Pfohl-Leszkowicz and Manderville, 2007]. The reabsorption of the secreted OTA occurs in the nephron segments (proximal tubules, distal tubules, and collecting ducts) by similar organic anion transport systems. The constant reabsorption leads to accumulation of OTA in renal tissue and may be an integral aspect to the nephrotoxicity of OTA [Anzai et al., 2010, Pfohl-Leszkowicz and Manderville, 2007]. OTA and its metabolite OT α can be eliminated in feces through biliary excretion. However, after the conjugated OTA compounds are secreted in the intestines they can be hydrolyzed by microflora, which allows the released OTA to participate in the enterohepatic circulation [Anzai et al., 2010, Ringot et al., 2006, Pfohl-Leszkowicz and Manderville, 2007]. Breast milk (pH 6.5) is more acidic than plasma, therefore xenobiotics can diffuse into the mammary glands. Many *in vivo* studies have described the lactational transfer of OTA in breast milk in animals and humans [Hallén et al., 1998, Kőszegi and Poór,

2016]. A study by Skaug et al., 2001 found a correlation between dietary intake of OTA and its contamination in human breast milk [Skaug et al., 2001].

OTA has been shown to be genotoxic, cytotoxic, carcinogenic, nephrotoxic (kidney is a major target organ), immunotoxic, neurotoxic, embryotoxic, and teratogenic in animal models and human cell lines [Patil et al., 2006, Ringot et al., 2006, Pfohl-Leszkowicz and Manderville, 2007, Kőszegi and Poór, 2016]. Ochratoxin a is classified as a group 2B carcinogen, showing that it is a possible human carcinogen based on several animal carcinogenicity studies in mice and poultry [Ostry et al., 2017]. OTA has both non-genotoxic mode of action and a direct genotoxic mode of action [Kőszegi and Poór, 2016, Wen et al., 2016]. OTA can i) inhibit protein synthesis, ii) disrupt phenylalanine metabolism leading to reduction in gluconeogenesis, iii) induce cell apoptosis, iv) produce reactive oxygen species (ROS) causing lipid peroxidation and cell membrane damage, v) interrupt hormonal calcium signaling and homeostasis, vi) cause DNA damage [Benesic et al., 2000, Stoev et al., 2001, Wen et al., 2016]. Since the 1970s, OTA has been regarded as causative factor in porcine nephropathy in Denmark, and since then has also been associated with human diseases from epidemiological studies like Balkan Endemic Nephropathy (BEN) and Chronic Interstitial Nephropathy (CIN) and urinary tract tumors (UTT) in Tunisia [Petkova-Bocharova et al., 1988, Stoev et al., 2001, Kőszegi and Poór, 2016, Ringot et al., 2006].

3.1.2 FB1

Absorption of fumonisins are relatively low, and in most species the bioavailability is less than 6% [Klarić et al., 2013, Voss et al., 2007]. Because of its low absorption rate, it has been suggested that enterohepatic cycling contributes to its intestinal absorption as well as incorporation into mixed micelles from cholesterol or bile salts [Grenier and Applegate, 2013]. Fumonisins are distributed throughout various tissues, however, they have species-specific target organs such as the brain in

horses or the lungs in pigs [Voss et al., 2007]. Information on biotransformation of fumonisins is relatively limited. However, Spotti et al., 2000 found that FB1 was able to alter CYP450 with *in vivo* and *in vitro* studies by inhibiting ceramide synthase [Spotti et al., 2000]. In addition to the distribution of the toxin, elevated levels of Sa and So can be found throughout the body [Voss et al., 2007, Voss and Riley, 2013]. It also interferes with lipid metabolism and lipid-dependent signaling pathways [Voss and Riley, 2013]. FB1 can be partially or fully hydrolyzed (losing one or both tricarballic acid side chains) in the gut by microflora [Voss et al., 2007].

The IARC classifies FB1 as a group 2B carcinogen [IARC, 1993]. FB1 has a non-genotoxic mode of action via sphingolipid metabolism disruption, DNA could be damaged when lipid metabolism is disrupted from the inhibition of ceramide synthase. Additionally, FB1 can induce global DNA hypomethylation which can affect the balance of DNA methylates/demethylases, and potentially cause genome instability and tumorigenesis [Wen et al., 2016].

Well known animal diseases attributed to FB1 exposure are porcine pulmonary edema (PPE), and equine leukoencephalomalacia (ELEM) [Smith et al., 2002, Gumprecht et al., 1998]. In humans, fumonisin exposure is associated with neural tube defects (NTDs) seen in Mexico-Texas, esophageal cancer in China, and decreased growth [CAST, 2003, Voss et al., 2007, Liew and Mohd-Redzwan, 2018].

3.1.3 ZEA

ZEA is readily absorbed after oral administration in the intestines. Several *in vivo* studies have been conducted on animal models to investigate ZEA absorption [Zinedine et al., 2007, Metzler et al., 2010, Fleck et al., 2016]. In rats, Ramos et al., 1996 used an *in situ* technique to find that ZEA was absorbed in the intestines by passive diffusion [Ramos et al., 1996]. Using cultured Caco-2 cells, Press & Di Grandi 2008, found that the permeability coefficient of ZEA and α -ZAL correlated with human absorption *in vivo* and are consistent with intestinal absorption of ZEA

and α -ZAL [Press and Di Grandi, 2008]. ZEA is distributed in different tissues (kidney, liver, adipose) and estrogen target tissues (uterus, testes and ovarian follicles) [Zinedine et al., 2007, Metzler et al., 2010]. The placenta has various CYP enzymes, reductases, and transferases and their expression and level is dependent on the stage of placental development [Pasanen, 1999, Partanen et al., 2009]. Placental transfer of ZEA and α -ZAL has been shown in rats and rabbits [Bernhoft et al., 2001, Lange et al., 2002]. Two well supported biotransformation pathways have been suggested by Olsen et al for ZEA in animals: i) enzymatic reduction via hydroxylation by hydroxysteroid dehydrogenase (HSDs) to α -ZOL and β -ZOL (Phase I), and ii) is a uridine diphosphate-glucuronosyltransferase (UGT) dependent conjugation of ZEA reduced with glucuronic acid (Phase II) [Olsen et al., 1981, Metzler et al., 2010]. Other animal studies have also suggested reduction of the keto group by hydroxysteroid dehydrogenases (HSD) to α -/ β -ZAL, cytochrome P450-catalyzed hydroxylation on aromatic and aliphatic positions, and Phase II conjugation to sulfates by sulfotransferases (SULT) [Metzler et al., 2010]. Pfeiffer et al., 2009 used rat and human hepatic microsomes to show that two of the major oxidative metabolites are the highly unstable catechols 13-hydroxy-ZEA and 15-hydroxy-ZEA hydroxylation of ZEA [Pfeiffer et al., 2009]. These catechol metabolites occur through aromatic hydroxylation and can be substrates for catechol-O-methyl transferase.

Note that it is important to consider the animal used in the study because there is some discussion and evidence showing different hepatic biotransformation of ZEA in different species [Malekinejad et al., 2006]. For example, pigs predominantly convert ZEA to α -ZOL while β -ZOL is the dominant hepatic metabolite in ruminants [Malekinejad et al., 2006, Zinedine et al., 2007]. Recently an *in vivo* study conducted by Fleck et al., 2017 used 2 month old female pigs as a sensitive model system for oral and intravenous dosing to further understand ZEA metabolism and competitive pathways [Fleck et al., 2016]. Their findings supported previous litera-

ture that Phase II conjugation and keto-reduction to α - and β -ZOL were the primary reactions observed. However, they found no LC/MS/MS evidence to support CYP-mediated hydroxylation reactions of aromatic to form catechols and methylcatechols after catechol-O-methyltransferase-mediated methylation [Pfeiffer et al., 2009, Fleck et al., 2016]. The ZEA glucuronide is excreted from the bile and then re-absorbed and metabolized again by intestinal mucosal cells leading to distribution in the liver. Urinary excretion occurs in conjugated and unconjugated forms and reductive metabolites. In humans, excretion via urine has been found to account for approximately 10% of the administered dose, with glucuronides of ZEA and α -ZOL as the main metabolites and minor amounts of β -ZOL [Metzler et al., 2010, Zinedine et al., 2007]. There is lactational transfer of ZEA in milk, however, it has a low transmission rate [Metzler et al., 2010]. ZEA and its metabolites competitively binds to estrogen receptors, is genotoxic, and may cause reproductive issues in a variety of animals [Metzler et al., 2010, Zheng et al., 2018]. *In vitro* studies with different cell lines (i.e. MCF-7, HEK293) have shown that the estrogen mimicking effects of ZEA at low doses stimulates cell proliferation and anti-apoptotic effects (carcinogenesis) while high doses of ZEA result in cell death from oxidative stress, DNA damage, mitochondrial damage, cell cycle arrest and apoptosis [Yu et al., 2005, Zheng et al., 2018].

3.1.4 DON

Bioavailability of DON has high variability amongst species due in part to the chemical and physical parameters of the GI tract; intestinal absorption and metabolism of ingested xenobiotics (DON) is heavily dependent on the ability of these bacteria to metabolize them [Maresca, 2013]. In ruminants (7%) DON is the least bioavailable, followed by chicken (20%) and rats (25%) and pigs (55-80%) [Goyarts and Dänicke, 2006, Grenier and Applegate, 2013, Bernhoft et al., 2017]. Mono gastric organisms have high bacterial content in the colon while polygastric animals (ru-

minants) have high bacterial content before and after the small intestines, this will determine where along the GI tract the mycotoxin will be metabolized, absorbed, or excreted [Maresca, 2013]. There have not been any reports on Phase I metabolism reactions involving DON, however, microflora have been shown to transform DON into de-epoxy DON (DOM-1) in the digestive tract [Maresca, 2013, Cirlini et al., 2014]. Various Phase II reactions have been shown with species specific site modifications [Goyarts and Dänicke, 2006]. Several toxicokinetic studies of radio-labeled DON in animals has shown tissue distribution was shown in all organs including the brain, reproductive organs, and fat tissue [Sobrova et al., 2010, Amuzie and Pestka, 2009b, Tola et al., 2015]. A more polar compound (like D3G) is less absorbed in the small intestines compared to parent [Maresca, 2013, Cirlini et al., 2014]. DON is able to pass through biological barriers and can affect cellular function and viability [Maresca, 2013]. The IARC classifies DON as a group 3 compound, meaning that its carcinogenic information in animals and humans is limited or inadequate [Sobrova et al., 2010]. Acute doses at low levels have been shown to cause nausea, diarrhea, and emesis while prolonged chronic exposure decreases weight gain, causes anorexia, decreases nutritional absorption, and increases immune dysfunction [Amuzie and Pestka, 2009b].

3.2 Endocrine Disrupting Chemicals & Effects on Endocrine/Neuroendocrine Systems

The endocrine system is an intricate network of glands that secrete various hormones and is controlled by the hypothalamus which is the base of operations for the neuroendocrine system by sending out neurotransmitters and by receiving target hormone feed-back information. Together, these neuroendocrine and endocrine pathways are responsible for orchestrating necessary life processes for the body such as the regulation of growth, metabolism, and reproductive function. There is a broad range of mechanisms for EDCs such as nuclear hormone receptor binding

to stimulate/antagonize, interference with enzymes involved in steroid biosynthesis, hormone degradation/elimination, and confound communication/signaling systems [Diamanti-Kandarakis et al., 2009, Gore, 2010]. Continued research has discovered more interactions for EDCs involving non-nuclear steroid hormone receptors and orphan receptors [Diamanti-Kandarakis et al., 2009]. This interconnected system relies on properly functioning components and clear communication, so imbalance in the level of hormones or disruption in this system to result in disease (i.e. diabetes, obesity), adverse reproductive affects, altered behavior, and disruption of homeostasis could also inhibit appropriate response to environmental stimuli [Gore, 2010].

Humans and animals can be exposed to EDCs though different routes - consumption, inhalation, indirect exposure via contaminated tissue and excretions, and through contaminated water [Diamanti-Kandarakis et al., 2009]. There are several factors that must be considered in order to understand both the chemical and the effect on the biological system. A review article by Diamanti-Kadarakis et al., 2009 lists a few of these critical factors that must be considered when understanding the nature and effect of EDCs: “i) age : there are different effects and consequences of exposure to a developing fetus or infant to that of matured adult, ii) sex : EDCs may have sexually dimorphic effects on the organisms reproductive system, iii) latency form exposure : there may be lag time between exposure and appearance of the disease/disorder, iv) mixture : individuals/populations are exposed to many compounds which may have synergistic or additive effects on each other, v) nontraditional dose-response dynamics: any level of exposure to EDCs can cause endocrine or reproductive abnormalities and may exert non-traditional dose-response curves (inverted U or U-shaped curves) , vi) transgenerational, epigenetic effects: effects might be transmitted by DNA methylation or histone acetylation which could modify factors regulating gene expression” [Diamanti-Kandarakis et al., 2009].

3.3 Gut Health, Liver Metabolism, and Inflammatory Processes

In many ways, the intestinal epithelium is the first target for mycotoxins and the first selective barrier against them [Grenier and Applegate, 2013, Liew and Mohd-Redzwan, 2018]. A healthy gut in animals and humans helps to determine the overall health of the host. There are many things that can be affected in the GI tract (the largest endocrine organ in the body) which cause overwhelming impacts for the rest of the body: such as alterations in enzyme activity, nutrient uptake, digestive microflora interaction, epithelial barrier integrity, mucosal immunity and more [Suzuki, 2013]. The transcellular pathway (transporters or channels on basolateral or apical membrane absorb and transport nutrients, amino acid, peptide, minerals, and vitamins) and the paracellular pathway (movement through intracellular space between cells) help create selective permeability [Suzuki, 2013]. The mucosal barrier acts as a selective barrier because it can regulate the paracellular permeability through its tight junction protein structures (i.e. claudins (CLDN), occludins (OCLN), cadherins, junction adhesion molecules (JAM), etc.) [Suzuki, 2013]. When the barrier function of the intestine is impaired this can mean increased permeability or translocation of dietary antigens, bacterial pathogens, or other harmful compounds (mycotoxins) which can then go on to over-activate the immune system or other cellular damage [Akbari et al., 2017, Suzuki, 2013]. This also means decreased nutrient absorption, which in the case of damage is in high demand [Grenier and Applegate, 2013].

It has been reported that approximately 70% of immune defenses are located within the GIT [Grenier and Applegate, 2013]. Intestinal epithelial cells and mucus themselves are apart of the guts innate immune system. An immune response can be induced by immunocomponent cells that are located on gut associated lymphoid tissues (GALT) such as Peyer's patch and, mesenteric lymph nodes [Grenier and Applegate, 2013]. Throughout the intestinal lymphoid tissue, dendritic cells (DCs) act

as a link between the innate and adaptive immune system by receiving, processing, and distributing antigens to T-cells. The two effector sites, the epithelial layer and the lamina propria (LP) have a range of different immune cells. Those within the LP are macrophages, plasma cells, memory B-/T-cells, mast cells, cytotoxic natural killer (NK) cells, and innate lymphoid cells [Grenier and Applegate, 2013, Maresca, 2013]. The innate lymphoid cells and other immune cells can produce cytokines which are small peptide proteins that act as signals for cellular communication thus stimulating the proliferation of antigen specific effector cells and regulating both local and systemic inflammatory pathways [Grenier and Applegate, 2013, Maresca, 2013]. In low doses, mycotoxins can stimulate immune response and induce CYP enzymes, while higher doses suppress immune system and the increased accumulation of toxins can lead to increased oxidative stress [Wen et al., 2016].

Experiments that show adverse effects on gut health can be seen with intestinal barrier breakdown through: i) decrease in transepithelial electrical resistance

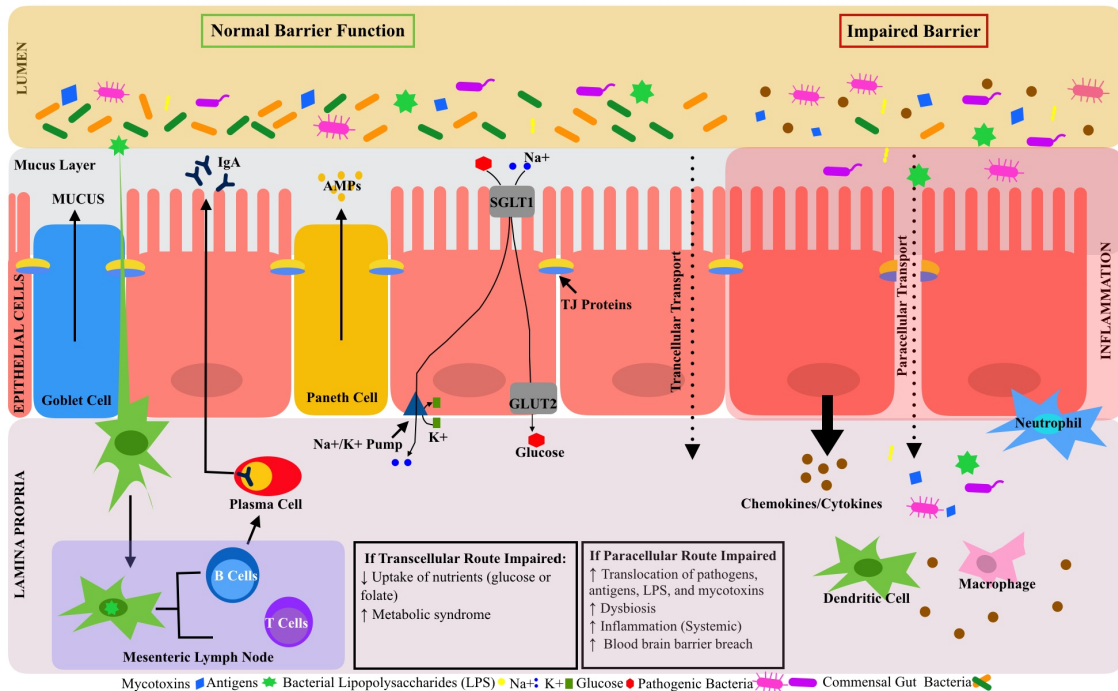


Figure 3.1: Normal vs. impaired barrier function (adapted and modified from [Grenier and Applegate, 2013, Akbari et al., 2017]).

(TEER) values, ii) increase in paracellular transport of a chosen compound/bacteria (i.e. dextran, antibiotics), and iii) modulation of the expression of TJ proteins [Akbari et al., 2017, Gao et al., 2017]. A decrease in TEER could be caused by an alteration in paracellular permeability or from differences in transcellular ion flux with alterations in plasma membrane channels and pumps [Akbari et al., 2017]. To further confirm the permeability tagged paracellular markers can be used [Akbari et al., 2017]. Alterations in expression and morphology of TJ proteins could be from the disruption of protein synthesis, oxidative stress, and the activation of MAPK pathways [Grenier and Applegate, 2013]. Additionally, studies have shown that co-contamination of mycotoxins will have greater impairment on barrier integrity [Gao et al., 2017, Bracarense et al., 2012].

Maresca et al., 2001 showed that Caco-2 (human epithelial colorectal adenocarcinoma) cells and HT-29-D4 (human adenocarcinoma) cells exposed to OTA had disrupted barrier function with decreased TEER values [Maresca et al., 2001]. A subsequent study with Caco-2 cells exposed to OTA up to 100 M had decreased TEER value along with increased permeability of the paracellular markers horse radish peroxidase (HRP 44kDa) and 4 kDa fluorescein isothiocyanate compound dextran (FITC-dextran), and increased translocation of *Escherichia coli* at concentrations beginning at 1 M [Maresca et al., 2008]. Marin et al., 2015 exposed IPEC-1 cells to ZEA and α -/ β -ZOL for 10 days at 25-50 M; the parent compound ZEA has no effect on TEER values, however, α -/ β -ZOL did induce decreases in TEER values [Marin et al., 2015]. Additionally, an *in vivo* study Liu et al., 2014 gave rats 0.3-146 mg ZEA /kg bw for 7 days and found it decreased the transcription levels of CLDN4 and OCLN transcription proteins [Liu et al., 2014]. Goossens et al., 2012 showed the T-2 decreased TEER values and increases permeability to doxycycline [Goossens et al., 2012]. Broiler chickens exposed to AFB1 (1.5 mg/kg bw) for 20 days showed increased transcription levels of CLDN1 and CLDN2 and increased in

plasma lactulose and rhamnose ratio with growth inhibitory effects on proliferating cells by altering the intestinal barrier and selected transport functions [Chen et al., 2016]. Loiseau et al., 2007 exposed IPEC-1 cells to 50-200 M of FB1 which resulted in decreased TEER values and increased permeability to FB1 [Loiseau et al., 2007]. Additionally, FB1 (0.5 mg/kg bw) given to piglets for 7 days significantly increased the translocation of *Escherichia coli* [Oswald et al., 2003]. Most food/feed are contaminated by more than one mycotoxin as well as the presence of masked mycotoxin, therefore, it is especially important to understand the combined effects of mycotoxins together [Grenier and Applegate, 2013, Pinton et al., 2012]. The effect of DON on the intestinal barrier has been shown in IPEC-J2 (porcine jejunal enterocytes) by Vandenbroucke et al., 2011 to increase translocation of pathogenic *Salmonella typhimurium* [Vandenbroucke et al., 2011]. Pinton et al., 2012 showed that 15ADON had greater toxicity than DON and 3ADON; all were able to decrease TEER values, increase permeability, and decrease protein expression of CLDN3 and CLDN4 [Pinton et al., 2012]. Bracarense et al., 2012 showed that exposure of 5 week old piglets to both DON + FB1 had various interactions (synergistic/additive/antagonistic) depending on the test parameter but in general significantly induces morphological and histological changes that suggested FB1 in the presence of DON could be absorbed more through intestinal barrier disruption [Bracarense et al., 2012]. Gao et al., 2017 showed that both AFM1 and OTA alone and combined can affect intestinal epithelial cells, however, when combined they have an additive/synergistic effect on barrier function [Gao et al., 2017].

Some mycotoxins can disrupt nutrient absorption directly, or indirectly. Stevens & Tang 1997 treated Caco-2 cells with FB1 which showed inhibited receptor-mediated uptake of folate by 50% [Stevens and Tang, 1997]. Other mycotoxins (i.e. DON and T-2) have been shown to interfere with glucose absorption by suppressing the expression of important transport proteins like the sodium glucose transport protein

1 (SGLT) and glucose transporter 2 (GLUT2) [Maresca et al., 2002, Maresca, 2013]. Also, morphological alterations to micro villi as well as decreased mucosal production can also interrupt nutrient absorption and cause inflammatory effects [Grenier and Applegate, 2013].

The liver plays an important role in metabolism (i.e. lipid, cholesterol, amino acid), nutrient and glycogen storage, and biotransformation/detoxification and removal of toxins, production of various proteins (i.e. albumin, clotting factor, transport, antimicrobial, lipoproteins), and immunologic response activity. Approximately 80% of hepatic blood supply comes from the portal vein after it picks up various dietary antigens, molecules from gut microflora, mycotoxins, cellular signaling molecules etc. The liver undergoes constant and highly regulated inflammation and resolution: i) hepatocytes detect cytokines from the blood stream and initiate

Table 3.4: Mycotoxin effects on intestinal barrier functions.

Mycotoxin	Species/Cell	Dose	Duration	Effect	Ref.
OTA	Caco-2-14 HT-29-D4	0.1-100 μ M	48 hrs	- decreased TEER	(Maresca et al., 2001)
OTA	Caco-2 cells	1-100 μ M	12 hrs	- decreased TEER values - increased permeability of HRP and FITC-dextran - increased translocation of <i>E. coli</i>	(Maresca et al., 2008)
ZEA α-β-ZOL	IPEC-1	25-50 μ M	10 days	- no effect on TEER values (ZEA) - decreased TEER values (α - β -ZOL)	(Marin et al., 2015)
ZEA	Rat	0.3-146 mg/kg bw	7 days	- decreased transcription levels of CLDN4 and OCLN	(Liu et al., 2014)
DON 3ADON 15ADON	IPEC-1	10-30 μ M	24-48 hrs	- decrease in TEER values - increased permeability of 4kDa FITC-dextran (15ADON>DON>3ADON) - decreased protein expression of CLDN3 and CLDN4	(Pinton et al., 2012)
DON	IPEC-J2	0.33-3.3 μ M	24 hrs	- increased translocation of pathogenic <i>Salmonella typhimurium</i> (strain 112910a)	(Vandenbroucke et al., 2011)
T-2	IPEC-J2	21 nM	-	- decrease in TEER values - increase in doxycycline permeability	(Goossens et al., 2012)
AFB1	Caco-2	1-100 μ M	7 days	- decrease in TEER values - decreased transcription levels of CLDN3 and OCLN	(Romero et al., 2016)
AFB1	Broiler chicken	1.5 mg/kg bw	20 days	- increase in transcription levels of CLDN1 and CLDN2 - increase in plasma lactulose and rhamnose ratio	(Chen et al., 2016)
FB1	IPEC-1	50-200 μ M	7 days	- decreased TEER values - increased permeability of FB1	(Loiseau et al., 2007)
FB1	piglet	0.5 mg/kg bw	7 days	- increase in translocation of pathogenic <i>E. coli</i>	(Oswald et al., 2003)
DON FB1	piglet	DON (3 mg/kg bw), FB1 (6 mg/kg bw), DON+FB1	5 weeks	- RT-PCR showed up regulation of cytokines (TNF- α , IL-1 β , IFN- γ , IL-6, and IL-10) - decreased expression of E-cadherin and OCLN - combined effects showed synergistic effect on immune cells, additive effect on cytokines and junction protein expression and histological lesion, and antagonistic effects of expression of immune cells and cytokines	(Bracarense et al., 2012)
AFM1 OTA	Caco 2	0.12, 12 μ M (AFM1) 0.2 and 20 μ M (OTA)	48 hrs	- decreased TEER - increased epithelial permeability - combined effects showed decreased expression of tight junction proteins (CLDN3, CLDN4, OCLN, ZO-1) and p44/42 MAPK	(Gao et al., 2017)

systemic response, ii) recruitment and production of more immune cells/cytokines (i.e. IL-6) occurs within the liver and leads to tissue fibrosis, iii) protection mechanism in place to reduce liver cell sensitivity and save undamaged cell tissue, iv) liver cell regeneration is activated by macrophage production of pro-resolution mediators [Robinson et al., 2016, Ramachandran and Iredale, 2012]. However, if liver homeostasis is dysregulated, chronic inflammation and fibrosis without regeneration could occur and lead to liver damage, chronic infections, autoimmunity, and metabolic disorders [Robinson et al., 2016, Kensler et al., 2010]. Aflatoxin has been shown to cause lipid accumulation in liver cells (steatosis) of animals because of its effects on liver enzyme activity and genes related to lipid metabolism and when in conjunction with Hepatitis B virus X antigen (HBx) and other dietary risk factors (i.e. high fat/cholesterol/alcohol) can lead to hepatocellular carcinoma [Groopman et al., 1985, CAST, 2003, Lu et al., 2013].

Growth hormone (GH) is a peptide hormone involved in growth promotion, cell division, and regeneration. It is released by the anterior pituitary somatotroph cells. Insulin like growth factors (IGF1) gets its name by its similarity with insulin. It is secreted in response to GH binding to the surface of liver cells, IGF1 then acts on growth plates at the end of long bones to promote growth. In addition to bone growth. IGF1 also stimulates differentiation and proliferation of myoblasts for muscle growth as well as stimulating amino acid uptake and protein synthesis in several tissues. The production of IGF1 occurs in the liver primarily, however, peripheral tissues (bone and cartilage) may also be synthesizers. Linear growth (height) in immature organisms is the main action of GH via IGF1, therefore a deficiency somewhere along the system could result in shorter stature or growth issues. Following subchronic and chronic DON exposure studies, it had been thought that feed refusal and thereby decreased food consumption was the cause for reduced weight gain [Prelusky, 1993, Akbari et al., 2017, Grenier and Applegate, 2013]. Prelusky,

1993 proposed that DONs accumulation in the brain and interactions with serotonin resulted in a behavioral modification through appetite control pathways [Prelusky, 1993]. However, DON also induces pro-inflammatory cytokines from multiple locations, and subsequent expression of suppressors of cytokine signaling (SOCS) which have the potential to interrupt GH signaling [Amuzie and Pestka, 2009b, Grenier and Applegate, 2013, Grenier and Applegate, 2013]. Amuzie & Pestka 2009 reported that 4-week old mice fed DON (20 ppm) for 2-8 weeks showed decreased weight gain, steady-state DON plasma concentrations, down regulated hepatic mRNA expression of insulin-like growth factor acid labile subunit (IGFALS), and decreased circulating IGF1 and IGFALS levels [Amuzie and Pestka, 2009b].

Calcium signaling is vital in immune cells (T lymphocytes) and tumor cells are regulated by continuous Ca^{2+} ion flux for survival, activation and proliferation. Disruption of calcium signaling can result in over activation of immune system, nephrotoxicity, and neurodegenerative diseases [Grenier and Applegate, 2013]. Immortalized human kidney epithelial cells (IHKE-1) treated with OTA resulted in disrupted Ca^{2+} hormonal signaling and altered cell proliferation effects [Benetic et al., 2000, Ringot et al., 2006].

3.4 Reproductive Toxicity

Phytoestrogens (estrogens produced from plants) and mycoestrogens (estrogens produced from fungi) are some examples of naturally occurring EDCs that can be regularly found in animal feed and human food [Patisaul and Jefferson, 2010]. The mycotoxins ZEA and its derivatives (α -ZAL, β -ZAL, α -ZOL, β -ZOL, and zearalanone/ZAN) are resorcylic acid lactones and fall under the category of mycoestrogens [Tatay et al., 2017, Bräse et al., 2013]. They are structurally analogous to estrogen and have shown estrogenic activity *in vitro* and *in vivo* [Tatay et al., 2017]. A review by Kowalska et al., 2016 discussed several ways that ZEA exhibits disrupting effects on the endocrine system in animals and humans [Kowalska et al.,

2016]. The ability of ZEA to competitively bind with estrogen receptors can lead to hyperestrogenism, and these abnormally elevated levels of estrogenic activity in the body can result in infertility issues such as the inhibition of oocyte maturation and the disruption of cortical granule free domains (CGFD) which could prohibit fertilization ability [Minervini and DellAquila, 2008, Demaegdt et al., 2016, Hou et al., 2015].

Several studies have found that placental tissue disturbed by AFB1 results in hormonal imbalance through the inhibition of estrogen synthesis or an increased expression of corticotropin-releasing hormone (CRH) [Wang et al., 2016, Zhu et al., 2016, Storvik et al., 2011]. *In vivo* studies with pregnant mice exposed to AFB1 late in gestation have shown earlier delivery times, and placental tissue with elevated levels of *crh* mRNA expression [Wang et al., 2016].

Storvik et al., 2011 treated JEG-3 cells with (0.3 - 1.0 M) AFB1 and found that it induced expression of CYP19A1, metabolized AFB1 to AFL (aflatoxinol), and also interrupted the expression of aromatase (enzyme needed for estrogen synthesis) [Storvik et al., 2011]. This could be one potential endocrine disrupting effect of AFB1 [Storvik et al., 2011]. Another study conducted by Zhu et al., 2016 looked at the effect of AFB1 in human placental JEG-3 cells also [Zhu et al., 2016]. They found that AFB1 treated JEG-3 cells showed a dose-dependent increase of COX-2 expression, with significant increases at 0.1 nM AFB1. The initial increase in intracellular calcium could then activate the PKC-ERK signaling pathway. It has been observed that COX-2 is necessary for reproductive functions, and that defective or absent *cox-2* genes could cause infertility. Other AFB1 effects have been shown to cause fetal growth retardation, increased fetus malformations/birth defects, and toxin accumulation in eggs from laying hens and AFM1 accumulation in milk for dairy cows [Roll et al., 1990, Jia et al., 2016, CAST, 2003].

In steroidogenesis, cholesterol is converted to testosterone by a cascade of en-

zymes. Luteinizing hormone (LH) is secreted by the pituitary gland under gonadotropin releasing hormone (GnRH) stimulus and the transcription of genes involved in testosterone biosynthesis in the (interstitial) leydig cells is induced. Steroidogenic acute regulatory protein (StAR) is a rate limiting protein that transports cytosol to the mitochondria. In the mitochondria cholesterol is converted to prenenolon and the transported to the smooth ER for remaining steps. Sertoli cells form the blood testis barrier (BTB) form around germ cells. If a toxin impairs/inhibits any of the steps in the biosynthesis processes involved in steroidogenesis, barrier cells involved, spermatogenesis could be disrupted resulting in adverse effects on reproductive capacity [Liu et al., 2014, Zheng et al., 2018]. Supriya et al., 2014, con-

Table 3.5: Toxicity of AFB1 and DON.

Mycotoxin	Study Type	Animal (n)/Cell line	Doses	Duration	Effect/end points	Ref.
AFB1	<i>in vivo</i>	Nile Tilapia (<i>Oreochromis niloticus</i>)	200 µg/kg	10 weeks	- immunotoxic; decreased total erythrocyte, total leukocyte, and hemoglobin counts - reduced weight gain - reduced survival rate	(Selim et al., 2014)
AFB1	<i>in vitro</i>	JEG-3	>1nM	-	- increase <i>cox-2</i> expression in placenta - increased intracellular calcium levels - TRP expression elevated (TRPC-3) and activate PKC β and ERK - may disturb physiological processes dictated by COX-2 in pregnancy	(Zhu et al., 2016)
AFB1	<i>in vitro</i>	JEG-3 cells	(0.3 - 1.0 µM)	24-96 hours	- induced expression of CYP19A1 - concentration >1 µM were cytotoxic - AFB1 metabolized to aflatoxicol (AFL) by JEG-3 - can effect expression of aromatase enzyme (chronic exposure to AFB1) cause endocrine disruption in fetoplacental unit	(Storvik et al., 2011)
AFB1	<i>in vivo</i>	ICR pregnant mice	>0.5 and 5 mg/kg bw	4 days	- time of delivery shortened - low birth weight in mice treated with 0.5 and 5 mg/kg - isolated placenta tissue showed mRNA expression of <i>cyp11a</i> increased - protein kinase C proteins were activated and Bel-2 () reduced, could affect morphogenesis of placenta	(Wang et al., 2016)
AFB1	<i>in vivo</i>	laying hens	89.5 µg/kg AFB1 (123 µg/kg Total AF)	-	- decrease egg production and reduced feed intake - poor egg shell quality - accumulation of AF residue in egg - might be effect of AF on liver metabolism and function	(Jia et al., 2016)
AFB1	<i>in vivo</i>	Chinese Hamsters	15, 45, and 90 mg/kg i.p	day 12 and 13 gestation	- retardation in fetal development - dose-related increases in cleft palates, wavy ribs, and diaphragm changes	(Roll et al., 1990)
AFB1	<i>in vivo</i>	Chinese hamsters	45 mg/kg p.o.	-	- reduced fetal weight - increased diaphragm changes and kidney malformations	(Roll et al., 1990)
AFB1	<i>in vivo</i>	Male Wistar Rats	10, 20, or 50 µg/kg bw	40 days of age (early puberty) - 100 days of age (sexual maturity)	- all doses lead to decreased body weight gain - reduced weight of testis (from degeneration of germinal epithelium) - reduced weight of epididymis (could be from decrease of sperm production) - decreases in testicular steroidogenic enzymes and serum testosterone levels = decreased steroidogenesis - reduced level of circulatory testosterone, possible inhibition of androgen synthesis endocrine disruption	(Supriya et al., 2014)
DON	<i>in vivo</i>	Carp (<i>Cyprinus carpio</i> L.)	352, 619, 953 µg/kg	6 weeks	- increased lipid peroxidation in liver, kidney, spleen (reversible) - increased liver damage at highest concentration had increased fat content suggesting altered nutrient metabolism - effected anaerobic metabolism from alteration of lactate dehydrogenase activity in kidney and high levels of lactate in serum - decreased serum albumin	(Pietsch et al., 2014)
DON	<i>in vivo</i>	Sprague-Dawley rats F	0.5, 1, 2.5, 5.0 mg/kg bw/day	days 6-19 of gestation	- decreased average fetal body weight, crown-rump length, and vertebral ossification - maternal toxicity is 0.5 mg/kg bw/day based on the dose-related increase in liver-body weight ratio observed beginning at 1 mg/kg bw - the NOEL for fetal toxicity was 1 mg/kg bw based on impaired fetal development at 2.5 and 5.0 mg/kg bw - teratogenic at 5mg/kg bw/day	(Collins et al., 2006)
DON	<i>in vivo</i>	rats M	0.5, 1.0, 2.5, and 5.0 mg/kg bw	28 days	- specimen in groups dosed with 2.5 and 5.0 mg/kg bw showed decreased weight gain, body, epididymal, seminal vesicle, and brain weight compared to control - dose-related decrease in spermatid numbers - doses of 5.0 mg/kg bw had sperm tail abnormalities - dose dependent increase in serum FSH and LH and decrease in serum testosterone, endocrine disruption	(Sprando et al., 2005)
DON	<i>in vitro</i>	pigs (cells from ovaries of prepubertal gilts)	3 µM	26 hr	- DON inhibited porcine oocyte maturation - disrupted meiotic spindle by reducing p_MAPK protein level leading to retardations of cell cycle - induced autophagy/apoptosis in porcine oocytes - increased DNA methylation by altering <i>DNMT3A</i> mRNA levels and histone methylation levels - increased levels of H3K27me3 and H3K4me2 protein levels epigenetic modification	(Han et al., 2016)

Table 3.6: Toxicity of various mycotoxins.

Mycotoxin	Study Type	Animal (n)/Cell line	Doses	Duration	Effect/end points	Ref.
T-2	<i>in vitro</i>	hCG-stimulated mouse Leydig cells	10^{-7} , 10^{-8} , 10^{-9} M	24 hrs	<ul style="list-style-type: none"> - inhibits mRNA gene expression of 3β-HSD-1, P450αcc, and StAR - decreases enzymatic activity of 3β-HSD-1, P450αcc, StAR, CYP17A1, and 17β-HSD - decreased testosterone production 	(Yang et al., 2015)
ZEA α-β-ZOL	<i>in vitro</i>	HepG2 cells	0, 6.25, 12.5, 25 μ M	-	<ul style="list-style-type: none"> - elevated ROS levels - increase DNA damage (measured by COMET assay) - decreased glutathione levels and catalase activity - increased glutathione peroxidase and superoxide dismutase - α-β-ZOL had greater capacity for oxidative stress than ZEA 	(Tatay et al., 2017)
ZEA ZAN	<i>in vitro</i>	JEG-3 Cells, Placental Subcellular organelles, and recombinant enzymes	10-100 nM (ZAN or ZEN)	2, 24, 72 hrs	<ul style="list-style-type: none"> - metabolized ZEA and ZAN to α or β-OH metabolites - α-OH metabolites have higher binding affinity for estrogen receptors than parent compound 	(Huuskonen et al., 2015)
ZEA	<i>in vitro</i>	TM4 (mouse sertoli cells)	0.1, 1.5, 10, 20 and 30 μ M	24 hrs	<ul style="list-style-type: none"> - cell viability decreased in dose dependent manner - f-actin filaments and α-tubulin microtubules destroyed, vimentin and N-cadherin expression decreased (cytoskeletal structure damage) - oxidative stress stimulated lysosomes and autophagosomes within cells via mTOR and MAPK signaling pathways with help of endoplasmic reticulum stress that inhibited ERK signaling pathway to suppress phosphorylation of mTOR - disruption of sertoli cells can disrupt spermatogenesis 	(Zheng et al., 2018)
PAT	<i>in vivo</i>	Chinese Hamsters	3.75 mg/kg p.o.	day 12 and 13 gestation	<ul style="list-style-type: none"> - increased occurrence of cleft palate, teratogenic 	(Roll et al., 1990)
OTA	<i>in vitro</i>	IHKE-1 (immortalized human kidney epithelial cells)	0.1-10 nmol/L	24 hrs	<ul style="list-style-type: none"> - concentration dependent increase in cAMP - increased epidermal growth factor (EGF) and angiotensin II (Ang II) induced cell proliferation - impaired hormonal Ca$^{2+}$ signaling 	(Benesis et al., 2000)
OTA	<i>in vitro</i>	HEK 293 (human embryonic kidney cells)	1.5, 9.4 μ M	24 and 48 hrs	<ul style="list-style-type: none"> - OTA caused significant DNA strand breaks; increased comet tail lengths and decreased expression of OGG1, and increased ROS while decreasing GSH concentrations in kidney cells, also mRNA expression of Nrf2 (an antioxidant defense regulator) was decreased - Resveratrol (polyphenol in injured grape skin and fruit) synthesized by plant defense mechanism with anti-inflammatory and antioxidant properties, helped reduce tail length in from OTA treated cells, and increased GSH (chemopreventative) 	(Raghubeer et al., 2015)
OTA	<i>in vivo</i>	Wistar rats	2 - 4 mg/kg bw (oral intubation)	one single dose on one day between gestation day 6 and 15	<ul style="list-style-type: none"> - maternal weigh reduction - external hydrocephaly (incomplete closure of skull and omphalocoele) - internal hydrocephaly, micropthalmia, enlarged renal pelvic and renal hypoplasia - skeletal defects and developmental defects: skull bones, sternebrae, vertebrae, and ribs - days 6 and 7 of early gestation were most critical at dose 2.75 mg/kg bw and above for teratogenicity in rats. (sing oral dose min. teratogenic dose in rats) 	(Patil et al., 2006)
OTA CPA	<i>in vivo</i>	Pig (18)	90 and 180 ppb	-	<ul style="list-style-type: none"> - Porcine nephropathy; renal damage morphology; atrophy of proximal tubules; interstitial cortical fibrosis; sclerotized glomeruli impaired tubular function (increase in glucose excretion) - increased water consumption by animals 	(Stoef et al., 2001)
FB1	<i>in vivo</i>	Male weanling pigs (24)	20 mg/kg bw	up to 5 days	<ul style="list-style-type: none"> - hepatic injury; lesions in liver - Porcine Pulmonary Edema (PPE); began day 3 (histologic alteration seen on day 2), - alveolar endothelial damage; heavy wet lungs; lesion on lungs 	(Gumprecht et al., 1998)
FB1	<i>in vivo</i>	Ponies (11)	0 - 0.20 mg/kg bw (i.v.)	7-28 days	<ul style="list-style-type: none"> - Equine Leukoencephalomalacia (ELEM); brain lesions and neurologic issues - Cardiovascular dysfunction; decreased heart rate, cardiac output, increased serum and myocardial sphinganine and sphingosine concentrations 	(Smith et al., 2002)
FB1	<i>in vivo</i>	LM/Be mice	5-20 mg/kg bw (i.p.)	day 7.5 and 8.5 of gestation	<ul style="list-style-type: none"> - dose-dependent increase in number of litters affected and number of fetuses with NTDs (ecencephaly) - accumulation of sphinganine and sa:so ratio in maternal liver and kidney, placenta, and embryo - folate treatment of 50mg/kg bw/day on days 0.5-9.5 of gestation reduced NTD incidence in FB1 treated dams - GM1 treatment of 10 mg/kg bw given on days 6.5-9.5 of gestation was more beneficial for reducing NTDs in dams treated with FB1 	(Gelineau van Waas et al., 2005)
FB1	<i>in vivo</i>	Steers	15, 31, 148 ppm	31 days	<ul style="list-style-type: none"> - Hepatic Damage; mild liver lesions - lymphocyte blastogenesis - elevated liver enzymes 	(Osweiler et al., 1993)
FB1	<i>in vivo</i>	Dairy cattle	100 ppm	70 days	<ul style="list-style-type: none"> - lower milk production from reduced feed consumption 	(Diaz et al., 2000)

ducted a study using male Wistar rats to determine the effect through a complete spermatogenic cycle [Supriya et al., 2014]. They found that AFB1 competitively binds to StAR proteins, and interrupts cholesterol transport into mitochondria and decreases the biosynthesis of testosterone which is required for spermatogenesis, and low levels affect quality of sperm. Alternatively, ZEA decreased TM4 mouse sertoli cell viability with increased doses [Zheng et al., 2018]. The authors found that, ZEA was able to cause cytoskeletal structural damage and possibly disrupt spermatogenesis [Zheng et al., 2018]. Yang et al., 2015 showed that T-2 was able to decrease testosterone production by inhibiting the expression and activity of key steroidogenic enzymes [Yang et al., 2015]. Sprando et al., 2005 gave male rates 0.5-5.0 mg DON/kg bw for 28 days, they showed that groups dosed with 2.5 and

5.0 mg/kg had characteristic male reproductive toxicity endpoints and sperm tail abnormalities (5.0 mg/kg bw) [Sprando et al., 2005]. There were dose-related decreases in spermatid number, increases in serum FSH and LH, and decreases in serum testosterone [Sprando et al., 2005].

3.5 Summary

The extent to which a mycotoxin effects an individual depends greatly on the species, sensitivity, age, gender, nutritional status, and any predisposed genetic condition or viral infection (i.e. HBV). Gut health is important to overall health of the organism's system it relies on structural barrier integrity, and immune responses (innate and adaptive). Interruption to barrier integrity with subsequent activation of inflammatory responses can lead to systematic inflammation. Furthermore, mycotoxins can interfere with critical metabolic processes and can alter nutrient absorption. Additionally, endocrine disrupting affects can be seen to alter growth and development and reproductive function.

Chapter 4

Diet Studies

4.1 Diet Studies

Despite the seemingly endless variety of food items available to us on a daily basis in developed countries there are really just a few staple crops that make up the human diet, regardless of the socio-economic standing of the country. Cereals, such as rice, wheat, maize, sorghum, and millet are estimated to make up 50 to 54% of calories consumed by humans alone in developed and developing countries respectively [Kearney, 2010]. Food and feed can be directly contaminated by the presences of mycotoxins in plant materials or products and can be indirectly contaminated through carry-over of the mycotoxin or metabolite into animal tissues, milk, and eggs from bioaccumulation [Yang et al., 2014, CAST, 2003, Escrivá et al., 2017].

Dietary intake studies can help identify health hazards and gives scientists, researchers, and policy makers a birds eye view of food consumption patterns and potential toxins. In order to characterize the mycotoxin associated risks to individuals or populations, a thorough exposure assessment must be performed evaluating food intake and mycotoxin induced health effect along with any available dose-response relationship (hazard characterization) information [Codex Alimentarius Commission, 2003, World Health Organization, 2009]. These studies can also help to identify populations at risk of mycotoxin exposure by collecting information on the kind of foods they are eating and in what quantities. Identifying people at risk to mycotoxin exposure is important for all populations, especially in areas where the diet consists of a single crop, the conditions are favorable for fungal growth and mycotoxin production, and very limited knowledge, regulation, or technology for proper handling procedures [CAST, 2003].

4.1.1 Food Consumption Patterns, Mycotoxin Contamination, and Exposure Assessments

The basis of an exposure assessment is to evaluate the probable ingestion of a contaminant with either qualitative or quantitative methods [World Health Organization, 2009]. For humans or animals, researchers combine food consumption data with available information on the occurrence of the contaminant in question from tested samples [De Nijs et al., 2016]. Data will need to include in depth information about the food product, the food chain, the microbiological hazard, and the consumer. Information on food products would include geographical location of origin, seasonal variety, storage time, and processing if any. Food chain information should include descriptions from every step of the production process as it pertains to production practices, hygiene practices, and environmental conditions [World Health Organization, 2009, CAST, 2003]. Prevalence and level of contamination for hazard data should also reflect relevant factors for onset, growth, survival, distribution, and any decontamination measure. Consumption data can be characterized by social, economic, geographic, gender, or age related factors as well as the frequency or handling practices of products [World Health Organization, 2009].

There are many challenges associated with data from consumption surveys, particularly when predicting patterns for whole populations without considering variations among groups of people who adhere to different diets due to age, disease, cultural or religious customs. Although it is often difficult to obtain, data can be found from a variety of methods and sources. Data can be collected from questionnaires in epidemiological surveys such as case-control and cohort studies, however, they are limited by their costliness, small sample group, and are not readily accessible by the public due to the inability of the studies to disclose personal information on subject. When data is limited or contains extreme gaps, modeling techniques can be applied to help fill in information (i.e. Monte Carlo Method) [CAST, 2003, De Nijs et al., 2016].

4.1.2 Surveillance Systems

There are a number of national and international surveillance systems in place for general food consumption as well as toxin contamination. Many agencies help support surveillance programs and have different duties.

The USDA partners with other agencies such as the National Center for Health Statistics (NCHS), Centers for Disease Control and Prevention (CDC), Department of Health and Human Services (DHHS), Agricultural Research Services (ARS), and the Food and Drug Administration (FDA) to run the surveillance program What we eat in America (WWEIA) and the National Health and Nutrition Examination Survey (NHANES), which collect and quantify dietary data on nutrient composition and food consumption [Ahuja et al., 2012]. See figure 4.1 for food consumption for total population. The databases associated with these are National Nutrient Database for Standard Reference (SR) and the Food and Nutrient Database for Dietary Studies (FNDDS) [Ahuja et al., 2012]. From WWEIA and NHANES, total diet market basket studies can be conducted that evaluate many contaminants and nutrient intake [Ahuja et al., 2012]. It should be mentioned that the USAs TDS surveillance focuses mainly on toxic elements, pesticide residues, and radio nucleotides, not mycotoxins; however, the data can be used in conjunction with information from other regulatory bodies that do monitor mycotoxins.

For European countries, the European Food Safety Authority (EFSA) set up a data collection and analysis system on the food supply chain in EU member states [Szűcs et al., 2013]. To obtain more accurate and reliable exposure assessment information, they developed the Comprehensive European Food Consumption Database and a hierarchical food classification system (FoodEx) [Szűcs et al., 2013]. The FoodEx 2 is even more advanced and allows for food classification and description at a global level and could act as a common language for all databases. Continuing their theme for a more harmonized approach, the EFSA launched the EU

Table 4.1: Surveillance Systems for Food Consumption and Contamination (abbreviations National Health and Nutrition Examination Survey (NHANES), What we eat in America (WWEIA), National Nutrient Database for Standard Reference (SR), Food and Nutrient Database for Dietary Studies (FNDDS), Joint Expert Committee of Food Additives (JECFA), Global Environmental Monitoring System (GEMS/Food), Chronic Individual Food Consumption Database and Summary Statistics (CIFOCCOSS), Global Individual Food Consumption Data Tool (GIFT). [Ahuja et al., 2012,Ahuja et al., 2017,EFSA, 2015a,EFSA, 2013,EFSA, 2014,EFSA, 2015b,FAO, 2018].

Country	Agency	Surveillance System	Data/Database	Purpose	Myco. Data	Start Date	Other Agencies
U.S.A	USDA	National Health and Nutrition Examination Survey, 'What we eat in America'	SR, FNDDS	Collect dietary data and quantify nutrient/food consumption.	No	1960, 2002	NCHS, CDC, DHHS, and ARS
		Total Diet (Market Basket) Study	Based on WWEIA and NHANES	Determine contaminants and nutrient intake of food.	No	1961	FDA and DHHS
Europe (EU-states)	EFSA	Comprehensive European Food Consumption Database	FoodEx1, FoodEx 2	Food data collection and analysis for EU-member states.	Yes	2011	Agencies of EU member states
		"What's on the menu in Europe?" EU Menu Project	-	EU-wide survey covers consumption data over all 4 seasons	-	2012 - 2017	
WORLD WIDE	WHO	Food Contamination Monitoring and Assessment Programme with GEMS/Food	GEMS/Food Consumption Database and Contamination Database	Collect, analyze, and assess global food intake of food and contaminants using cluster diets	Yes	1976	FAO, GEMS/Food, and 30+ collaborating institutions
		Chronic Individual Food Consumption Database and Summary Statistics	Individual Food Consumption Database, Global Individual Food Consumption Data Tool	Summary Statistics of consumption surveys (duration >2 days) from 26 countries.	No	2013	FAO
	FAO	Joint Expert Committee of Food Additives	GEMS/Food data, scientific literature, JECFA Database	Evaluate the safety of food additives, contaminants, and residues. Establish acceptable daily intake (ADI) and guidance.	Yes	1956	WHO

Menu project (Whats on the menu in Europe?), this EU wide survey is proclaimed as more age appropriate and covers all four seasons throughout the 2012-2017 collection period [Szűcs et al., 2013].

In 1976, Global Environmental Monitoring System - Food Contamination and

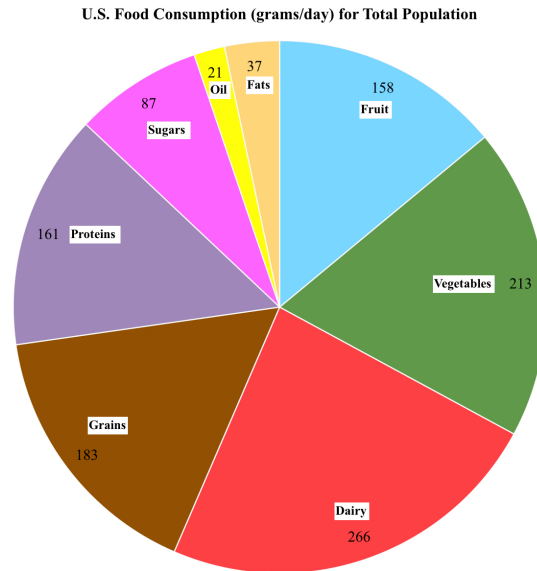


Figure 4.1: Total foods consumed in g/day for total U.S. population. For Child consumption vs Adult consumption see figure A.11 Source: 2007-10 National Health and Nutrition Examination Survey

Monitoring (GEMS/Food) was established to help provide information and trends to governing bodies about food consumption and contamination. They collect data for hazard occurrence, food composition, and food consumption for sharing information on international risk [World Health Organization, 2009]. As part of a collaboration with the WHO working group, and 30 more collaborative agencies and center. Using data from food balance sheets, WHO and GEMS/Food created 5 regional diet patterns (Middle Eastern, Far Eastern, African, Latin American, and European), this was later transformed in 1997 into 13 cluster diets for 183 countries [World Health Organization, 2009, Szűcs et al., 2013, World Health Organization, 2014, Sy et al., 2013]. In 2012, GEMS/Food introduced 4 additional clusters (17 cluster diets total) [Szűcs et al., 2013, World Health Organization, 2014, World Health Organization, 2012, Sy et al., 2013]. In this cluster method, the US falls into G10 along with Belarus, Bulgaria, Canada, Croatia, Cyprus, Estonia, Italy, Japan, Latvia, Malta, New Zealand, Republic of Korea, and Russia [World Health Organization, 2012, Sy et al., 2013, World Health Organization, 2014]. See figure 4.2 for products consumed

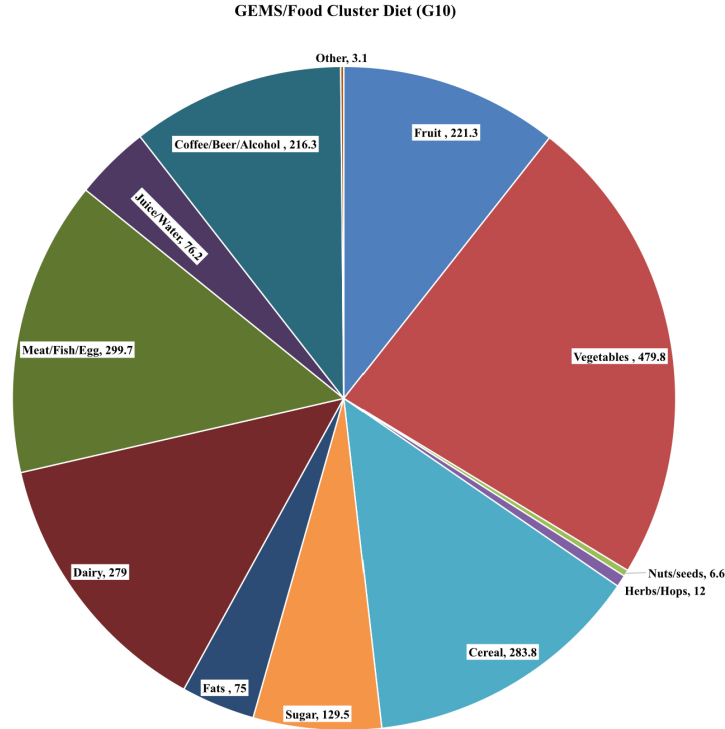


Figure 4.2: Products consumed in g/day data from Group 10. To see complete breakdown of food items see appendix. Data from [World Health Organization, 2012].

in grams per day for GEMS/Food 2012 Cluster Diet G10.

Chronic Individual Food Consumption Database and Summary Statistics (CIFO-COss) is a newly developed database by WHO and FAO that aims to harmonize data and make it publicly accessible [De Nijs et al., 2016, FAO/WHO, 2017]. The criteria for its data are food consumption surveys that have a duration of 2 or more days, thus representing a more chronic range of consumption. Currently, they have the summary statistics from 37 surveys of 26 countries (17 within the EU). The dissemination platform meant to make data accessible is the Global Individual Food Consumption Data Tool (GIFT) [FAO, 2018].

The Joint FAO/WHO Expert Committee of Food Additives (JECFA) is responsible for evaluating food additives as well as naturally occurring contaminants and veterinary drug residues. They develop and assess principles for quantifying

risk, analytical methods, and guidance values of exposure for reliable and harmonized information [FAO/WHO, 2017]. Through toxicological evaluations, they establish acceptable daily intakes (ADI) or tolerable intakes for acute and chronic exposure [FAO/WHO, 2017]. Still there are many more food consumption surveys conducted by national bodies that are not listed here. The main goal of these international organizations is to develop a harmonized approach that allows for comparisons and easy access for scientific evaluations and risk assessment decisions.

4.1.3 Food Monitoring Surveys, Total Diet Studies, and Duplicate Diet Studies

Several different types of data collection strategies can be used for assessing food consumption and exposure to mycotoxins. A review article by de Nijs et al., 2016 concisely describes established exposure assessment strategies using various data collection methods that can make up food consumption surveys (FCSs). These strategies include the use of food monitoring studies, total diet studies, and duplicate diet studies along with developing methods in human biomonitoring. Many FCSs are based on questionnaires or surveys that try to estimate the amount and the frequency of foods consumed that are suspected of being contaminated by mycotoxins [De Nijs et al., 2016]. These are compiled into databases by national and international organizations for further assessment.

Mycotoxin occurrence data can be collected using food monitoring studies, total diet studies, and duplicate diet studies. All of which can be used for chronic and

Table 4.2: Range of mycotoxin concentration levels that may occur in nature (ng/g) (adapted from [Grenier and Applegate, 2013]).

Mycotoxin	Usual (ng/g)	Worst Case (ng/g)
Aflatoxins	< 300	2000
Ochratoxins	< 300	2000
Fumonisin	< 10000	40000
Zearalenone	< 1000	5000
Deoxynivalenol	< 5000	25000
T-2 toxin	< 500	2000

Table 4.3: Contamination levels of mycotoxins in assorted food and feed products from North America only.

Mycotoxin	Commodity	% Incidence	M./Avg. (ng/g)	Range (ng/g)	Ref.
AFB1	Peanut butter	16%	14	-	Wood, 1989
AFB1	Peanuts	10%	68	-	Wood, 1989
AFB1	Corn	47%	30	-	Jelinek, 1987
OTA	Wine	85%	1.3	0.3 - 8.6	De Jesus et al., 2017
OTA	Blue Cheese	-	-	0.2 - 0.3	Dall'Asta et al., 2008
FB1	Corn grain	-	37973	0.1 - 93060	Abbas et al., 2006
FB1	Corn based breakfast food	-	59	5 - 237	Park et al., 2004
FB1	Corn chips	-	40	5 - 134	Park et al., 2004
FB1	Beer	41%	-	0.3 - 12.7	Hlilywka & Bullerman, 1999
FB1	Animal feed	37%	280	20 - 2120	Yu et al., 1999
FB1	Maize silage	97%	615	21 - 1824	Kim et al., 2004
FB1	Hay/silage	52%	120	20 - 450	Yu et al., 1999
ZEA	Corn based breakfast food	21%	6.1	up to 21	Roscoe et al., 2008
ZEA	Wheat breakfast cereal	38%	2.4	up to 5.5	Roscoe et al., 2008
ZEA	Multigrain breakfast cereal	30%	15	up to 100	Roscoe et al., 2008
ZEA	Animal feed	32%	220	120 - 320	Yu et al., 1999
DON	Animal feed	100%	730	340 - 6020	Yu et al., 1999
DON	Hay/silage	100%	610	570 - 720	Yu et al., 1999
CIT	Cereal grains	-	-	70 - 80000	Scott et al., 1972
PAT	Apples	40.8%	-	8.8 - 417.6	Harris et al., 2009
PAT	Apple juice	22.7%	-	8.8 - 2700.4	Harris et al., 2009

acute exposure assessment with strategies such as point estimates, observed individual mean, or a probabilistic approach [De Nijs et al., 2016]. In food monitoring studies (FMS), random samples from any point along the food supply chain can be collected/tested to assure compliance within regulatory and legal limits [De Nijs et al., 2016]. Table 4.2 shows a range of mycotoxin concentration level that can be found in nature, these ranges come from studies from multiple countries and help illustrate what may usually occur vs. the worst case scenario from bad weather conditions or something enhancing fungal ability to produce mycotoxins [Grenier and Applegate, 2013].

Mycotoxin occurrence data can also be found via food recall databases, such as the Rapid Alert System for Food and Feed (RASFF) or WHO's Global Environ-

Table 4.4: Contamination levels in border detection of US products. There are several hundred more examples, these are just show the kinds of mycotoxins are common occurrences/ issues [RASFF, 2018].

Product	Contaminant Conc.	Classification	Notifying Country	M-D-Y
spaghetti	OTA: 7.2 mg/kg	border rejection	Finland	12-18-12
pistachios	OTA: 32 µg/kg	alert	Netherlands	8-9-16
pistachios	OTA: 44 µg/kg (AFB1: 18; AF Tot: 20 µg/kg)	border rejection	Netherlands	1-18-17
pistachios	OTA: 94 µg/kg	border rejection	Netherlands	2-24-17
soft wheat	DON: 1676.6 µg/kg	border rejection	Italy	7-1-16
common wheat	DON: 3.138 mg/kg	border rejection	Italy	7-7-16
maize	DON: 2688 µg/kg	information	Ireland	8-28-17
soya hulls (feed)	ZEA: 0.76 mg/kg	alert	U.K.	8-27-07
dog food	AFB1: 22 µg/kg	info	Finland	2-22-06
peanut butter	AFB1/AFTot: 3.7/4.4 µg/kg	border rejection	Cyprus	4-7-17
groundnuts for birdfeed	AFB1: 220 µg/kg	border rejection	Netherlands	11-16-17
unshelled pistachios	AFB1/AFTot: 270/290 µg/kg	alert	Netherlands	1-12-18
groundnuts	AFB1/AFTot: 4.7/14.92 µg/kg	border rejection	Netherlands	1-17-18
unshelled pistachios	AFB1/AFTot: 8/10 µg/kg	border rejection	Italy	1-18-18
pistachios in shells	AFB1/AFTot: 45.3/51.3 µg/kg	border rejection	Lithuania	1-19-18
pistachios in shells	AFB1/AFTot: 16/17 µg/kg	border rejection	U.K.	1-31-18

ment Monitoring System/Food Contamination Monitoring and Assessment Program (GEMS/food) [World Health Organization, 2009, De Nijs et al., 2016]. Food monitoring studies are helpful in providing point estimates with an overall exposure estimated for entire geographic regions, which are help initial risk identification [De Nijs et al., 2016].

Total diet studies (TDS) sample food from diets that are prepared as they would be consumed [Tanase et al., 2011]. The market basket approach can parallel the dietary habits of a given population [Raad et al., 2014, Tanase et al., 2011]. Processed and cooked food items are analyzed, not just single food items. Mitchell et al., 2017 conducted a risk assessment for dietary OTA in the USA [Mitchell et al., 2017]. They compiled occurrence data from previous studies that collected food and drinks

from grocery stores throughout the US during 2012 and 2013. In total, 2296 food susceptible to OTA contamination were randomly selected at different times and from 9 different locations (IL, CA, TX, PA, ND, ID, MN, MI, NE); these samples were then analyzed to determine contamination values. Consumption information by age group (<12 mo, >12 mo-5yrs, >5yrs-18yrs, and >18yrs) from What we eat in America was used to determine dietary exposure for each commodity by multiplying daily consumption (ng/kg bw) with the related median OTA contamination levels from the samples for an observed individual mean. In Fig. 4.5 the tolerable daily intake (TDI) or the provisional maximum tolerable daily intake (PMTDI) which is set by JECFA and based on the most sensitive no-observable-adverse-effect-level (NOAEL) studies is shown for several mycotoxins, except for CIT which only has a level of no concern. The estimated dietary intake (EDI) (see equation 4.1) of different mycotoxins for average U.S. adult were compiled from different studies with U.S. data (AFB1, OTA, ZEA) or from JECFA global averages. There is no EDI average for CIT so one was calculated using both GEMS/Food Cluster Diet (283.8 g/day) and USDA/NHANES (183 g/day) food consumption data estimates with the lowest CIT occurrence level of 19 $\mu\text{g/kg}$ in cereal grains [World Health Organization, 2012, World Health Organization, 2014, EFSA, 2012]. Both EDIs for CIT were calculated to show the difference in exposure estimates given the difference in consumption data. The percentage of the TDI/PMTDI consumed with the EDI is given for each mycotoxin; the average EDI for AFB1 in the average American adult is almost double the TDI and DON is 100% of health based guidance value.

Another TDS was conducted by Sirot et al., 2013 in France using food data from the second national individual dietary consumption survey (INCA2) that occurred from 2006-2007, and required a 7-day food diary and questionnaire [Sirot et al., 2013]. From the survey, 212 core foods were selected based on their consumption popularity and possible contributions to exposure. All samples were prepared as

Table 4.5: Estimated dietary intake of mycotoxin by U.S. adult and the percentage of the PMTDI/TDI that they comprise or exceed. The EDIs were collected from different studies involving U.S. population data or calculated [Mitchell et al., 2017, Zinedine et al., 2007, EFSA, 2012, CAST, 2003](Wogan et al., 1974, Kuiper-Goodman et al., 2010, EFSA 2011, Cressey 2014)

Mycotoxin	PMTDI/TDI (ng/kg bw/d)	ng Mycotoxin Consumed (ng/d)	EDI for Avg. Adult (ng/kg bw/d)	% of Tolerable Intake Consumed
AFB1	0.15	18.2	0.26	173%
FB1	2,000	5,600	80	4%
OTA	17.1	8.4-123.9	0.12 - 1.77	0.5-9.94%
DON	1,000	70,000	1,000	100%
T-2/HT-2	60	1,190	8 and 9 (ind.) 17 (comb.)	28.3%
ZEA	500	2,100	30	6%
CIT	200	3,477* 4,539**	49.7* 64.8**	24.8%* 32.4%**
PAT	400	7,000	100	24%

*data USDA Consumption Information (183 grams of grain per day) used in EDI calculation considering 19 ng/g CIT
**data from GEMs/Food Cluster Diet G10 (283.8 grams of grain per day) used in EDI calculations considering 19 ng/g CIT

would be cooked described in the INAC2 survey and then subjected to analysis for 25 mycotoxins; levels were determined with lower bound (LB) and upper bound (UB) approach. From this data, they found that adults and children exceeded the guidance values for DON (1000 ng/kg bw/d) and for T-2 and HT-2 (60 ng/kg bw/d) [Sirot et al., 2013].

Alternatively, a duplicate diet study (DDS) will sample duplicates of the same foods that are consumed by an individual over a 24 hour period by an individual in the study [De Nijs et al., 2016]. Sizoo & Van Egmond 2005 analyzed a 24 hour DDS for AFB1, AFM1, and OTA in 123 participants from a small Dutch village [Sizoo and van Egmond, 2005]. To represent seasonal fluctuations, dietary collection periods were separated and obtained in March and then in September of 1994. The authors stated that the results of their DDS were comparable to those of another study determined via probabilistic exposure assessment that used food consumption data and mycotoxin concentration from individual food product [Sizoo and van Egmond, 2005].

From 2012 to 2016, Frances ANSES led a 4 year collaborative research project

with 26 partners from 19 different countries called the TDS-Expsoure project which focused on many food contaminants and mycotoxins [ANSES, 2018a]. The project was funded by the EU, the projects objectives were to “i) identify how TDSs could benefit exposure information, ii) develop TDSs throughout Europe by use of small scale studies, iii) harmonize the TDS method and use a Quality Management System, iv) create a database that is available for further assessment and management” [ANSES, 2018b]. Through collaboration and evaluation, countries that have high levels of mycotoxin contamination could establish TDSs that focus on whichever mycotoxin is most prevalent. The outcome of this project is slowly trickling into scientific reports and will hopefully expand the available information on mycotoxin contamination to different populations and can increase the available data about mycotoxin contamination.

4.1.4 Equations and Calculations

In order to calculate the exposure estimates, both occurrence data and consumption data is combined. The different approaches that can be used are either probabilistic or deterministic methods. The general equation for assessing the dietary exposure is given by:

$$\text{Dietary Exposure} = \frac{\Sigma \text{ conc. of mycotoxins in food} \times \text{food consumption}}{\text{Body Weight}}, \quad (4.1)$$

where the dietary exposure is defined as the concentration of mycotoxin in the food multiplied by how much food is consumed divided by the weight of the individual [World Health Organization, 2009, De Nijs et al., 2016].

A deterministic approach uses known/available data measured from the food product as well as the data gathered from food consumption studies [World Health Organization, 2009]. A point estimate would be a value from one mycotoxin and one input of consumption [De Nijs et al., 2016]. The observed individual mean

would use the mean mycotoxin concentration per food product multiplied by the food consumed per day per consumer that has been averaged over the amount of days shown in the survey [De Nijs et al., 2016, Boon et al., 2015]. For both of these deterministic methods, either means, minimum/maximums, or 95th percentile values can be used [World Health Organization, 2009, Assunção et al., 2016]. Alternatively, the probabilistic method, can utilize mathematical models to determine the probability of an exposure estimate [World Health Organization, 2009, De Nijs et al., 2016, Assunção et al., 2016]. In place of having any one value input, these models use distribution functions. The most used model for this approach is Monte Carlo simulations [CAST, 2003, World Health Organization, 2009, De Nijs et al., 2016]. There are relative uncertainties with each method.

4.1.5 The downside of memory-based assessment methods

Alternatively, nutrient and food consumption intake methods based on memory-based dietary assessment methods (M-BM) could lead to misrepresented inferences based on flawed data. These M-BMs such as FFQs or 24 hour recalls which are taken in the form of interviews and surveys are open to error and flawed in their designs. A review by Archer et al., 2012⁵ suggests that M-BM data does not realistically represent the actual energy and nutrient consumption of the individuals studied [Archer et al., 2015]. Their reasoning for the inadmissibility of this nutritional epidemiological research data is rooted in the flaws of the collection methods used due to the inaccuracy of human memory, adherence to protocols that illicit false recalls, and the subjective nature of the data in general. Some of the evidence they give in support of their theory, is the disparity between NHANES M-BM data and USDA Food Availability economic data, where trends in one have been historically opposite of the trends in the other [Crane et al., 1992, Archer et al., 2015].

Considering this, it would stand to reason that mycotoxin exposure estimates based off of M-BMs may be widely inaccurate. These inaccuracies may be further

compounded by the questionable reliability and difficulties in sampling procedures and analytical methods used to obtain occurrence data [Miraglia et al., 1996]. This could potentially lead to both drastic over and underestimations. Biomonitoring of mycotoxins in tissues and fluids of animals and humans offers an appealing and more accurate approach to exposure and risk assessments.

4.2 Biomarkers and biomonitoring

Biomarkers offer a more accurate measure of individual level exposure that can relate to the internal dose [Routledge and Gong, 2011]. They can help uncover various aspects of its metabolism as well as determine the mechanism of action; excreted mycotoxins and their metabolites, interaction products, or DNA adducts can be important biological markers of exposure [Routledge and Gong, 2011].

Biomarkers can be divided into three groups: exposure, effect, and susceptibility. Exposure biomarkers help to determine the presence of toxins and their metabolites as well as the severity of recent or prior exposure [Miraglia et al., 1996]. These can be used in health risk assessments, occupational safety, and epidemiological studies which can help guide and influence public health regulations [Kensler et al., 2010, Groopman et al., 2005]. Examples of biomarkers of exposure are conjugated or unconjugated mycotoxins found in plasma and tissue or excreted in urine, feces, milk [De Nijs et al., 2016]. The presence of a mycotoxin in a biological sample matrix is not necessarily indicative a certain risk. Therefore, biomarkers of effect maybe more useful when conducting risk assessments. Biomarkers of effect show the occurrence and severity of any biological response from the toxin exposure and can be applied to hazard identification [Kensler et al., 2010]. Examples of biomarkers of effect are DNA mutations, DNA adducts, altered mRNA expression, or interrupted metabolic pathways.

Aflatoxin biomarkers of effect can offer insight into exposure and the passage of time; AFB1N7-Guanine is used for recent exposure studies and detection of AFB1-

albumin or AFB1-lysine can be used for longer exposure studies (few months) due to the longer half life of albumin [Scholl and Groopman, 2008]. Fumonisin inhibits ceramide synthase and disrupts the sphingolipid metabolism which then leads to build up of sphinganine-1-phosphate (Sa1P) and sphingosine-1-phosphate (So1P) levels, therefore, Sa:So ratios may be useful in evaluating the effect but also contend with a great degree of variation [Voss and Riley, 2013, Riley et al., 2015].

The final biomarker category is that of susceptibility, which aims to provide information about inter-individual sensitivity, this can be an inherent or acquired trait that can initiate associations between environmental toxins and the onset of a clinical disease [Groopman, 2017, Kensler et al., 2010]. Basically, the ability of an organism to respond based on differences in different genotype/phenotype, environmental exposure, and modified diet. Examples of susceptibility biomarkers can be a genetic polymorphism or altered metabolic enzymes [Kensler et al., 2010].

4.2.1 Current biomarker/biomonitoring

Absorption, distribution, metabolism/biotransformation, and excretion of the mycotoxin are part of the foundation for developing a specific biomarker [Routledge and Gong, 2011]. Different matrices such as blood, urine, milk, or tissue samples will have different biomarkers. Various fluid, tissue, and organ samples of animals can be analyzed to determine adverse effect, bioaccumulation and subsequent potential for contamination up the food chain. Many methods and studies are developed and validated on animal species such as pigs and lab rats before being used in human studies. Additional factors that need to be considered are "i) sample collection method, ii) sample extraction and preparation, iii) sensitivity of assays, iv) cost, and v) reliability," [Routledge and Gong, 2011, Turner et al., 2015]. Sometimes the methods that work for *in vitro* or *in vivo* studies may not be applicable to human population studies, because of either detection sensitivity or species differences in metabolism [Escrivá et al., 2017]. Noninvasive fluid or tissue collection methods

may be preferred for human studies because invasive collection methods might limit a sample pool and increase costs. Methods that minimize analyte losses during extraction and cleanup and from matrix effect, have increased sensitivity, and that conserve time are the most effective methodologies. Validation criteria biomonitoring methods are defined by the EU commission regulations.

Parameters of quantification and detection are very important to method validation and can greatly impact the accuracy of risk assessments [World Health Organization, 2009]. The Limit of Detection (LOD) is the lowest concentration of the analyte that can be detected without quantification under the stated experimental conditions [FAO/WHO, 2017]. The Limit of Quantification (LOQ) is the lowest concentration of an analyte determined with acceptable levels of compounds in sample matrix [World Health Organization, 2009]. These (LOQs) should be as low as possible and much lower than any regulatory limit. Contaminants, especially mycotoxins, are present in foods at very low levels which can be easily missed if the detection techniques are not sensitive enough and data without a quantification (censored data) may then become a source of misrepresentation for exposure calculation [World Health Organization, 2009, FAO/WHO, 2017]. The resulting risk can then become either an underestimation or an overestimation [FAO/WHO, 2017].

Sample extraction and clean up and analytical detection methods used for human biomonitoring are similar to those used for detection in other food matrices, and will only be briefly discussed here. Previous analytical methods used to detect mycotoxins in human biological samples were liquid chromatography coupled to a fluorescence detector (LCFD) or enzyme-linked immunosorbent assays (ELISA), however, these were not without their issues. Extensive sample preparation for LC-FD and the potential for target compounds to cross react with metabolites or matrix components in ELISA methods make them less optimal when compared to more recent analytical methods using LC systems coupled with mass spectrometers

(LC-MS) [Capriotti et al., 2015, Turner et al., 2015].

In the late 1980s Fenn et al., 1989 developed electrospray ionization (ESI) that ionizes intact (no fragmentation) chemical species through multiple charging and helped pave the way for proteomics [Fenn et al., 1989, Banerjee and Mazumdar, 2012]. This analytical process has greatly enhanced the ability of LC-MS methods to further elucidate information and understanding about the structure of mycotoxins and their interactions [Banerjee and Mazumdar, 2012]. Additionally, the recently developed ultra high performance liquid chromatography (UHPLC) has the advantage of smaller incorporation of tandem quadrupole mass spectrometers (MS/MS) or high resolution mass spectrometry (HRMS) there is enhanced specificity, reliability, and sensitivity and therefore become more common in research [Gumustas et al., 2013, Turner et al., 2015]. That being said, LC/ESI-MS/MS system are vulnerable to sensitivity and selectivity issues from matrix effect, which is the differential response of an analyte in a standard solution compared with the same analyte in a biological matrix [Kearle and Tang, 1993]. A recent article by Shaw et al., 2014 describes how these effects interrupt chromatographic behavior and ionization and possible ways to overcome these issues (i.e. use of a internal standard) [Shaw et al., 2014].

One of most important ways to not only prevent interfering matrix compounds but to also increase sensitivity and reproducibility of the analysis is through careful sample preparation and extraction methods [Shaw et al., 2014]. When combined with LC-MS, either a direct or indirect approach can be taken. A direct approach called dilute and shoot (DaS) does not require enzymatic hydrolysis of the sample but instead relies on commercial reference standards for a specific mycotoxin and major urinary metabolites and other synthesized standards for glucuronide conjugates [Huybrechts et al., 2015]. It can forgo the added costs of sample cleanup and the risk of losing biomarkers in those processes, however, this can be problematic

due in part to the limited amount of available commercial reference standards and the costly and time consuming requirements for a lab to make their own [Huybrechts et al., 2015]. For example, Warth et al., 2011 developed a direct method for DON exposure using DON and an in house synthesized DON-3-O-glucuronide (D3GlcA) because there were no commercially available standards for conjugated mycotoxins like DON-GlcA [Warth et al., 2016].

An indirect approach will determine total mycotoxins once the sample has been subject to enzymatic deconjugation and sample clean-up. This can be done through a variety of methods such as immunoaffinity clean up (IAC), solid-phase extraction (SPE), liquid-liquid extraction (LLE) [Mally et al., 2016]. The effectiveness of the methods for clean-up and detection depend on the nature of the target mycotoxin and the matrix. For example, Blaszkewicz et al., 2013 developed a sensitive method for analyzing CIT in human plasma and urine [Blaszkewicz et al., 2013]. The authors compared the sensitivity of two analytical methods, HPLC-FD and LC-MS/MS. Results indicated that LC-MS/MS was more sensitive and could detect the lowest amounts of CIT and was therefore considered the better method. They used protein precipitation to clean up plasma before analysis with LC-MS/MS. Only CIT was measured in the plasma matrix, with an LOD of 0.07 ng/mL and an LOQ of 0.15 ng/mL. Two clean-up methods of sample prep were tested on urine, SPE with a C18 cartridge and IAC. Due to the matrix effect, detection using SPE was difficult and therefore IAC was more suitable for extraction of CIT and HO-CIT in urine samples. CIT had a LOD and LOQ in urine of 0.02 ng/mL and 0.05 ng/mL respectively. The LOD and LOQ for HO-CIT in urine was determined to be 0.05 ng/mL and 0.10 ng/mL respectively.

In addition to the technological advancements of liquid chromatography tools, trends in sample preparation and extraction methods have evolved to combat earlier disadvantages with traditional methods such as LLE and SPE which are more

time consuming and require large volumes of solvent and sample [Huybrechts et al., 2015, Escrivá et al., 2017, Cigić and Prosen, 2009]. These trends generally looked toward greater efficiency of technique and time which could arise from method simplification and conservative volumes of solvent and samples [Cigić and Prosen, 2009, Escrivá et al., 2017]. Escrivá et al., 2017, used urine to help develop a sensitive and fast analytical method for 11 mycotoxins (AFB1, AFB2, AFG1, AFG2, OTA, ZEA, BEA, EN A, EN B, EN A1 and EN B1). They compared three different extraction methods: salting-out liquid-liquid extraction (SALLE), miniQuEChERS (quick, easy, cheap, effective, rugged, and safe), and dispersive liquid-liquid microextraction (DLLME) to determine the most efficient one based on LODs and LOQs and validation results from recovery, reproducibility, and repeatability. The sample size In regards to their findings, DLLME was the most appropriate and had to lowest LODs/LOQs of the three (0.005-2/0.5-4 $\mu\text{g/L}$) [Escrivá et al., 2017]. In a study conducted by Andrade et al., 2013, breast milk was simultaneously analyzed for AF(B1, B2, G1, G2, M1) and OTA with HPLC-FD. They optimized and validated a this methods using LLE followed by low temperature purification for clean up and separation (LLE-LTP), which was found to require less time and cost and better recoveries when compared with the use of an SPE column [Andrade et al., 2013].

4.3 Matrices

4.3.1 Blood

According to WHO, blood is the most common matrix used for human biomonitoring studies, primarily because blood has continuous contact with mycotoxins throughout the whole organism and is in equilibrium with tissue and organs [WHO, 2015]. Overarching disadvantages to the use of blood is that it is invasive, requires specially trained individuals, and needs special transport/storage. When using blood, there are well established protocol for matrix preparation and either plasma or serum can be used. A study conducted by Yu et al., 2011 investigated the differences between

metabolite profiles in plasma and serum, they concluded that both matrices showed similar results, however, higher metabolic concentrations found in serum make it a better option for more sensitive results with biomarkers [Yu et al., 2011]. Jager et al., 2016 conducted a pilot study from June 2011 to March 2012 in São Paulo, Brazil that analyzed the biomarkers in serum and urine for AFB1 and AFM1. They tested for serum AFB1-Lysine adducts and urinary AFB1-N7-Guanine adducts but no urine sample was positive [Jager et al., 2016]. However, 74 out of the 113 (65%) urine samples had detectable amounts of AFM1 which had a positive correlation with to the PDI they determined from food questionnaires and AFB1 and AFM1 detection levels in foods they tested [Jager et al., 2016]. This just further shows that biomarkers are matrix specific also.

4.3.2 Urine

Urine is useful for multiple mycotoxin biomarker analysis and is readily available. It is non-invasive and can be collected easily and in large amounts. Animal and human urine can help with the accuracy of exposure assessment at both individual levels as well as establish population reference ranges to help identify vulnerable groups [WHO, 2015]. It can be used for rapidly metabolized and excreted mycotoxins, however, some issues or challenges can that arise with urine are the low analyte concentration [Escrivá et al., 2017]. Biomarkers that are excreted in urine can be the parent compound, conjugated/metabolized compounds, or DNA adducts. For example most ingested DON is eliminated in the urine, therefore biomarkers used for detection are DON or its glucuronidated form DON-GlcA [Warth et al., 2011, Ali et al., 2015]. Egner et al., 2006 validated a method using isotope dilution mass spectrometry for detecting a major aflatoxin DNA adduct, AFB1-N7-Guanine that is excreted in urine. They used and internal standard, triple-quadruple mass spectrometry, SPE, and IAC to improve the accuracy, sensitivity, and specificity of the method to detect the biomarker, and was validated for a range of 0.8-25 pg/

20 mL urine. Twenty samples were collected from an AF contaminated region to assess the method, and 80% of the samples were positive at concentrations above the LOQ [Egner et al., 2006].

4.3.3 Breast milk

Breast milk is a complex matrix that can be used to monitor the presence of environmental pollutants and exposure to lactating mother and infant child [Capriotti et al., 2015, Mally et al., 2016]. Biomonitoring of breast milk can help to ascertain the risks of consumption and exposure to contaminated food products as well as helping determine the efficiency of intervention programs [Routledge and Gong, 2011, Fenton et al., 2005]. It is especially important to monitor for biomarkers of exposure in breast milk because it is a primary food source for infants and therefore represents their primary source of exposure to a contaminant; infants exclusively breastfeed for up to 6 months. Substitution of human breast milk with infant formula produced from cow milk or soy protein may also pose a risk depending on the level of contaminants that may persist through those processing methods which will be discussed further below.

There are many methods for determining mycotoxin concentration levels in milk and other dairy products such as cheese, yogurts, etc. and are similar to those used for biomonitoring [Capriotti et al., 2015, Turner et al., 2015]. Though, transfer rates and data is limited, mycotoxins that are excreted in breast milk with considerable (enough to cause alarm in highly contaminated diets) carry-over concerns are aflatoxins, ochratoxins, zearalenone, and fumonisins [Capriotti et al., 2015, Warth et al., 2016, Escrivá et al., 2017, Mally et al., 2016]. Composition of milk changes at different stages of breastfeeding so timing of sample collection will influence the level of contamination present in the sample matrix [Fenton et al., 2005]. To understand the full scope of exposure and effect it is helpful to collect more than one matrix and decipher a relationship. Although some relationships between breast milk and other

biological matrices (plasma, urine) have been observed more are necessary [Capriotti et al., 2015].

Like urine, breast milk offers a promising potential for multi-analyte detection. Rubert et al., 2014 modified a QuEChERS-UHPLC-HRMS to unambiguously identify target mycotoxins and screen for non-target mycotoxin compounds. Detection of the parent compounds and their metabolized products (DON, 3-ADON, NIV, FUSX, NEO, DAS, HT-2, T-2, ZEA, α -/ β -ZOL, FB1, FB2, FB3, EN (A, B, A1, B1), BEA, AF(B1, B2, G1, G2, M1), STE, OTA, OT α) were effectively quantified with LOQs ranging from 1-50 $\mu\text{g/L}$ [Rubert et al., 2014].

4.3.4 Alternative Matrices

Alternative matrices besides urine, serum, and breast milk may offer additional opportunities to look at long term effect [Mupunga et al., 2017]. Hair, nails, teeth, and saliva each represent their own advantages and disadvantages for use with appropriate biomarkers. Sewram et al., 2003 used human hair to show the possibility of long term accumulation assessment for FB1, FB2, and FB3. They collected hair samples from 5 population clusters in South Africa exposed to contaminated maize then used methanol extraction and strong anion exchange (SAX) cleanup followed by detection with HPLC-ESI-MS. Using hair is noninvasive and offers a long term window for chronic exposure assessment [Sewram et al., 2003, Mupunga et al., 2017].

4.4 Summary

In this section, different types of diet studies (food monitoring surveys, total diet studies, duplicate diet studies) and how they can be used to help characterize risks associated with mycotoxin exposure at the individual and population level was discussed. Food consumption patterns combined with contamination and occurrence data help determine populations at risk. In the absence of data, modeling methods can be used. Most food consumption data is obtained by memory based methods and can give unreliable estimates that are over or under the actual values, however,

when combined with biomonitoring, approaches the estimates can be more accurate. Several agencies such as WHO, FAO, USDA, EFSA, etc. run international and national surveillance and biomonitoring programs that monitor food consumption and mycotoxin/ chemical intake, however, the US program does not monitor for mycotoxin intake. More biomonitoring programs using different biomarkers: i) exposure (presence of toxin or metabolite), ii) effect (occurrence and severity of biological response), and iii) susceptibility (predisposition and onset of disease) can help and should be considered for areas of concern within the US. Of the programs that are in place, they lack standardization and comparability, therefore, group efforts like ANSES can help achieve a more coherent regulation. When analyzing samples, LOD and LOQ need to be as low as possible, because mycotoxins can be present in matrix samples in very low levels and can have an effect on the outcome of the study estimated exposure. Analytic methods and the sample preparation methods depend on the target mycotoxin and the matrix that it is in. Biomarkers can be matrix specific also. Moving forward, the incorporation and use of -omics technology into exposure-response health risk assessments has the potential to unveil new biomarkers [WHO, 2015]. The abbreviation of -omics incorporates many fields of study including genomics, metabolomics, lipidomics, transcriptomics, and proteomics. With -omics tools, researchers can simultaneously screen vast amounts of targets and alterations from a particular environmental influence at different levels of cellular organization [Kyrtopoulos, 2013]. These global profiling techniques can further help elucidate the exposome which is defined as “the measure of all exposures of an individual in a lifetime and how those exposures relate to health,” [DellaIra and Dall’Asta, 2017].

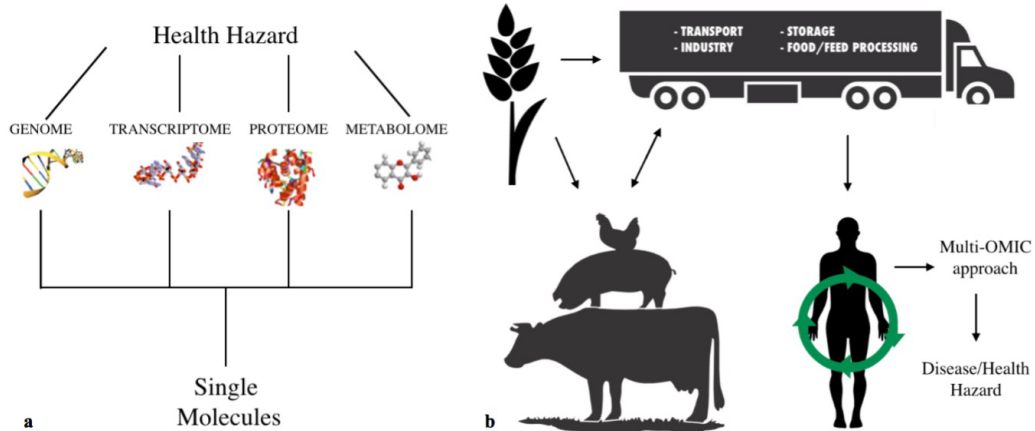


Figure 4.3: a) Molecular perspective of OMIC technology b) The exposome as it pertains to mycotoxin contamination (adapted from [Dellafiora and DallAsta, 2017] (Arbona et al.,2013))

Table 4.6: A variety of validated or potential biomarkers for use in human and animal biomonitoring and the analytical methods used to detect them.

Matrix	Biomarker	Prep.	Method	LOD	LOQ	Reference
URINE	AFB1-N7-Gua	IAC, SPE: C ₁₈	HPLC-ESI-MS/MS	0.02 pg/mg	0.07 pg/mg	(Egner et al.2006)
SERUM	AFB1-N7-Gua	SPE	UPLC-ESI-MS/MS	3 pg/mL	10 pg/mL	(Jager et al.2016)
URINE	UAFM1	IAC	UHPLC-ESI-MS/MS	0.075 pg/mL	0.25 pg/mL	
SERUM	AFB1-lys	SPE C ₁₈	HPLC-FD	10 pg/mL	-	(Johnson et al.2010)
URINE	UAFM1	IAC	HPLC-FD	0.5 pg/mL	-	
MILK	AFM1	-	ELISA	0.92 ng/L	2.79 ng/L	(Cantú-Cornelio et al. 2016)
URINE	DON, DOM-1	IAC	LC-MS/MS	0.16, 0.10 ng/mL	-	(Ali et al. 2015)
	DON, D3GlcA	DaS	LC-MS/MS	6, 3 µg/L	20, 10 µg/L	(Warth et al. 2011)
SERUM, URINE, MILK	OTA	LLE	HPLC-FD	30, 70, 10 ng/L	50, 100, 30 ng/L	(Muñoz et al. 2014)
URINE	OTA , OT α	IAC	HPLC-FD	0.05 µg/L	0.1 µg/L	(Ali et al. 2017)
SERUM	OTA	IAC, SPE: C ₁₈	HPLC-FD	-	0.1 µg/L	(Malir et al. 2013)
PLASMA	CIT	-	HPLC-MS/MS	0.07 ng/mL	0.15 ng/mL	(Błaszczewicz et al. 2013)
URINE	CIT, HO-CIT	SPE vs IAC	HPLC-MS/MS	0.02-0.05 ng/mL	0.05 - 0.1 ng/mL	
URINE	ZEA, α - β -ZAL, α - β -ZOL, ZAN	LLE	UHPLC-MS/MS	0.02-0.03 ng/mL	0.1-1.0 ng/mL	(Belhassen et al. 2014)
MILK	ZEA, α - β -ZAL, α - β -ZOL, ZAN	SPE	UHPLC-ESI-MS/MS	0.004-0.1 ng/mL	0.01 - 0.2 ng/mL	(Capriotti et al. 2015)
URINE	FB1, FB2, FB3, HFB1	SPE	LC-MS	0.04 - 0.18 ng/mL	-	(Riley et al. 2012)
MILK	FB1	LLE, SAX	HPLC-FD	5500 ng/L	19500 ng/L	(Magotha et al. 2014)
HAIR	FB1, FB2, FB3	SAX	HPLC-ESI-MS	25 pg	60 pg	(Sewram et al. 2003)
MULTIPLE MYCOTOXIN MONITORING						
SERUM/ URINE	EN (A, A1, B, B1), BEA	SPE: GCB	LC-ESI-MS/MS	0.01 - 0.04, 0.02 µg/L 0.0025 - 0.02, 0.005 µg/L	0.02 - 0.04, 0.04 µg/L 0.005 - 0.02, 0.01 µg/L	(Serrano et al. 2015)
MILK	AF(B1, B2, G1, G2, M1), OTA	LLE-LTP	HPLC-FD	-	0.005 - 0.03 ng/mL	(Andrade et al. 2013)
SKIN	EN(A,A1, B, B1, D, E, C/F) , BEA	-	UHPLC-MS/MS	10 - 17 pg/mL	-	(Taevernier et al. 2014)
URINE	AF(B1, B2, G1, G2) OTA, ZEA, BEA, EN (A, B, A1, B1)	SALLE, miniQuEChERS, DLLME	HPLC-MS/MS	0.1 - 10; 0.1 - 2; 0.05 - 2 µg/L	0.5 - 40; 0.5 - 6; 0.5 - 4 µg/L	(Escrivá et al. 2017)
MILK	DON, 3-ADON, NIV, FUSX, NEO, DAS, HT-2, T-2, ZEA, α - β -ZOL, FB1, FB2, FB3, EN (A, B, A1, B1), BEA, AF(B1, B2, G1, G2, M1), STE, OTA, OT α	QuEChERS	UHPLC-HRMS	-	1 - 50 µg/L	(Rubert et al. 2014)

Chapter 5

Epidemiological Studies with Dietary Perspective: Exposure Groups

There are many populations which require special consideration, in part due to their specialized diets along with other genetic or physical conditions. As previously discussed, diet plays a large role in how humans are exposed to mycotoxins. This particular section will focus on how populations with diets different than the average individual for some biologically or personally related reason can be a cause of concern for chronic exposure and adverse health effects. Some of the populations that will be discussed here are pregnant women, infants/young children, vegetarians/vegans, and immune compromised individuals.

5.1 Pregnant Populations

Pregnant populations in particular are important high risk associated group to observe for chronic exposure. During pregnancy, the human body undergoes chemical, physiological, and behavioral changes at different stages [Groopman et al., 2014, Klaassen et al., 1996]. Aside from the obvious physiological changes of another life growing inside the human body, there are minor and major changes that affect the cardiovascular, respiratory, gastrointestinal, immune, endocrine systems, and more [Groopman et al., 2014]. These changes can result in alteration of toxicokinetics of xenobiotics in the maternal system, particularly mycotoxins. An example of these changes, such as the increase of blood volume leading to a decrease in plasma protein concentration levels, could mean more unbound mycotoxins. Another example is the rising levels of progesterone reducing intestinal mobility, which then delay gastric emptying and prolongs the toxicants retention time in upper gastrointestinal tract [Klaassen et al., 1996, Groopman et al., 2014]. Additionally, the slight immune suppression and modulation of the mothers system which occurs to allow for the fetus to develop, can further increase adverse risk. Finally, internal

and environmental factors influence gut microbiota which might alter bacterias role metabolizing mycotoxins [Nuriel-Ohayon et al., 2016].

Behavioral changes in regards to diet and health during pregnancy could stem from conscious behavior, as well as internal cues from the body [Nuriel-Ohayon et al., 2016]. Pregnant women have increased needs for energy and nutrients and therefore consume a greater amount of food. They may also have a behavioral incentive to follow a healthier diet during their pregnancy for the best outcome of their progeny. A study conducted by Verbeke and Bourdeaudhuij 2007, investigated the dietary behavior of pregnant women versus women that were not. Primarily their findings inferred that pregnant women are more conscious about their diet and their food choices are strongly motivated by food safety concerns. According to the data, many pregnant women have a higher fruit, beef, dairy, and fat intake. However, contrary to initial assumptions, very few women from the study observed health and safety guidelines like avoiding alcohol, tobacco, and raw foods [Verbeke and De Bourdeaudhuij, 2007]. A majority of pregnant women do not follow prescribed health and food safety guidelines. The reasons that prevent them from doing so could depend on the food availability or a variety of socio-economic factors (i.e. lack of access to health care, education).

Current research also highlights the importance of diet and the effects of epigenetic controls for future generations [Hernandez-Vargas et al., 2015, Han et al., 2016]. It is not just what a mother eats or is exposed to before or during pregnancy, but also the pregnancy diet itself that may effect the reproductive outcome, as well as progeny fitness. These epigenetic changes from environmental stresses and exposures may lead to, predispose, or contribute to the development of immunological and chronic diseases of the offspring later in life [Hernandez-Vargas et al., 2015].

Table 5.1: Mycotoxins ability to affect reproductive health [CAST, 2003, Collins et al., 2006, Piekola et al., 2012, Capriotti et al., 2015, Wang et al., 2013, Han et al., 2016, Yu et al., 2017]

	Endocrine Disrupter	Toxic Maternal Sytem	Crosses Placental Barrier	Embryotoxic/ Fetal Toxicity	Teratogen Potential	Adverse Birth Outcome	Lactational Transfer	Epigenetic Effects
AFB1	Y	Y	Y	Y	Y	Y	Y	Y
OTA	Y	Y	Y	Y	Y	-	Y	Y
ZEA	Y	-	Y	-	Y	-	Y	Y
DON	Y	Y	Y	Y	Y	Y	Y	Y
T-2	Y	Y	Y	Y	Y		Y	Y
FB1	-	Y	Y	Y	Y	Y	Y	Y
Ergot	Y	Y	Y	Y	Y	Y	Y	-

5.1.1 Mycotoxin Exposure and Risk to Maternal-Fetal Unit

Over the last two decades several biomonitoring studies (cohort, case control, cross-sectional studies) have been carried out on pregnant women all over the world. The goal has been to help determine potential risk from mycotoxin exposure to both the mother and the fetus. Pregnant women are mainly exposed to mycotoxins through contaminated food, however, some may have been exposed through the use of medical treatments originating from a specific mycotoxin (i.e. ergotamine for headaches) [Hughes and Goldstein, 1988, Acs et al., 2006]. Mycotoxins have been linked to or associated with Neural Tube Defects (NTDs), growth stunting, immune system impairment, late term abortions, and congenital abnormalities [Kristensen et al., 2000, Marasas et al., 2004, Missmer et al., 2006, Acs et al., 2006, Hernandez-Vargas et al., 2015, Gupta et al., 2017]. There is still a great uncertainty regarding the impact of mycotoxins on birth outcomes and defects in humans. Biomonitoring studies and use of laboratory animal studies could help to fill these gaps. These biomonitoring studies use a combination of food frequency questionnaires (FFQs) and biological samples (serum, urine, cord blood, milk, placenta) collected at different stages during pregnancy to see when the greatest exposure and potential risk may coincide.

5.1.2 OTA

Reproductive and developmental toxic effects of OTA have been observed in research and farm animals. Not only can OTA pass through the placental barrier, it is also transferred via lactation [Gupta et al., 2017]. Studies indicate OTA exposure in pregnant research animals results in birth defects of the central nervous system (Neural Tube Defects) [Gupta et al., 2017].

OTA has a high binding affinity for protein albumin, since 99.8% of OTA in the human circulatory system is bound to plasma proteins (albumin) with a very small amount available in its freely unbound state [Kőszegi and Poór, 2016]. Evidence suggests that during pregnancy there is a decrease in serum albumin concentration and chemical-binding protein, resulting in reduced protein binding availability [Groopman et al., 2014, Zimmerli and Dick, 1995]. This could mean that drugs and other

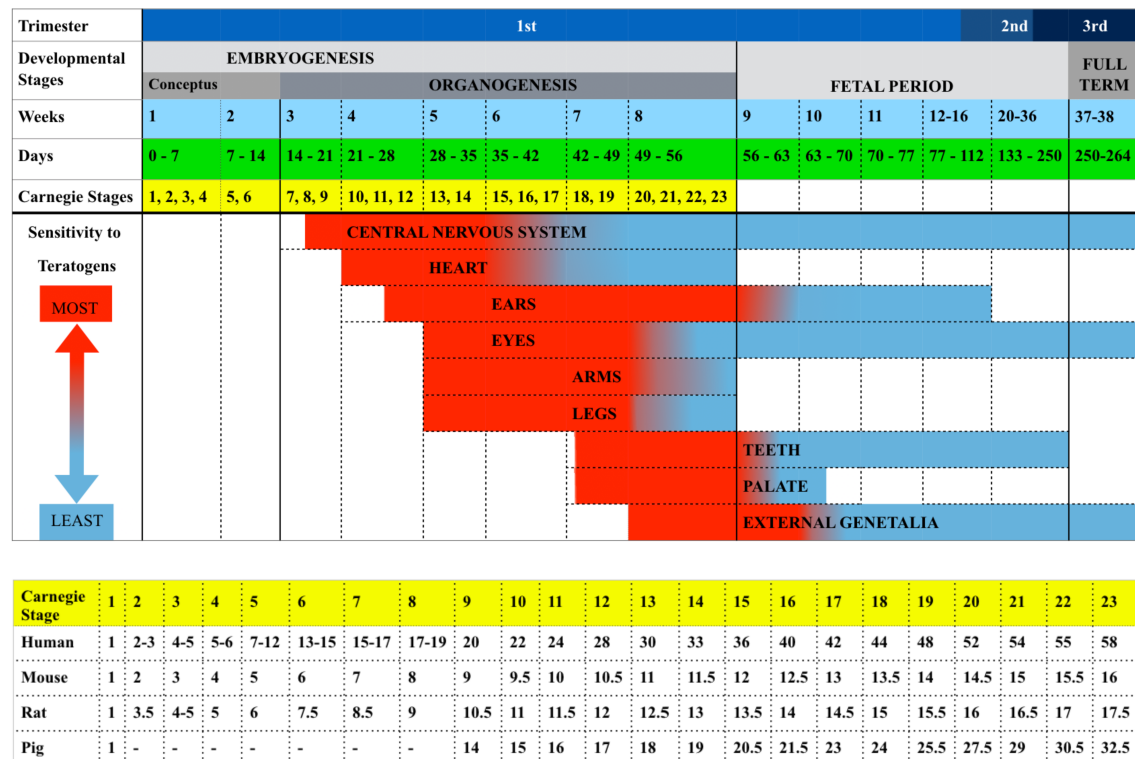


Figure 5.1: Reproductive stages of development and sensitivity to teratogens with Carnegie stage comparison for human, rat, mouse, and pig. Adapted from [O’Rahilly, 1979, Witschi, 1962, Pérez-Cano et al., 2012].

Table 5.2: Reproductive biomonitoring studies. (NTD = Neural Tube Defect Cases, Cont. = Control, CB = Cord Blood, NSA = Non South Asian, SA = South Asian, Sub = Suburban, alb = albumin, cr = creatinine).

Location	Mycotoxin	Time Period of Sampling (wks)	Individuals	Matrix, Biomarker (+/n)	Concentration (Mean, Avg., or Range)	EDI (Mean, Avg., or Range)	< or > TDI (% of TDI)	Ref.
Czech Republic	OTA	1 - 11	100	S, OTA (96/100)	M: 0.15; R: 0.10 - 0.35 µg/L	M: 0.58 ng/kg bw	<	(Malir et al., 2013)
USA (Tx.-Mexico Border)	FB1	1 - 11	NTD: 137/225 Cont: 185/378	S, Sa:So	R: 0.07 - 0.35	M: NTD 172, Cont. 156 R: 30 - 9,441 ng/kg bw	< (max > 470%)	(Missmer et al., 2006)
Africa (Gambia)	AFB1	20 - 32, CB at birth, Infants at 16 wks	Maternal: 119 CB: 99 Infant: 118	S, AF-alb: Maternal (119/119), CB (48/99), Infant (13/118)	M(R): Maternal 40.4 (4.8-260.8), CB 10.1 (5-89.6), and Infant 8.7 (5-30.2) pg/mg alb	-	-	(Turner et al., 2007)
	AFB1	1 - 16	115	S, AF-alb (115/115)	R: 3.9-458.4 pg/mg alb	-	-	(Hernandez-Vargas et al., 2015)
USA (Salt Lake City, Utah)	ZEA	15 - 35	30	U, ZEA (41/30)	A(R): 0.10 (LOD - 0.3) µg/L	-	<	(Fleck et al., 2016)
	α-ZOL			U, α-ZOL (11/30)	A(R): 0.11 (LOD - 0.25) µg/L	-	<	
U.K. (Bradford)	DON	28 - 38	Total: 85 NSA: 53/85 SA: 29/85	U, DON (85/85)	M: ALL 10.3, NSA 8.6, SA 15.2; R: 0.5 - 116.7 ng/mg cr	M: ALL 10.1, NSA 8.4, SA 13.7; R: 1.4-58.8 µg/kg bw	>	(Hepworth et al., 2012)
Africa (Egypt)	OTA	28 - 38	98	S, OTA (80/98)	M: 0.33; R: 0.20 - 1.53 ng/mL	R: 0.43 - 3.26 ng/kg bw	<	(Woo & El-Nezami, 2016)
	AFB1	28 - 38	100	S, AF-alb (34/98)	M: 2.25 (3.0 - 35.1) pg/mg alb	-	-	(Piekkola et al., 2012)
	AFM1			U, AFM1 (44/93)	M: 5.48 (4.12 - 408.6) ng/mg cr	-	-	
	DON			U, DON (63/93)	M: 1.11 (0.5 - 59.9) ng/mg cr	-	-	
	DOM1			U, DOM1 (2/93)	M: - ; R: 0.10 - 0.12 ng/mg cr	-	-	
India (Bangladesh)	DON	28 - 38	Total: 54 Rural: 32/54 Sub.: 22/54	U, DON (28/52)	M: 0.86 ± 1.57; R: Rural 0.47 ± 0.73, Sub. 1.44 ± 2.20 ng/mL cr	M: ALL 0.05 µg/kg bw M: Rural 27, Sub. 90 ng/kg bw	<	(Ali et al., 2015)
Croatia (Osijek)	DON	28 - 38	Total: 40 Rural: 20/40 Urban: 20/40	U, DON (31/40)	A: 18.3 µg/L	M: ALL 2.5, Rural 3.1, Urban 1.8; R: 0.1 - 33.1 µg/kg bw	> (183 - 775%)	(Šarkanj et al., 2013)
	DON-15-GlcA			U, DON-15-GlcA (39/40)	A: 120.4 µg/L	-	-	
	DON-3-GlcA			U, DON-3-GlcA (33/40)	A: 28.8 µg/L	-	-	

natural chemicals such as OTA, which usually have higher concentrations of OTA-plasma bound proteins molecules, might exhibit different concentration profiles in pregnant women. The concentration of plasma protein bound OTA may decrease and unbound, free OTA molecules may increase during pregnancy [Groopman et al., 2014, Zimmerli and Dick, 1995]. As a free molecule, OTA can pass through the placental barrier, a greater portion may impact the embryo. The detection of higher amounts of OTA in human fetal serum and cord blood confirms *in utero* exposure [Woo et al., 2012, Zimmerli and Dick, 1995].

As term approaches, the placenta thins and may become more susceptible to environmental toxins, which may suggest that OTA exposure is greater in late in pregnancy [Zimmerli and Dick, 1995, Woo and El-Nezami, 2016]. However, results from human placental perfusion study by Woo et al., 2012, suggests that is not the case and OTA can reach the fetus just as well [Woo et al., 2012]. Because of this,

exposure to OTA during early gestation may have an increased teratogenic potential and developmental effects (see Fig. 5.1).

A study conducted by Malir et al., 2013 used blood samples from 100 pregnant Czech women in the first trimester of pregnancy, that were obtained and evaluated using HPLC-FD to determine OTA serum level [Malir et al., 2013]. Of the 100 samples, 96% tested positive for OTA (contamination $> 0.1 \mu\text{g/L}$). A dietary exposure estimate for pregnant women was estimated based on food consumption data from a national food survey and food samples were analyzed with validated ultra-trace HPLC. For pregnant Czech women (age 19-40), the average daily intake from a combination of all cereals and cereal products, baked goods, spices, coffee, pork meat, juices, and chocolates was 0.58 ng/kg bw . The intake assessment based on OTA level in blood serum, which had a mean of $0.15 \mu\text{g/L}$ (range of $0.10 - 0.35 \mu\text{g/L}$) was calculated to be between $0.2 - 0.5 \text{ ng/kg bw/day}$ [Malir et al., 2013]. For this study, the estimated intake was significantly less than the TDI for OTA, which is $17.1 \text{ ng/kg bw/day}$, however, the potential toxicity to the fetus may be higher considering its lack of development (metabolism, immune) and small size [Malir et al., 2013]. Monitoring OTA intake through diet adjustments during the first trimester, when the fetus is most sensitive to teratogens could be an important consideration for researchers and pregnant women.

Woo & El-Nezami 2016, conducted a study to assess the maternal-fetal cancer risk of OTA in Egyptian women during their third trimester of pregnancy [Woo and El-Nezami, 2016]. A total of 98 serum samples and FFQs were obtained, OTA was detected in 80 urine samples. The mean and range for serum OTA concentrations was 0.33 ng/mL and $0.20 - 1.53 \text{ ng/mL}$ respectively. Using the Klaassen equation, the derived maternal estimated dietary intake was in the range of $0.43 - 3.26 \text{ ng/kg bw/day}$, however, the authors suggest that the *in utero* exposure to the fetus was twice that of the maternal exposure ($0.86 - 6.52 \text{ ng/kg bw/day}$). The estimates

from this study show that the mothers intake of OTA was less than the TDI (17 ng/kg bw/day). However, based on a tumorigenic study in the kidneys of male rats, a negligible cancer risk intake (NCRI) was calculated to be 3.38 ng OTA/kg bw/day [National Toxicology Program, 1989, Woo and El-Nezami, 2016]. Thus, the fetus exposure was greater than the pregnant Egyptian women, but less than the TDI and NCRI, except for the high exposure group (6.52 ng/kg bw/day) which exceeded the NCRI [Woo and El-Nezami, 2016]. It is important to mention that, although all of these estimates are highly uncertain, they can help identify potential hazards.

FB1

As mentioned before, disease outbreaks among animals can be an indicator for contamination in food. Equine leukoencephalomalacia (ELEM) is an indicator for fumonisin contamination and was linked to several clusters of neural tube defects (NTDs) amongst hispanic population in border regions of Texas/Mexico [Gelineau-van Waes et al., 2005, Gelineau-van Waes et al., 2009, Voss et al., 2017, Voss et al., 2005, Missmer et al., 2006]. The incidence rate for NTDs went from 15/10,000 live births to 27/10,000 live births [Marasas et al., 2004, Voss et al., 2005, Missmer et al., 2006]. The incidence rate for NTDs on average are 10/10,000 live births globally, however, areas of the world that rely on maize as a primary staple have rates 6 - 11 times higher (i.e. China, Guatemala, Mexico, South Africa ranging between 35 -106/10,000) [Bolger et al., 2001, Marasas et al., 2004, Gelineau-van Waes et al., 2005, Gelineau-van Waes et al., 2009, Voss et al., 2005].

Initially, several *in vivo* studies suggested that fumonisins could not cross the placental barrier and were not teratogenic in animals that were not sensitive to FB1. However, a model using LM/Bc mice (folate and GM1-responsive animal model for FB1-induced NTDs), injected with FB1 on gestation days 7.5 and 8.5 with doses from 5 -20 mg/kg bw, demonstrated dose dependent increases of affected litters and

exencephaly [Gelineau-van Waes et al., 2005, Voss et al., 2005]. Maternal livers, placentas, and embryos showed increased concentrations in sphinganine (sa) and sphinganine to sphingosine (sa:so) ratios with increasing FB1 treatments [Gelineau-van Waes et al., 2005]. In mice treated with FB1 at doses of 20 mg/kg bw, 80% of the fetuses in those litters were NTD positive [Voss et al., 2005]. To further assess the biodistribution potential, ^{14}C -FB1 was administered on gestation day 10.5 and confirmed that FB1 does cross the placenta in this sensitive strain of mice [Gelineau-van Waes et al., 2005, Voss et al., 2005]. Supplementation with folate and GM1 (deficiency for beta-galactosidase) in the FB1-treated mice helped to reduce the amount of NTD positive fetuses in the litters [Gelineau-van Waes et al., 2005, Voss et al., 2005].

To address strain susceptibility FB1-treated CD1 mice and LM/Bc mice were compared. The mice were given FB1 contaminated feed with doses of 50 or 150 mg/kg five weeks prior to mating and throughout the study and the corresponding EDI to these amounts were 25 (LM/Bc) and 38 (CD1) mg/kg bw/day [Voss et al., 2005]. Authors observed that fetotoxicity and NTDs can develop at maternally toxic doses and that there are strain/species dependent differences in sensitivity (CD1 are less sensitive and have a stronger placental barrier) [Voss et al., 2005]. A preliminary dietary NOAEL for FB1-induced NTDs is $< 50 \mu\text{g}/\text{kg}$ [Voss et al., 2005, Gelineau-van Waes et al., 2005].

A case-control study conducted along the Mexico-Texas border by Missmer et al., 2006, looked into fumonisin exposure from periconceptional (time before and during early pregnancy) intake of maize tortillas. As a biomarker for fumonisin exposure, sphinganine:sphingosine (sa:so) ratio in serum from the first trimester and from postpartum serum were analyzed and compared. The results from the data have inverted U-shape responses. Women with a daily exposure to FB1 of 0.15 - $0.65 \mu\text{g}/\text{kg}$ bw have more NTD cases [Missmer et al., 2006, Voss et al., 2005].

This suggests that the risk for NTD increases with increased exposure to fumonisins during the first trimester, especially in weeks 3 and 4 of gestation, which is suspected to occur in diets with high consumption of maize tortillas [Missmer et al., 2006, Riley et al., 2015, Voss et al., 2017].

There is speculation on the validity of using sphinganine concentrations and sa:so ratios in epidemiological studies and there are suggestions that studies should consider the metabolized sphengoid base-1 phosphate compounds, which are bioactive and play an important role in signaling pathways [Voss et al., 2005]. Sphinganine (sa) is phosphorylated by sphingosine kinases (SphK1 and SphK2) into the active sphingaonine-1-phosphate (Sa1P) [Voss et al., 2005]. Two important effects are that Sa1P binds and saturates cell surface G protein coupled receptors (interferes with signaling pathways, especially ones in neural tube closure) and elevated Sa1P interferes with actions of histone deacytlases (HDACs) that affect epigenetic regulation of gene expression [Gelineau-van Waes et al., 2012].

Gelineau-van Waes et al., 2012 treated pregnant SWV dams and inbred LM/Bc mice-strains with injections of FB1 at 20 mg/kg/day on embryonic days (ED) 7.5 and 8.5 or orally gavaged with FTY720, a S1P receptor agonist, at 10 mg/kg/day on ED 6.5 to 8.5 [Gelineau-van Waes et al., 2012]. Blood spots and embryonic tissues from each group were collected on various days from ED 9.0 - 12.5. Mouse embryonic fibroblasts (MEFs) and serum free mouse embryo (SFME) neural progenitor cells were treated with 40 μ M of FB1 for 24 hours. All samples were analyzed with LC-ESI-MS/ MS for sphengoid bases, sphengoid base-1-phosphates, FTY720, and FTY720-1-phosphate. The rationale was that prolonged exposure to elevated levels of sphengoid base-1-phosphates following FB1 or FTY720 treatment would activate S1P receptor signaling pathway and result in failed neural tube closures [Gelineau-van Waes et al., 2012]. Their findings confirmed early studies, suggesting differential susceptibility to FB1-NTDs between LM/Bc and SWV, and the potential use of

Sa1P as a biomarker of effect for FB1-NTDs research. Additional studies with FB1 treated LM/Bc MEFs conducted by Garnder et al., 2016 suggests that the FB1-induced disruption of sphingolipid metabolism leads to Sa1P accumulation, HDAC inhibition, and histone hyperacetylation is the potential mechanism for FB1-NTDs [Gardner et al., 2016].

Guatemala has an NTD incidence rate of up to 106/10,000 live births in certain areas [Voss et al., 2005, Bolger et al., 2001]. Riley et al., 2015 (Guatemala study) conducted a series of studies that looked at consumption of tortillas and NTDs [Riley et al., 2015]. After developing a urinary exposure biomarker and blood spot mechanism-based biomarker method, they then conducted a year long study to determine potential correlations [Riley et al., 2012, Torres et al., 2014]. Every 3 months (during 2011-2012), samples of maize from community resources and biological samples of urine and blood spots from 1240 women in 3 areas in Guatemala (Chimaltenango, Escuintla, and Jutiapa) with varying degrees of fumonisin exposure were collected [Riley et al., 2015]. A biomarker validation response study was also performed, in which sampling sites across Guatemala were chosen in order to replicate the results of the 2011-2012 biomarker study. From February to March 2013, samples of maize, urine, and blood (female participants n=299) were collected from Sacatepquez, Santa Rosa, and Chiquimula [Riley et al., 2015]. Important information for consideration from these studies are: UFB1 levels of Guatemalan women with 0.5 ng/mL corresponds to intake of 1.67 $\mu\text{g/kg bw/day}$, which is less than the PMTDI. However, women with UFB1 > 0.5 ng/mL will exceed the PMTDI (2.0 $\mu\text{g/kg bw/day}$) and increased ratios of Sa:So or Sa1P:So1P (by fumonisin inhibition of ceramide biosynthesis) positively correlates with fumonisin intake based on levels of UFB1 [Riley et al., 2012, Riley et al., 2015].

AFB1

It has been suggested that *in utero* exposure to AFB1 early in life can negatively impact immune health and growth and increase the chance of developing diseases later in life [Turner et al., 2007, Piekkola et al., 2012, Hernandez-Vargas et al., 2015]. The ability of AFB1 to cross the placental barrier has been shown in many animal studies, as well as in the detection of AF-alb adducts in human cord blood [Turner et al., 2007, Partanen et al., 2009]. Further evidence of this transfer has been shown with human placental perfusion models. Partanen et al., 2009 used 8 human placentas in a placental perfusion model to demonstrate both the transfer and the biotransformation of AFB1 into aflatoxinol (AFL). The adverse effects of exposure are dependent on the timing. Essentially, exposure at different time points during gestation may have different effects. Both AFB1 and DON transfer through the placenta at a faster rate than OTA [Nielsen et al., 2011, Woo et al., 2012].

Given that the placenta itself acts as an endocrine gland during pregnancy, the disrupting effects of AFB1, such as altering gene expression can result in various developmental and birth outcomes [Mesiano and Jaffe, 1997, Wang et al., 2016]. Wang et al., 2016 studied the effect of AFB1 exposure in late gestation on ICR pregnant mice. Different groups were treated with 0, 0.05, 0.5, and 5 mg/kg/d. Compared to the control group, dams given doses of 0.05 mg/kg and above gave birth sooner, and dams given the highest dose of 5 mg/kg/d had pups with lower birth weight. Higher doses of AFB1 were also found to have increased placental *cox-2* (cyclooxygenase-2) and *crh* (corticotropin) mRNA expression and decreased *15-hgpd* (15-hydroxyprostaglandin dehydrogenase) mRNA expression. The higher dosage of AFB1 given late in gestation caused an increase of CRH and estrogen in the plasma that may have led to the onset or premature delivery [Wang et al., 2016].

Roll et al., 1990 investigated the reproductive and teratogenic effects of AFB1

using pregnant Chinese hamsters [Roll et al., 1990]. On days 12 and 13 of gestation, AFB1 doses of 15, 45, and 90 mg/kg were injected intraperitoneally while another group received 45 mg/kg orally. The AFB1 injected hamsters demonstrated reduced fetal development and dose-related increases of teratogenic effects (cleft palates, wavy ribs, and diaphragm changes). The orally dosed hamsters resulted in pups with lower fetal weights, diaphragm changes, and kidney malformations [Roll et al., 1990].

A cohort study of 115 pregnant women in Gambia conducted by Hernandez-Vargas et al., 2015 investigated the effects of AF exposure during the embryonic development stage and epigenetic changes that occur as a result [Hernandez-Vargas et al., 2015]. The plasma samples were collected from these women between 1-16 weeks and analyzed for aflatoxin albumin (AF-alb) adducts as a biomarker for exposure. The range of AF-alb adducts found in the women was between 3.9-4 58.4 pg/mg albumin. White blood cell samples from their infants were then collected between 2-8 months of age for genome-wide DNA methylation as an epigenetic marker. One analysis used the AF-alb data as a continuous variable to determine the association of methylation patterns between a single locus and regional DNA, resulting in the identification of 71 aflatoxin-associated methylation sites (AfMSs), CpG sites that correlate with AF-alb, and a 1.7% alteration in methylation. A large portion of these CpG sites have been previously annotated and involved in genes with important immune and growth functions [Hernandez-Vargas et al., 2015]. Though more studies are needed, these results suggest that differential DNA methylation patterns resulting from aflatoxin exposure may affect growth and immune response [Hernandez-Vargas et al., 2015].

This information also further supports an earlier study in Gambia conducted by Turner et al., 2007 that investigated a connection between exposure to low levels of AFB1 *in utero* and growth faltering of those infants during their first year of

life [Turner et al., 2007]. The study collected blood samples from 119 women at two collection times (4.5 and 1 months prior to birth), 99 cord blood samples, and 118 blood samples from the corresponding infant at 16 weeks. Analysis of the samples by ELISA methods found that mothers, cord blood, and infants had AF-alb levels of 40.4 (4.8-260.8), 10.1 (5-89.6), and 8.7 (5-30.2) pg/mg respectively. Measurements of height and weight from 138 infants (born to women in the study) were taken monthly. The authors found that levels of AF-alb in maternal samples acted as a predictor for both the height and the weight of the infants [Turner et al., 2007]. Thus an inverse relationship was detected where infants born to mothers with higher levels of AF-alb adducts showed reduced height and weight gains. They also suggested that by reducing maternal AF-alb from 110 pg/mg to 10 pg/mg there could be a corresponding increase of 0.8 kg in weigh and 2 cm increase in height during the first year, [Turner et al., 2007]. Biomonitoring studies that look at the effects of aflatoxins in Africa often deal with consumption of corn and groundnuts which are critical dietary staples and are often contaminated with aflatoxins. Therefore the results of theses studies should stimulate further research by looking at other geographical areas (even in developed countries) that also produce and consume large portions of groundnuts like China and the United States (especially in major peanut producing states like GA, TX, AL, FL, etc.) [CAST, 2003, USDA-NASS, 2010].

ZEA

From the onset of pregnancy, estrogen plays many roles, from regulating embryogenesis, cell proliferation and differentiation, organogenesis, to parturition (birth) [Kaludjerovic and Ward, 2012]. As mentioned in earlier sections, mycoestrogens can have numerous endocrine disrupting effects on the reproductive systems of exposed male and female organisms. Several *in vivo* studies have demonstrated that ZEA can cross the placental barrier of animals (rats, rabbits, and pigs) and indicate

its potential in doing so in the human placenta [Bernhoft et al., 2001, Lange et al., 2002, Goyarts et al., 2007, Gao et al., 2017]. In pigs, placental transfer of ZEA results in teratogenic effects that manifest as various abnormalities of the genitals [Minervini and DellAquila, 2008, Dmello et al., 1999]. An *in vitro* study using both JEG-3 cells and human placental sub-cellular organelles showed that the human placental enzymes metabolize ZEA and ZAN into primary OH-metabolites (especially α -ZAL) which have even greater binding affinity for estrogen receptors than the parent compound [Huuskonen et al., 2015]. This metabolic proclivity toward α -ZAL may increase the risk of disruption to placental estrogen signaling, which becomes the primary source of estrogen during the 8th week of pregnancy [Mesiano and Jaffe, 1997, Kaludjerovic and Ward, 2012, Huuskonen et al., 2015]. The fetus is developing its own endocrine system prior to and during this time period, and could be interrupted by hormonal imbalance [Kaludjerovic and Ward, 2012]. Also, it has been hypothesized that exposure to estrogenic compounds in the perinatal phase (weeks leading up to birth) could result in altered epigenetic gene regulation, resulting in postponed effects (positive or negative) or even trans-generational inheritance or toxicity [Kaludjerovic and Ward, 2012, Gao et al., 2017]. This suggests it might be perfectly acceptable to blame your mother for everything that's wrong with you. Therefore, mycoestrogens in pregnant women could induce long-term programming effects on developing fetus as well as modulate adrenal gland growth and development through DNA methylation [Kaludjerovic and Ward, 2012]. It is important to mention that these mycoestrogens do not directly donate/accept a methyl group, but may open a region for methylation by promoting histone acetylation from its interaction with estrogen receptors [Kaludjerovic and Ward, 2012].

To ascertain this risk, Fleck et al., 2016 used a cohort of 30 pregnant women from the metropolitan area of Salt Lake City, Utah to estimate the internal exposure to dietary estrogens (including ZEA and its metabolites) during a period of fetal

development (15-35 weeks) that is uniquely sensitive to estrogen [Fleck et al., 2016]. Urine was collected over a 6 hour field exposure and a 24 hour clinical period, during which subjects ingested food items that consisted of corn. The urinary elimination of ZEA is approximately 10-20% of the administered dose and can be used to ascertain the estimated daily intake [Šarkanj et al., 2013]. Though, no consistent dietary source of ZEA was determined for the subject cohorts, both ZEA and α -ZOL were detected in urine samples with an average of 0.10 $\mu\text{g/L}$ (range of LOD - 0.31 $\mu\text{g/L}$) and 0.11 $\mu\text{g/L}$ (range of LOD - 0.25 $\mu\text{g/L}$), respectively [Fleck et al., 2016]. In comparison to a study with ZEA consumption at 83% of the TDI (0.25 $\mu\text{g/kg bw/day}$) and corresponding urinary concentrations of 0.3-0.6 $\mu\text{g/L}$, it can be concluded that the lower urinary concentrations from the Fleck et al., 2016 were significantly lower than the TDI [Šarkanj et al., 2013, Fleck et al., 2016].

Trichothecenes

Trichothecene such as DON, T-2, and their metabolites have been shown to generate negative or toxic effects on maternal and fetal systems. When cold and wet climate conditions promote the growth of *Fusarium graminearum* and the production of DON, its effects can be seen in the reactions and effects in animals. In 1981, midwestern states around Illinois were subject to such conditions, which resulted in animal feed with DON concentrations ranging from 0.1 to 41.6 ppm [Cote et al., 1985]. Of the swine fed with this contaminated feed, Côté et al., 1985 observed that half of all veterinary clinical complaints made about the pigs were reproductive related problems [Cote et al., 1985].

Ndossi et al., 2012 investigated the endocrine disrupting effects of DON, T-2, and HT-2 *in vitro* with H295R cell model subjected to various assays [Ndossi et al., 2012]. Specifically, this cell model was used to show alteration of gene expression in steroidogenesis process and the reporter gene assay (RGA) to detect any steroid hormone (androgen, estrogen, and glucocorticoid) agonist or antagonistic behavior

on behalf of trichothecene exposure [Ndossi et al., 2012]. Unlike the estrogenic activity shown with ZEA, trichothecene endocrine disrupting potential does not occur through direct interaction with steroid hormone receptors [Doi et al., 2008, Ndossi et al., 2012, Yu et al., 2017]. Instead, the endocrine disrupting nature of these trichothecenes relies on processes that reduce cell viability, inhibit DNA synthesis and interrupt steroidogenesis by altering gene expression or hormone synthesis [Ndossi et al., 2012, Yu et al., 2017].

Collins et al., 2006 studied the developmental effects of DON *in utero* in female rats (Sprague-Dawley) [Collins et al., 2006]. During gestational days 6-19 pregnant females were gavaged with doses of DON between 0 - 5 mg/kg bw. Maternal toxicity was established at 1, 2.5, and 5 mg/kg bw where dose-related increases in maternal liver weight correlated with cytoplasmic alteration of hepatocytes. Fetal size was reduced significantly at 2.5 mg/kg bw, while doses of 5 mg/kg bw were teratogenic with many skeletal malformations [Collins et al., 2006]. Other issues related to high doses were increased deaths per litter and number of runts in litter [Collins et al., 2006]. Studies using female pigs have shown placental transfer and effects of DON on maternal and fetal toxicity [Diaz-Llano and Smith, 2006, Goyarts et al., 2007, Yu et al., 2017]. Diaz-Llano & Smith 2006, demonstrated the effects of DON with pregnant sows that were fed naturally contaminated feed with 5.5 mg/kg bw in the last 3 weeks of gestation, which resulted in maternal weight loss and stillborn piglets. A review article by Doi et al., 2008 discusses the maternal and fetal toxicity in pregnant mice and rats due to T-2 and HT-2 toxins. Many of the toxic effects reported in this article are a result of apoptosis in maternal, placental, and fetal tissues, induced by oxidative stress from alterations in genes being expressed for specific metabolic pathways (i.e. lipid) [Doi et al., 2008].

In addition to crossing the placental barrier in animals, DON and T-2 have the potential to transfer through human placentas as shown by *in vitro* modeling

methods [Collins et al., 2006, Diaz-Llano and Smith, 2006, Nielsen et al., 2011, Doi et al., 2008, Wang et al., 2013]. Nielsen et al., 2011 used both an in vitro study with BeWo b30 Transport Assay and an *ex vivo* dual placenta perfusion model with 5 term placentas to demonstrate the ability of DON to transfer across the human placenta. Data results from the placental perfusion model demonstrated that after 4 hours of incubation 21% of the initial DON concentration was transported to the fetal reservoir [Nielsen et al., 2011]. Wang et al., 2014 used BeWo cells to study the transport kinetics of T-2 and HT-2 toxins across the placental barrier. They hypothesized that the uptake of T-2 and HT-2 toxins into fetal circulation was by transporter proteins (i.e. multi-drug resistance-associated protein, MRP; organic anion transporter, OAT; organic anion-transporting polypeptide B, OATPB; organic cation transporter, OCT) and passive diffusion respectively, while the efflux of the toxins back into the maternal circulation was through simple diffusion [Wang et al., 2013]. This study also suggests that T-2 and HT-2 induced toxicity are associated with increased cellular accumulation of the toxins and membrane transport could be the first fundamental stage in embryotoxicity and teratogenicity, [Wang et al., 2013]. All together, these studies indicate that maternal dietary exposure to DON and T-2 toxin can result in fetal exposure and detrimental effects from either indirect or direct interaction of the toxins with important systems. Therefore, implementing programs for biomonitoring trichothecenes for pregnant women who reside in areas that have history of toxicosis may be beneficial for evaluating the public health of that area and to determine the need of interventions.

A cohort study in Bradford, UK conducted by Hepworth et al., 2012 analyzed the urine of 85 pregnant women in their last trimester along with a FFQ that characterized the traditional British diet as well as a British-Asian diet [Hepworth et al., 2012]. Based on the FQQ food groups of cereals, breads, pastas, baked goods, and snacks, the authors suggest that the greatest exposure of DON is through white

bread. A urinary creatinine analysis was utilized to detect both free DON and DON-glucuronide (fd-DG). Urinary DON levels were found in all 85 samples and had a mean of 10.3 ng/mg creatinine and ranged between 0.5 - 116.7 ng/mg creatinine. The overall and combined estimated daily intake based on the FFQ for the pregnant UK women was 10.1 $\mu\text{g/kg bw}$, however when categorized via ethnicity, it was found that intake of DON at 8.4 $\mu\text{g/kg bw}$ for non South Asian women was significantly less than the 13.7 $\mu\text{g/kg bw}$ seen in South Asian women. The dietary intake of DON by South Asian women was supported by the higher amount of UDON (15.2 ng/mg creatinine) found in urine [Hepworth et al., 2012]. These findings highlight the importance of understanding different dietary habits and their association with mycotoxin exposure based on a region as well as different ethnicity within that region.

Other important comparisons that can help understand diet and exposure are the difference between people living in rural vs suburban areas. In a cohort study of pregnant women in Bangladesh, India, conducted by Ali et al., 2015, 32 urine samples from a rural setting and 22 urine samples from a suburban setting were collected during their third trimester [Ali et al., 2015]. Food consumption data was acquired through a FQQ. The mean level of UDON ranged from 0.86 ± 1.57 ng/mL creatinine, with a significant difference between the mean urinary level of the suburban (1.44 ± 2.20 ng/mL) and rural (0.47 ± 0.73 ng/mL). The provisional daily intake calculated based on UDON concentrations had a total mean of 52.5 ± 94.4 ng/kg bw, a rural mean of 26.8 ± 39.0 ng/kg bw, and suburban mean of 89.9 ± 133.2 ng/kg bw. All of these findings are still less than the TDI, however the data do not fit the trend of having higher mycotoxin exposure in more rural and lower income areas.

In comparison, Šarkanj et al., 2013 conducted a pilot study on 40 pregnant women in Croatia to determine exposure. The women were sub-categorized based

on location and then education level; urban (n=20), rural (n=20), university (n=18), high school (n=22). Urine was collected during the third trimester and analyzed for multiple mycotoxins including two glucuronidated forms of DON (DON-15-GlcA and DON-3-GlcA). Positive samples for DON (31/40), DON-15-GlcA (39/40), and DON-3-GlcA (33/40) had a average concentration of 18.3, 120.4, and 28.8 $\mu\text{g/L}$ respectively. Estimated equivalents of DON intake was calculated for the total, urban, rural, university, and high school groups to be 2.5, 1.8, 3.1, 1.8, 3.0 $\mu\text{g/kg}$ bw/ day and the averages ranged between 176 - 775% of TDI [Šarkanj et al., 2013].

Though these are only a few examples DON biomonitoring, a majority of them are conducted later on in the pregnancy during the third trimester. This may indicate concern for growth and fetal development issues or adverse birth outcomes (preterm birth, low birth weight, late term abortions, etc). For example, Kristensen et al., 2000, found a correlation between the increased ratio of late term abortions among grain farmers in Norway during seasonal warnings for blight [Kristensen et al., 2000]. It should also be noted that during seasonal climate periods favorable to fungal growth, pregnant woman may be exposed to both increased pesticides, especially fungicides as well as mycotoxins [Kristensen et al., 2000]. This may lead to increased adverse reproductive outcomes. Up to the time of birth as well as after, neural, endocrine, immune, and gastrointestinal micro flora systems continue to develop and can be negatively affected by DON or T-2 (more information section 5.2).

5.1.3 Additional Exposure Through Medication

Historically, ergot had been used to help speed up pregnancy but soon shifted to primarily help control bleeding during miscarriages and postpartum hemorrhaging [Bove, 1970, Raymond, 1995, CAST, 2003]. Recent use of its purified form, ergotamine is widely accepted to treat migraine headaches but may have adverse health effects *in utero* [CAST, 2003, Acs et al., 2006]. Ergotamine has the ability to

cross the placental barrier and has a variety of effects on the vascular system and will increase uterine motor activity [Rall and Schleifer, 1985, Raymond, 1995]. Pregnant rats were used in an animal study by Sch/'o et al., 1975 to determine the effect of ergotamine. Between days 4 and 19 of gestation, 10 mg/kg bw ergotamine tartrate was orally administered; resulting effects between day 11 to 14 (max) showed increased prenatal mortality, while days 13 to 16 presented with cleft palate and limb defects. The deformities are suggested to be characteristically similar to the anomalies that occur as a result of uterine vessel clamping [Schon et al., 1975].

A case study/clinical report by Hughes & Goldstein 1988 hypothesized that birth defects may result from vascular occlusion due to ergotamine therapy during pregnancy. The subject of their study was an infant born at term with microcephaly and underdeveloped, contracted lower limbs to a mother who was treated for migraine headaches with a concoction of ergotamine and caffeine during the first 14 weeks along with propranolol during and up to 20 weeks. This case study was small and based on a combination of toxins, however, it provides some insight into the contraindication for using ergotamine itself or with other drugs due to potential synergistic activity during pregnancy [Hughes and Goldstein, 1988].

There have also been studies that look at ergotamine exposure from an epidemiological view. Bánhidý et al., 2007 used data from Hungarian Case-Control Surveillance of Congenital Abnormalities (HCCSCA) from 1980 to 1996 to evaluate the potential associations between ergotamine treatment during pregnancy and the incidence of low birth weight and preterm births [Acs et al., 2006]. The study found a significant decrease in gestation time and birth weight and an increase in frequency of low birth weight newborns and preterm births of those treated with ergotamine compared to controls. Additionally, researchers found the greatest of these instances to occur with ergotamine treatment in the third trimester and with male fetuses. They suggest low birth weight and preterm birth as a result of ergotamines

dose-dependent effect on the placenta [Acs et al., 2006].

Live stock and other animals may still be exposed to ergot alkaloids through contaminated feed and tall fescue grasses and as such can experience neurological effects and abortions [Klotz, 2015]. Fortunately, there is very little chance for ergot exposure through human diet today, however, its daily use in the form ergotamine as medicine poses its own risks. Based on circumstantial evidence from animal studies and available clinical/case studies, it has been suggested that ergotamine has teratogenic potential and, therefore, should be avoided throughout the duration of pregnancy, if possible, in order to avoid birth defects [Rall and Schleifer, 1985, Raymond, 1995, Schon et al., 1975, Acs et al., 2006].

5.2 Infants and Children

Mycotoxin exposure in infants and young children is different than that of adults. They are in an important phase of life for growth and development and, as such, could be more at risk to certain mycotoxins than adults due to many physiological differences. Infants and children are still developing critical pathways for metabolism and their higher food consumption to body weight ratio may increase their exposure and risk to negative health outcomes [CAST, 2003, Klaassen et al., 1996, Cappozzo et al., 2017]. The diet of infants and young children revolve heavily around milk which can be potentially contaminated with several mycotoxins, especially AFM1. Most experiments with young animals have shown higher sensitivity to mycotoxins [Smith et al., 2012]. Several studies suggests that aflatoxins and trichothecenes can affect growth and immune function [Smith et al., 2012].

5.2.1 Infants, Breast Milk, and Infant Formulas

It is strongly recommended that infants should consume breast milk exclusively for the first 6 months. Breastfeeding should then continue for up to a year or more with the addition of complementary foods [Rossen et al., 2016]. Evidence for this advisory has been supported by many studies that compare health outcomes of

infants who were breastfed exclusively and those that were never breastfed or had supplemental formulas and food too early [Rossen et al., 2016]. Breastfed infants are associated with lower risk of developing atopy, GI disorders (inflammatory bowel disease and celiac), obesity, allergies and diabetes mellitus. Prior to the 19th century, the alternative to breast milk for infants was animal milk, however, technological advancements in food preservation lead to the development of evaporated milk in the mid to late 1800s [Stevens et al., 2009]. Soy infant formula was later developed and produced in the 1920s as an alternative for infants that exhibited allergies towards lactose [Fomon, 2001, Stevens et al., 2009]. Over the past several decades, globalization, industrialization, unregulated marketing, and government control of food (i.e. the Special Supplemental Nutrition Program for Women, Infants, and Children 'WIC'), helped artificial infant formula rise in opposition to breastfeeding [Kent, 2015, Stevens et al., 2009].

There are different kinds of artificial infant formulas commercially available which are milk (cow, goat) based or soy based and come in liquid (ready to use) or powdered form. The processing of milk products through pasteurization, concentration, or spray drying for infant formulas has conflicting results on its effect on the concentration of AFM1, and those showing alteration to concentration levels have been reported to be insignificant [Fink-Gremmels, 2008]. According to the CDC Breastfeeding report card, the percentage of breast fed infants in the U.S. has increased by almost 20% over the past two decades, as breastfeeding rates at 60% in 1994 have risen to 81.1% in 2013 [U.S.EPA, 2011, Li et al., 2002, CDC and National Immunization Survey, 2016]. It is important to mention that these estimates can obscure socio-demographic and cultural differences and represent the percentage of women that have partially breastfed and not necessarily those sticking to the strict 6 month exclusion period [Rossen et al., 2016]. For milk, the carry-over of mycotoxins from feed to milk is the percentage of the recoverable toxic compound that has been

excreted in the milk. Reviews by Fink-Gremmels 2008 and Becker-Algeri et al., 2016 reported the feed to milk carry-over percentages in cows for DON (0.0001 - 0.0002%), T-2 (0.05 - 2.0%), FB1 (0.05%) ZEA (0.06 - 0.08%), OTA (0.03%), and AFM1 (2.0 - 6.2%). Aside from AFM1, the carryover is generally considered to be very low, and therefore not regulated in milk products, however, AFM1 is regulated by the FDA (0.5 $\mu\text{g}/\text{kg}$) and the EU (0.025 $\mu\text{g}/\text{kg}$ for infant formula and 0.05 $\mu\text{g}/\text{kg}$ other) in milk products sold [Fink-Gremmels, 2008, Becker-Algeri et al., 2016].

This carry-over occurs through lactational transfer where lipophilic chemicals can penetrate membrane barriers and concentrate in milk fat globules [EFSA Scientific Committee et al., 2017]. Fat content is a major determinant of mycotoxin level in milk, so the concentration of mycotoxins can vary at different periods of lactation in addition to other factors like diet, season, and volume production [Fink-Gremmels, 2008, Warth et al., 2016].

The milk to plasma ratio (M/P) for human or cow is the concentration of the mycotoxin in milk divided by the concentration of mycotoxin in maternal plasma. This ratio can help find the concentration of mycotoxin in milk and can be used to estimate the infants mycotoxin dose excreted from their mother [Warth et al., 2016]. It is important to consider the difference between ruminants and humans for milk carryover in respect to metabolism and milk processes. However, given that information on food to milk mycotoxin carryover in humans is more limited, the percentages for cow milk can provide a guiding estimate on what to expect [Warth et al., 2016]. Mycotoxins that have a longer half-life, less protein binding, lower molecular weight, high bioavailability, greater tissue distribution, and an overall higher M/P ratio are more likely to have greater transfer into milk [Fink-Gremmels, 2008, Degen et al., 2017].

Degen et al., 2017 recently assessed how maternal intake of mycotoxins at TDI relates to a nursing infants exposure [Degen et al., 2017]. This is important to

understand because while the TDI may be acceptable for the mother, it may not be for infants who are consuming excessive quantities of milk as their only form of nutrition. Through a series of calculations they determined the concentrations of mycotoxins AFB1, CIT, DON, FB1, OTA, PAT, T-2/HT-2, and ZEA in breast milk at TDI for single-dose and AFB1, CIT, OTA, and ZEA for a continuous daily intake dose. The concentrations in breast milk were then multiplied by the average amount of milk (0.185 L) consumed per kg of body weight for the given age range to give infant daily dose (IDD) values for a mothers single dose and continuous daily dose [Degen et al., 2017]. Given the relatively undeveloped excretory function of an infant (up to 16 weeks of age), the IDD was then divided by a factor of 3 to adjust for age [Degen et al., 2017]. The IDD/corrected TDI (ratio) values for the single maternal dose and of AFB1, CIT, DON, FB1, OTA, PAT, T-2/HT-2, and ZEA are 0.01/0.228 (0.04), 0.1/66.6 (0.002), 651/333 (1.96), 0.23/666 (0.0003), 1.33/5.8 (0.23), 296/133 (2.2), 2.55/33 (0.08), and 1.2/83 (0.02) ng/kg bw/day respectively. The IDD/infant corrected TDI (ratio) for the continuous daily maternal doses of AFB1, CIT, OTA, and ZEA were 0.04/0.228 (0.17), 0.09/66.6 (0.001), 314.2/5.8 (54.2), and 0.08/83 (0.001) ng/kg bw/day. From their data, the authors indicate that the IDD for DON and PAT exceed the acceptable TDI for single doses, while continuous maternal intake of OTA means an IDD that greatly exceeds the corrected infant TDI [Degen et al., 2017]. It is therefore suggested that the TDI for OTA

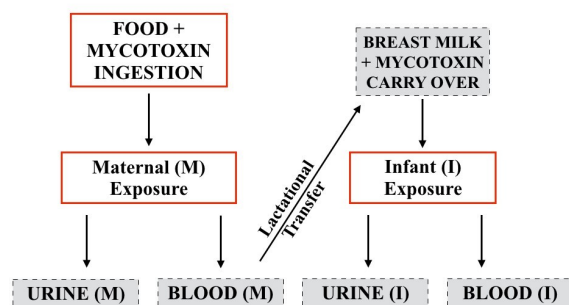


Figure 5.2: Lactational transfer (adapted from [Warth et al., 2016])

should be re-evaluated for nursing mothers [Malir et al., 2013, Degen et al., 2017].

Not unlike the idea of wet nurses from previous centuries, human milk banks have been increasing over the past decades, with 27 in North America and several hundred internationally (locations can be found and explored [HMBANA, 2016]). First donors are thoroughly tested and screened for a variety of physical health factors and then the milk is also regularly tested and processed for bacterial pathogens. However, there is very little information at all on whether or not they test for mycotoxins in donated breast milk [for Clinical Practice at NICE (UK et al., 2010)]. Donor milk is generally received by preterm infants and neonates (less than 4 weeks), who may be at an increased risk of exposure to mycotoxins. Therefore, it would be beneficial to incorporate biomonitoring and detection methods for mycotoxins into the donated milk screening process (and of the donors, as well), particularly in areas that are known to have higher incidences of exposure. A study by Navas et al., 2005 used HPLC-FD to detect and quantify AFM1 and OTA in samples of milk from the Human Milk Bank of the Southern Regional Hospital, So Paulo, Brazil. Of the 20 samples they analyzed, only 1 had detectable levels of AFM1 (20 ng/L) and 2 for OTA (10 and 20 ng/L). They suggested that other Human Milk Banks throughout Brazil should also be tested [Navas et al., 2005]. A follow up to this study was conducted by Iha et al., 2014 with 100 samples from a Human Milk Bank of the Hospital das Clinicas - USP, in Ribeiro Preto City during June 2011 to Aug 2012. This is predominantly agricultural region of So Paulo, Brazil [Iha et al., 2014]. For this study they used LC-FD and the LOD and LOQ for both mycotoxins was 0.3 ng/L and 0.8 ng/L respectively. The analysis showed 66 out of 100 samples had OTA ranging from 1 - 21 ng/L with an average of 4 ng/L. Only 2 out of 100 samples had AFM1 at 0.3 and 0.8 ng/L with one of them also containing OTA at 1.1 ng/L. These concentrations were within legal limits.

As mentioned previously, formula cow milk and soy milk are alternatives. It has

been reported that 25% of U.S. new borns consume soy infant formula, which may pose chronic health issues due to a combination of phytoestrogens, mycoestrogens (ZEA) and estrogenic isoflavins [Patisaul and Jefferson, 2010]. There are very few reports or information on occurrence or detection of ZEA in soy based infant formula, however, the EU guidance level for ZEA contamination in infant formula/food should not exceed 20 ng/mL [Warth et al., 2016, EFSA CONTAM Panel et al., 2017].

Cappozzo et al., 2017 and Mitchell et al., 2017 conducted a two year survey that investigated the occurrence of OTA in infant cereal and formula in the US market. They collected both conventional and organic forms of milk, soy based formula, and cereals (barley, rice, oat, wheat, and mixed grain) to be analyzed. Based on LC-MS/MS, the LOD and LOQ for infant formula was 0.1 ng/g and 0.25 ng/g while infant cereals was 0.1 ng/g and 0.5 ng/g. Over the two year period, there was no OTA detected in US infant formulas. However, OTA was detected in 30% (first year) and 31% (second year) of infant cereals at levels above the European Unions established limit (0.5 ng/g) for this food type. Similarly, oat and rice based breakfast cereals were found to exceed the EU limit for cereal products not intended for infant. There was no significant difference between the contamination levels in organic versus conventional foods. Oat based infant cereals and oat based breakfast cereals were found to have the highest occurrence of OTA (30/51 and 142/203) [Cappozzo et al., 2017, Mitchell et al., 2017]. In Brazil, AFM1 was detected in 43.8% of the infant formula powder samples 0.024 ± 0.01 (n.d. - 0.046) ng/g which just barely falls below the EU limit (0.025 ng/g) [Ishikawa et al., 2016]. This study also showed that there was a lower occurrence of AFM1 in breast milk at much smaller concentrations 0.018 ± 0.005 (n.d.- 0.025) ng/g. Areas of West Africa and the Middle East had relatively high levels of AFM1 in most of their samples tested at levels that exceeded the EU and US (0.5 ng/g) regulations [Ware et al., 2017, Raad et al., 2014]. In Serbia, Torović et al., 2018 tested samples of juices and purées

intended for infants and children for PAT. 44% of the juice samples and 16% of the purée samples were contaminated with 8.3 and 7.7 ng/g respectively and falls just below the EU regulation of 10ng/g for PAT in any product intended for infants and children, while 43% of the juice samples for children had around 30.2 ng/g which exceeds 10 ng/g [Torović et al., 2018]. The PMTDI for PAT is 0.4 $\mu\text{g/kg}$ bw/day, however, WHO/JECFA estimates that the daily intake for children is 0.2 $\mu\text{g/kg}$ bw/day which is 50% of what is deemed tolerable More examples are shown in the table 5.3; and two studies show the occurrence of multiple mycotoxins in formula and cereal which is a cause for more concern due to possible synergistic activity of mycotoxins [Ware et al., 2017, Assunção et al., 2016].

Table 5.3: Mycotoxins in food meant for infants and young children. Occurrence and concentration of mycotoxins in food products meant for infants and young children.

Region	Toxin	Matrix (Occ.)	Conc. in Matrix Mean/Avg. (Range)	EU limit ng/g	EDI of infant/child Mean/Avg. (Range) ng/kg bw/d	Ref.
Central Mexico	AFM1	Breast Milk ALL: (100/112) Winter (20/20) Spring (35/42) Summer (45/50)	ALL: 10.35 (3.01-34.2) ng/L 12.78 ng/L 12.09 ng/L 7.91 ng/L	-	2.35	(Cantú-Cornelio et al., 2016)
Brazil	AFM1	Breast Milk (5/94)	0.018 \pm 0.005 (n.d.- 0.025) ng/g	-	0.018-0.069	(Ishikawa et al., 2016)
		Infant Powder Formula (7/16)	0.024 \pm 0.01 (n.d. - 0.046) ng/g	0.025	0.078-0.306	
USA	OTA	Infant Formula: Milk (0/53) Soy (0/45)	-	0.5	0.007	(Cappoozzo et al., 2017, Mitchell et al., 2017)
		Infant Cereal: Barley (1/9) Oat (30/51) Rice (2/54) Mixed (2/6)	Barley: 14.4 ng/g Oats: 0.6 - 22.1 ng/g Rice: 1.4 ng/g Mixed: 1.2 ng/g	0.5	ALL: 1.77 Barley: 0.02 Oats: 0.81 Rice: 0.09 Mixed: 0.042	
		Breakfast Cereal: Corn (15/103) Oat (142/203) Wheat (38/117) Rice (10/66)	Corn/Wheat 0.1-0.5 ng/g Oat: 0.1-9.3 ng/g Rice: 0.1-1.5 ng/g	0.5 (infants) and 3	Corn: 0.01 Oat: 0.43 Wheat: 0.02 Rice: 0.002	
Jordan	AFM1	Infant formula (20/20)	0.12026 (0.01655-0.15414) ng/g	0.025	-	(Omar, 2016)
Serbia	PAT	Infant Fruit Juices: 21/48 Infant Purée: 11/66 Children's Juice: 43/100	8.3 $\mu\text{g/kg}$ 7.7 $\mu\text{g/kg}$ 30.2 $\mu\text{g/kg}$	10	-	(Torović et al., 2017)
Africa, Ouagadougou (Burkina Faso)	AFB1 OTA FB1	Infant Formula: AFB1 (83.9%) OTA (7.5%) FB1 (1.5%)	AFB1: 3.8 (0-87.4) ng/g OTA: 0.1 (0-3.2) ng/g FB1: 30.3 (0-672.9) ng/g	AFB1: 0.025 OTA: 0.5 FB1: 200	-	(Ware et al., 2017)
Portugal	PAT OTA	Cereals: PAT (15/20) OTA (10/20) PAT+OTA (8/20)	PAT: 2.33 (n.d.-4.50) ng/g OTA: 0.061 (n.d.-0.263) ng/g	PAT: 10 OTA: 0.5	PAT: 3.59 OTA: 0.06	(Assunção et al., 2016)

5.2.2 Growth and Development in Children

Growth and development in children is dependent on several interrelated factors such as nutrition, hormones (i.e. GH, insulin), environment, exposure to toxins and medications, and recurrent or chronic illness [Smith et al., 2012]. Thus the pathogenesis of growth stunting in children has been tied to environmental enteropathy which may be caused by intestinal disruption from chronic exposure to AFB1, DON, and FB1 amongst other things [Smith et al., 2012]. Several studies have shown that AFB1, DON, and FB1 alone and together are able to decrease TEER (transepithelial electrical resistance) values, transcription and expression levels of TJ (tight junction) proteins and increase permeability, translocation as well as up-regulate immune cell and cytokine expression [Bracarense et al., 2012, Gao et al., 2017, Chen et al., 2016, Vandenbroucke et al., 2011]. Stunting can be associated with chronic malnutrition while the occurrence of being underweight can indicate acute malnutrition [Gong et al., 2002]. Enteropathy in young children has been associated with chronic immune stimulation and is inversely correlated with growth [Smith et al., 2012, Turner et al., 2007].

A study conducted by Gong et al., 2002 studied 480 children (9 months to 5 yrs) from 16 villages in West Africa. They detected AF-albumin adducts in 475 samples that had a GM of 32.8 pg/mg. From ages 1-3 they found a steady increase in AF-alb concentration, and that children who were weaned had almost 2.5 times the concentration of those who were still being breastfed. Underweight children with stunting displayed 30-40% greater AF-alb concentration as well. The authors suggest that the weaning process and transition to solid foods in AFB1 prevalent area and co-exposure with other infectious issues may result in growth faltering [Gong et al., 2002]. Castelino et al., 2015 conducted a study using samples from 199 schoolchildren in Kenya that were analyzed for AF-alb, IGF1, and IGF-binding protein-3 (IGFBP3) with ELISA. They found that levels of AF-alb were inverse

to IGF1 and IGFBP3, IGF1 and IGFBP3 was associated with height and weight, and those with highest AF-alb levels (>198.5 pg/mg) were shorter than children with the lowest level of exposure (<74.5 pg/mg). They then conducted an *in vitro* test using HHL-16 (human hepatocyte cell line) treated with $0.5\text{--}20$ $\mu\text{g/mL}$ for 24–48 hours and measured the IGF1 and IGFBP3 gene expression by qPCR, which revealed that AFB1 down-regulated the genes and protein levels of IGF [Castelino et al., 2015]. A study conducted by Kimanya et al., 2010 revealed that children with FB1 intake that exceeded the PMTDI had reduced height and weight than those who had consumed less FB1 [Kimanya et al., 2010]. Several studies have shown that DON can decrease growth and down regulate IGF1 and IGFBP3 (insulin like growth factor binding protein acid labile subunit) [Amuzie and Pestka, 2009a]. Studies have also shown that DON decreases glucose absorption by inhibiting the specific transport proteins as well as reducing the uptake and transfer of vitamin B12 in different animal models [Hunder et al., 1991, Maresca et al., 2002, Amuzie and Pestka, 2009a]. No studies have been conducted that correlate child stunting with DON, however, in Belgium, the UK, and China children exposure to DON ranges from 33–69.7% [Heyndrickx et al., 2015, Papageorgiou et al., 2018, Deng et al., 2018]

Some of the major effects of ZEA in children target the reproductive systems and interrupt organ structure and function. In the late 70s and early 80s there were increased epidemics of central precocious puberty and premature thelarche (onset of breast development) in Northern Italy and Puerto Rico that was attributed to the consumption of meat tissue and dairy that was contaminated with anabolic es-

Table 5.4: Urinary DON biomarker analysis for children and adolescents.

Country	Study Sample	Matrix	Occurrence	Concentration Mean (Range)	EDI Mean (Range)	% in excess of TDI	Ref.
Belgium	Child (3–12 yrs): 155	U OTA	OTA: 79/155 (51%)	OTA: 83.2 ng/mg creatinine	OTA: 5.41 (0.2 - 100) ng/kg bw/d	1% (1/71)	(Heyndrickx et al., 2015)
		U DON	DON: 109/155 (70%)	DON: 5.5 ng/mg creatinine	DON: 3.26 (0.17–19.57) $\mu\text{g/kg bw/d}$	69% (100/144)	
UK	Child (3–9 yrs): 40	U DON	~74/79 (>95%) over 2 days	41.6 (8–219) ng/mg creatinine	0.97–1.95 (0.14–9.28) $\mu\text{g/kg bw/d}$	33–63% (13–25/40)	(Papageorgiou et al., 2017)
	Adol (10–17yrs): 39			21 (1.8–95.6) ng/mg creatinine	0.47–0.95 (0.03–2.44) $\mu\text{g/kg bw/d}$	5–46% (2–18/39)	
China	Child (2–12 yrs): 33	U DON	33/33 (100%)	63.2 (7.42–224) ng/mL	2.09 (0.23–7.57) $\mu\text{g/kg bw/d}$	69.7% (23/33)	(Deng et al., 2018)
	Adol (13–17 yrs): 14		14/14 (100%)	73.1 (11.4–240) ng/mL	3.08 (0.58–9.62) $\mu\text{g/kg bw/d}$	85.7% (12/14)	

trogens such as zeranol (α -zearalanol) that is used to increase the growth rate in animals [Fara et al., 1979, de Rodriguez et al., 1985, Massart et al., 2008]. Bandera et al., 2011 conducted a cross-sectional analysis of 163 healthy girls from 9 to 10 years of age that were participating in a Jersey Girl Study [Bandera et al., 2011]. They used data from questionnaires, anthropometric measurements, puberty staging, dietary intake and assessments to find a relationship with the presence of ZEA mycoestrogens in urine. A subset ($n=58$) of girls had dietary recalls the day before their urine was collected. Interestingly, they found that when urinary levels were compared to food consumption on the previous day, beef and popcorn were the only food products that were related to urinary ZEA and total mycoestrogens. The GM of total mycoestrogens was 7.11 ng/mL for those who consumed beef and popcorn before urine collection and was 0.07 ng/mL for those who did not consume those products. When they evaluated anthropometric characteristics with urinary mycoestrogen, they found that mycoestrogen-negative girls were taller, had adiposity (more fat), and had reached the onset of breast development compared to mycoestrogen-positive girls who were found to be shorter and less likely to have reached the onset of breast development. These findings suggest that ZEA may have an anti-estrogenic effect [Bandera et al., 2011]. The authors reviewed the importance of this study due to evidence suggesting that the peri-pubertal period is a critical window for the susceptibility and exposure to endocrine disrupting agents at this time may lead to breast cancer [Bandera et al., 2011].

5.3 Obesity, Diabetes, and other Immunocompromised Individuals

On the opposite side of the spectrum from reduced weight, being overweight and obese are risk factors for diabetes (Type 2 Diabetes Mellitus), cardiovascular disease, chronic kidney disease (CKD), cancer, and non-alcoholic fatty liver disease (NAFLD). In the US, Louisiana, Arkansas, Mississippi, Alabama, and W. Virginia have some of the highest rates of obesity [CDC, 2016a].

The average American diet (aka Western Diet) is high in fat, alcohol, meat, cholesterol and carbohydrates [Martins, 2015, Chen et al., 2016]. Glucose is taken up through cells via facilitated diffusion with glucose transporters that is stimulated by insulin binding to membrane receptors. Insulin resistance is an inability to respond to insulin which means that glucose cannot be taken into the cell or that there is excess blood glucose because hepatic glucose output cannot be turned off. Insulin resistance is a common occurrence for obese and diabetic individuals [Kahn and Flier, 2000]. As mentioned earlier, trichothecenes (DON and T-2) have been shown to affect the expression for glucose transport proteins which could be another mechanism in insulin resistance [Maresca et al., 2002, Amuzie and Pestka, 2009a]. Additionally, diets that are high in cholesterol or foods high in bacterial LPS (lipopolysaccharides are components of the outer membrane in gram-negative bacteria) and mycotoxins may promote increased absorption of mycotoxins and LPS, which can then lead to or influence hypercholesterolemia (which negatively influence cholesterol synthesis and liver cholesterol metabolism), dyslipidemia (elevated levels of plasma cholesterol that can lead to atherosclerosis), and NAFLD [Martins, 2015, Anyanwu et al., 2006]. Cholesterol is a necessary membrane component that is important to hepatocytes and neurons for proper function, as well as maintaining

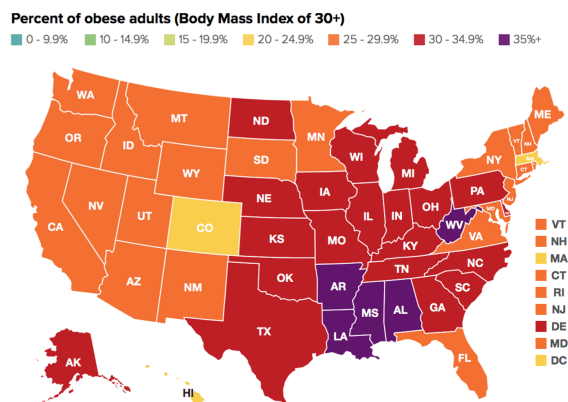


Figure 5.3: A look at obesity in the US. Percent of Obese Adults having a body mass index (BMI) greater than 30 [Trust for America's Health et al., 2017, CDC, 2016a].

synaptic connections. Therefore disruption of cholesterol homeostasis can result in blood brain barrier issues or increase neurotoxicity in the form of neurodegeneration and possible Alzheimers Disease (which is also referred to as Type 3 Diabetes) [Martins, 2015, Mittal et al., 2016]. Exposure to certain xenobiotics such as pesticides could have additive or synergistic effects that might interfere with hepatic elimination of mycotoxins, therefor preventative measures in encouraging diets that are healthy and that optimize lower absorption of LPS and mycotoxins into may help reduce neurotoxic effects [Martins, 2015].

In order to assess the effect of DON exposure in obese humans, Kobayashi-Hattori et al., 2011 used high fat diet-induced obese mice to characterize DONs impact on body weight and composition. Two subsets of high fat (HF) diets (45% and 60% kcal) were fed to female B6C3f1 mice for 94 days, then each group was divided with one half given 10 mg/kg DON added to their HF diet for 54 more days. A control group had a low fat diet (10% kcal) for 148 days. Their results showed that within 11 days of the addition of DON to the HF diets, the treated mice had decreased body weight and fat mass to a level comparable to control group, along with decreased food consumption. Using ELISA and RT-PCR they found that DON reduced plasma insulin, leptin, IGF1, IGFALS, and increase hypothalamic mRNA levels of orexigenic peptide agouti-related protein (AgRP) [Kobayashi-Hattori et al., 2011].

Individuals with inflammatory diseases such as Celiac disease (CD), irritable bowel syndrome, and gluten sensitivity (GS) have been linked with impaired intestinal barrier from decreased amounts of tight junction proteins or misconfiguration of their proper distribution [Akbari et al., 2017]. Gluten is a protein found in varieties of wheat, barley, and rye. For CD, the ace in glutens tool box is the immunogenic peptide gliadin, because of its resistance to pancreatic and enzymatic digestion [Proietti et al., 2013, Hausch et al., 2002]. Under healthy conditions, gliadin is unable

to pass into the lamina propria, however when tight junction proteins are modified they can pass through. When this happens, enterocytes will secrete immune cells signals and thus a cascade of events ensues resulting in the secretion of cytokines and chemokines and the recruitment of more immune cells which go on to attack the enterocyte cells resulting in an inflammatory reaction [Vanga and Kelly, 2014]. It has been reported that if left undiagnosed or untreated, CD could precipitate more autoimmune diseases, diabetes, osteoporosis, reproductive issues, neurological issues, and possibly cancer [Rubio-Tapia et al., 2012]. In the U.S., it is estimated that 3 million (1%) have CD [Rubio-Tapia et al., 2012]. Thus, it is important to understand what mycotoxins individuals who follow gluten free diets are exposed to. Dall'Asta et al., 2012 conducted a study to determine the fumonisins exposure risk in a gluten-free diet followed by adult celiac patients as opposed to non-celiac patients [Dall'Asta et al., 2012]. They collected data on dietary habits and analyzed the fumonisin level in the most frequently consumed corn-based products. Their findings indicated a higher intake of fumonisins daily for celiac patients ($0.395 \pm 0.049 \mu\text{g/kg bw}$) than non-celiac patients ($0.029 \pm 0.006 \mu\text{g/kg bw}$). The take away points from this study was the increased potential of exposure risk to gluten-free diets may consume greater amounts of maize-based products could potentially put individuals with celiac disease (or individuals who follow gluten-free diets for other health reasons) at risk. However, a case-study based in Italy conducted by Cirlini et al., 2016 collected urine samples and 7 day dietary dairies from 55 celiac patients and 50 non-celiac individuals. The urine was analyzed for DON, ZEA, and FB1 with ZEA being the most detected in both subject groups. No significant difference was found between celiac and non-celiac groups for mycotoxin exposure and both were below the TDI values for each of the detected mycotoxins. However, there are differences in diet and mycotoxin levels depending on geographical location, so the results for Italy may not represent other regions [Cirlini et al., 2016]. There is a

need for more data on nutritional intake for celiac and gluten-free sub-populations.

As mentioned previously, Hepatitis B virus (HBV) is a co-etiological agent with AFB1 for the development of hepatocellular carcinoma (HCC) [Groopman et al., 1985, CAST, 2003, Liu and Wu, 2010]. For every ng AF/kg bw/day for individuals without HBV the incidence rate is 0.01 cases of HCC per year per 100,000 people, however, there is a 2900% increase for individuals with HBV to 0.3 cases of HCC per year per 100,000 people [Groopman et al., 2005, Liu and Wu, 2010]. For the United States, the average rate of HBV per 100,000 people is just at 1.0 (s.d. 0.08), however, some states like West Virginia, Kentucky, and Tennessee had rates of 9.8, 4.0, 3.3 respectively [CDC, 2016b]. For more information on HBV rate see figure 5.4. This could mean that individuals within theses states are at an even higher risk for HCC from AFB1 and HBV exposure.

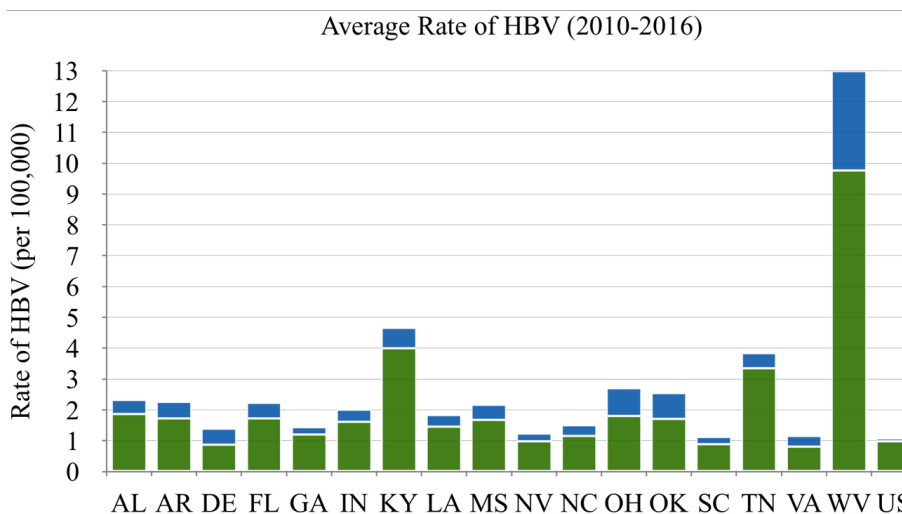


Figure 5.4: Average rate (green) and standard deviation (blue) of HBV per 100,000 people over 7 year period (2010-2016) in AL=Alabama, AR=Arkansas, DE=Delaware, FL=Florida, GA=Georgia, IN=Indiana, KY=Kentucky, LA=Louisiana, MS=Mississippi, NV=Nevada, NC=North Carolina, OH=Ohio, OK=Oklahoma, SC=South Carolina, TN=Tennessee, VA=Virginia, WV=West Virginia, and US=United States. Data from [CDC, 2016b].

5.3.1 Altered diet - Vegetarians

There are many environmental benefits associated with a vegetarian diet as it is environmentally sustainable and conscious efforts to reduce certain meat consumption (demitarian) could also improve individuals health. The production behind a vegetarian diet demands less phosphorous and nitrogen. It has been suggested that changing from the average western diet to a vegetarian diet may reduce phosphorous consumption by 20-25% [Cordell et al., 2009]. However, it would be beneficial to know the degree to which vegetarian individuals are exposed to mycotoxins and if it is within guidance values.

There are several kinds of vegetarian diets, which are devoid of flesh products but may or may not contain egg or dairy products. Lacto-ovo-vegetarian eat both egg and dairy products while lacto-vegetarians eat dairy but no eggs and ovo-vegetarians eat eggs but no dairy. Vegans on the other hand do not eat eggs or dairy products and raw vegans consume between 75-100% of uncooked vegetables, fruits, nuts/seeds, legumes and sprouted grains [Melina et al., 2016].

In the United Kingdom, a recent cohort study was conducted by Wells et al., 2017, which compared urinary DON levels and food consumption data of vegetarians and non-vegetarians over the course of two days. Specifications for vegetarians to be used in this study required that the subjects had followed this dietary pattern for more than 12 months and that they were lacto-ovo-vegetarian. Lacto-ovo-vegetarian do not eat meat or fish but do consume grains, nuts, seeds, fruits, vegetables, dairy products, and eggs. In the study, DON tested positive in 100% of non-vegetarian adults while only 77.8-90.5% for vegetarians, which the authors suggested was counter intuitive being that vegetarians are considered to be a high risk group. Additionally, they reported that female vegetarians tested positive for urinary DON more often than males. On day 1 and 2 of the study, non-vegetarians had mean levels of 3.05 ng DON/mg creatinine and 2.98 ng/mg respectively, with

an estimated intake of 422 ng/kg bw/day and 537 ng/kg bw/day which was within TDI levels. Vegetarians on the other hand, had 6.69 ng DON/mg creatinine followed by 2.42 ng/mg, this meant that intake of DON was 855 ng/kg bw/day of DON and 654 ng/kg bw/day, suggesting that 32% of UK vegetarians exceed the TDI for DON [Wells et al., 2017].

There are no biomonitoring studies that monitor mycotoxin exposure in America. Based on data from the Continuing Survey of Food Intake by Individuals (CSFII) in 1994-1996 and 1998, around 13,341 participants aged 6 and older were asked if they define themselves as vegetarians [Haddad and Tanzman, 2003]. Approximately 2.5% of the respondents identified as vegetarians. According to 2016 online poll conducted by the Vegetarian Resource Group approximately 3.3% of the American population identifies as vegetarian (which also includes vegan) based on 2,015 responses from adults over the age of 18 (for more details on this online poll, see [Vegetarian Resource Group, 2016]). Food consumption studies have shown that vegetarians may consume more grains and nuts than non-vegetarians [Haddad and Tanzman, 2003]. Additionally, the increased consumption of soy based products could also increase exposure to estrogenic chemicals like ZEA and isoflavones [Messina and Messina, 2010]. There is an ongoing dispute between the benefits of soy rich diet versus chronic health risks from endocrine disrupters. However, there is not enough information and more research is required [Messina and Messina, 2010, Patisaul and Jefferson, 2010]. More biomonitoring studies should be conducted on vegetarian populations for DON, AFB1, FB1, OTA, and ZEA in order to determine if there are significant exposure related differences that could lead to health problem and to inform people of possible preventative measures that could alleviate them (chemoprotection, additives, or binding agents).

5.4 Summary

Several factors determine an individual's dietary habits such as age, geography, culture, socioeconomic standing, or disease. Diets in conjunction with various differences in physiological processes can show a myriad of issues from toxic exposures. Multiple animal studies have shown that mycotoxins can be reproductively toxic both prior to conception and during gestation. Epidemiological studies where pregnant women have been exposed to doses above TDI/PMTDI have been correlated with adverse birth outcomes. Chronic low levels of exposure may result in epigenetic modifications that could lead to defects and diseases later in the life of the progeny exposed to mycotoxins during gestation. The timing that a lactating woman is exposed to mycotoxins may result in different concentrations of excreted metabolite in milk depending on milk fat content, individual clearance rate, and prior exposures. Regulations and MLs have been imposed by the FDA and EU to monitor AFM1 in dairy products that are sold on the market, however there is no such regulation of AFM1 in breast milk from human milk banks. Adding mycotoxin testing to breast milk donated to human milk banks could provide a platform in which regional monitoring for an area could inform the population of regular mycotoxin exposure in infants. There should also be more occurrence data and studies for soy milk infant formula and the potential occurrence of mycoestrogens. In light of these issues: i) more clearance and half-life data is necessary for better understanding and estimation, ii) breast milk in milk banks should be regularly tested/monitored for mycotoxins, iii) areas where foods and diets are uncharacteristically high in mycotoxins should help educate breast feeding mothers so they can adjust their intake or take precautionary measures. Young children also consume a great amount of milk and milk alternatives so occurrence studies exposure assessments in relation to other milk alternatives such as almond milk, rice milk, flax milk would also be beneficial. In children and adults, mycotoxin induced alterations in metabolic en-

ergy and nutrient uptake processes, GIT disruption, and endocrine disruption could negatively affect growth, reproductive development and processes, chronic immunodeficient complications, neurodegenerative disorders, or cancer (breast/hepatic). Public education and outreach regarding such contamination in foods is critical so that the individual be informed enough on the topic help them plan their diet or monitor their intake in such a way that prevents significant exposure that may lead to adverse health affects in the future.

Chapter 6

Conclusions

A significant chronic health risk exists due to multiple mycotoxin levels in the American diet. Human exposure to mycotoxins present in food poses a greater health risk than presently acknowledged due to combinations of different mycotoxins, which harbor potential synergistic adverse health impacts.

One of the goals of this document is to paint a solid picture of just how ubiquitous mycotoxins are in our world and how constant they are in our diet. Humans and animals are generally exposed to multiple mycotoxins in chronic low doses daily. For the most part, it is assumed that a majority of mycotoxin exposures occurs through dietary ingestion. According to a worldwide survey that looked at samples from 72 countries, 71% were co-contaminated with more than one mycotoxin detected [BIOMIN, 2018]. From the limited but increasing information available for fungal genomics, it is understood that some genes involved in mycotoxin synthesis are clustered together on chromosomes and are co-regulated under similar mechanisms [Keller and Hohn, 1997, Keller et al., 2005, Woloshuk and Shim, 2012]. Thus, multiple mycotoxins can actually be expressed under the same regulatory conditions and factors [Keller et al., 2005]. Additionally, as food items continue along the food or feed processing chain, they can find themselves infected by different fungal species (i.e. *Aspergillus spp.* and *Penicillium spp.*) that can produce more and different mycotoxins. Table 4.2 shows a range of mycotoxin concentration levels averaged from multiple studies around the world with usual conditions and worst case scenario conditions [Grenier and Applegate, 2013]. Table 4.3 and 4.4 show mycotoxin levels detected in food/feed samples after some kind of processing or transportation. The ability to detect mycotoxins depends on the sample preparation and detection method used, due in part to the chemical properties of different mycotoxins and if they are in conjugated form. Also the LOD matters a great deal because mycotox-

ins can be present in very low levels which may elude detection. There have been advances in recent years that allow multiple mycotoxin to be detected in a sample (food matrix or biological matrix) at once (see Table 4.4), however techniques for detection still need to be improved.

Diet studies and food monitoring surveys can help to understand food consumption trends at an individual and national level, and when combined with mycotoxin occurrence data can provide estimates of daily exposure intake. In Table 4.5, average adults were evaluated based on whether they consumed more than the health based guidance values. Most mycotoxins EDIs for the average American adult are below acceptable limits, (except for AFB1 and DON), however, vulnerable groups such as pregnant woman and children could be exposed to almost a double the average adult intake (see Table 6.1).

Current diet and food monitoring studies are still very limited in their ability to predict accurate estimates, and can be severely over or under estimated. Biomonitoring studies using mycotoxin biomarkers from human and animal samples can further improve the mycotoxin exposure estimates and the potential effects on the human body (see Table 5.2) with more evidence from the biological sample testing.

Acute high doses of mycotoxins that can lead to drastic mycotoxicosis diseases or high mortality rates have been seen throughout the 20th and 21st centuries in multiple countries for both animals and humans. Some of the reported outbreaks and cases have occurred in countries such as Russia (Toxic Alimentary Aleukia),

	EDI for Avg. Adult (ng/kg bw/day)	% of TDI/PMTDI for Avg. Adult	EDI for Vulnerable group (ng/kg bw/day)	% of TDI/PMTDI for Vulnerable group	Vulnerable group ID
FB1	80	4%	152-172	7.6-8.6%	NTD cases of woman along Texas-Mexico Border
DON	1000	100%	1500	150%	Child average consumption of DON
DON	1000	100%	2500	250%	Croatian pregnant woman (GEMS/Cluster G10)
PAT	100	24%	200	50%	Child average intake of PAT

Table 6.1: Comparison of Mycotoxin Exposure for Avg. Adult and Vulnerable Groups (U.S. centered information or GEMS/Food cluster alternative G10 or world wide estimates) [].

Japan (Akakabi-byo), Denmark (porcine nephropathy), Africa (aflatoxicosis), India, France, and the U.S. [CAST, 2003, Desjardins, 2003]. Acute high doses are representative of unusually high concentrations of mycotoxin level in food due to an assortment of factors that optimize mycotoxin(s) production. Different regions may have higher prevalence of certain mycotoxins synthesized over the others depending on climate, crop, or ecology, however, we live in such a globalized economy and we often eat food from different regions and places. This means that its very hard to assume that one country is only being exposed to certain contaminants. Further, even if a country or region is not exposed to acute high doses, these compounds can still be present in chronic low daily doses. It is important to realize that regardless of where an individual lives, they are very likely getting chronic low daily doses of multiple mycotoxins.

The mycotoxin itself can have different mechanisms of action such as inhibition of protein, DNA, or RNA synthesis, or the inhibition of critical enzymes (i.e. ceramide synthase) and these mechanisms generally all lead to adverse effects at the cellular level (see Table 3.2). Further complicating matters are the interactions between co-contaminating mycotoxins and other chemicals that an individual can be exposed to on a daily basis (i.e. residual pesticides, pharmaceuticals, lead). Interacting effects can be described as additive (combined effect equal to sum of the individuals), synergistic (combined effect greater than the individuals), and in some cases antagonistic (combined effect less than the individuals). Alarmingly, The most common interactions between mycotoxins are additive and synergistic; figure 6.1 shows the combined toxicity and interactive effects between several mycotoxins in different experimental systems for cytotoxic and genotoxic endpoints. It is important to point out that major mycotoxins such as AFB1, OTA, FB1, and DON all have strong synergistic interactions with each other.

The chronic exposure to small doses of synergistic mycotoxins in the American

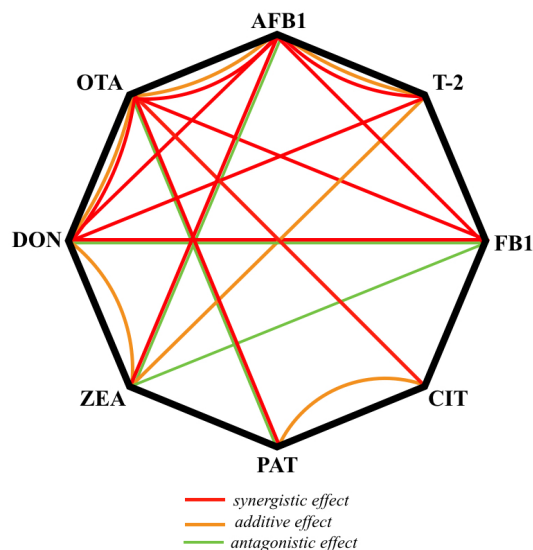


Figure 6.1: Combined toxicity and interactive effects of multiple mycotoxin studies in different experimental systems (see appendix Table A.2 for references).

diet is very alarming. However, compared to other organizations, such as the European Commission of the EU, the FDA's regulatory agenda for mycotoxins is lacking. Action levels are set limits that, if surpassed, the FDA will remove a product or substance from the market. There are action levels set for aflatoxins (see Table 6.2) and patulin (see Table 6.3). The EU, has much lower maximum limits for total aflatoxins, AFB1, and especially AFM1 in milk and dairy products for both the general public and an even lower dose for anything intended for infants and children. The action levels for animal feed in the US range from 20-300 ppb, with 20 ppb for feed intended for dairy cows and immature animals and 300 ppb for finishing feed. The EU on the other hand sets the maximum AFB1 level in any feed at 20 ppb. Patulin has similar levels in both the FDA and the EU, however, the EU requires any product designated for the consumption of small infants and children to be below 10 ppb.

The FDA currently has advisory levels for DON (see Table 6.4), which means that should the contamination level exceed a certain limit, they can take action, however, they also might not. This is not exactly reassuring. There are also guidance levels

Table 6.2: Regulatory Levels for Aflatoxins by FDA and EU [Council of European Union, 2006].

USA: Action Levels (ppb)	Tot. AF	AFB1	AFM1
All Food:	20	-	-
Nut, peanut, and peanut products:	20	-	-
Milk/Dairy:	-	-	0.5
Corn/peanut products for finishing feed for beef cattle and cottonseed meal beef cattle, swine, poultry:	300	-	-
Corn/peanut products for finishing feed for swine less than 100 lbs:	200	-	-
Corn/peanut product for breeding/mature animals:	100	-	-
Corn/peanut/cottonseed meal products for immature animals and all feeds/ingredients for dairy animals:	20	-	-
European Union: Maximum Levels (ppb)	Tot. AF	AFB1	AFM1
All cereals (maize and rice), groundnuts, tree nuts, oilseeds, dried fruit:	4	2	-
Milk/Dairy:	-	-	0.05
Infant formula; foods for infants/kids:	-	0.1	0.025
All feed material:	-	20	-
Complementary/complete:	-	10	-
Compound feed for dairy and immature animals:	-	5	5

for combined FB1+FB2+FB3 (see Table 6.5), but these guidance levels again have no enforcement behind them. The FDA only considers 4 mycotoxins (and ergot) to be of any importance, regulatory-wise. The EU, on the other hand, considers 10 different mycotoxins and are consistently updating their regulatory limits for mycotoxins as more information emerges. Further, they follow a consistent pattern of setting much lower limits for products destined for infants and children, as well as immature, breeding, and dairy animals.

The reason for the difference in the EU and US approach to mycotoxin regulation is how they consider the problem. The FDA is considering single mycotoxins by themselves, whereas the EU may be considering the whole picture, which allows for multiple exposures to multiple mycotoxins in chronic low doses every day. This

Table 6.3: Regulatory Levels for Patulin by FDA and EU [Council of European Union, 2006].

USA: Action Levels- Food (ppb)	PAT
Apple juice, apple juice concentrate, and apple juice as food ingredient:	50
European Union: Maximum Levels- Food	PAT
Fruit juices and drinks with apple juice:	50
Solid apple products (apple purée):	25
Apple juice and apple products for infants/young children:	10

Table 6.4: Regulatory Levels for Deoxynivalenol by FDA and EU [Council of European Union, 2016].

USA: Advisory Levels-Food, Guidance Levels-Feed (ppb)	DON
Finished wheat products for human consumption:	1000
Grain for ruminative beef and feedlot cattle > 4 months:	10000
Grain and by-product for swine:	5000
Grains and by-products for other animals:	5000
European Union: Maximum Levels- Food, Guidance Level-Feed	DON
Unprocessed cereals (excluding durum, oats, and maize):	1250
Unprocessed durum wheat, oats, and maize:	1750
Cereal flour, maize flour, grits, maize meal, and dry pasta:	750
Bread, biscuits, pastries, cereal snacks and breakfast cereals:	500
Processed cereal based baby/infant food:	200
Cereals and cereal products with the exception of maize by-products:	8000
Maize by-products:	12000
Complementary & complete feedstuffs:	5000
Complementary & complete feedstuffs for swine:	900
Complementary & complete feedstuffs for calves, lamb, kids, dogs:	2000

difference in approach could explain why the EU has much lower limits on mycotoxin exposure. Therefore, the FDA needs should update and reevaluate and consider lowering mycotoxin regulatory limits as well as add regulations for more mycotoxins.

Research funding for human biomonitoring could further help understanding the effects of life long exposure to chronic low doses of multiple mycotoxins and

Table 6.5: Regulatory Levels for Fumonisin by FDA and EU [Council of European Union, 2010].

USA: Guidance Levels (ppb)	FB1+ FB2+ FB3
Degermed dry milled corn products (i.e. flaking grits, corn meal) :	2000
Cleaned corn for popcorn :	3000
Whole/partially degermed dry milled corn products; dry milled corn bran; cleaned corn for mass production:	4000
Corn and corn byproduct for equids and rabbits:	5000
Corn and corn byproduct for swine and catfish:	20000
Corn and corn byproduct for breeding animals (ruminants, poultry, mink), dairy animals, and laying hens:	30000
Ruminants > 3 months raised for slaughter and mink for pelt production:	60000
Poultry for slaughter:	100000
All other species/classes of livestock and pet animals:	10000
European Union; Maximum Levels- Food, Guidance Level-Feed	FB1+ FB2
Unprocessed maize (not for wet milling):	4000
Maize foods for direct human consumption:	1000
Maize based breakfast cereals and snacks:	800
Processed maize based foods and baby foods for infant/young children:	200
Maize and maize based products:	60000
Complementary & complete feedstuffs for pigs, horses, rabbits, and pets:	5000
Complementary & complete feedstuffs for fish:	10000
Complementary & complete feedstuffs for poultry, calves (<4 months) , lambs, and kids:	20000
Complementary & complete feedstuffs for adult ruminants (>4 months) and mink:	50000

may be especially beneficial to pin point areas, populations, or both that are at higher risk from mycotoxin exposure. Southwest Texas along the Mexican border is such an area in the United States that have a routinely high precedence for mycotoxin occurrence and livestock disease from mycotoxin exposure. Populations that have been discussed in this thesis that are more susceptible to chronic low level mycotoxin co-exposure are infants, children, pregnant or lactating women, and individuals with viral infections (i.e. HBV) or disease (i.e Celiac disease). Important observational and biomonitoring areas should be states like West Virginia, Kentucky, and Tennessee because they exceed the national average rate of HBV incidence (see Figure 5.4). Obesity also enhances the deleterious effects of mycotoxin exposure, and therefore areas that are known to have high BMIs (see Figure 5.3) could benefit from mycotoxin biomonitoring. There are human milk banks all around the U.S. that take in breast milk and screen the mothers and test the milk for various contaminants already, therefore its seems prudent to add an additional mycotoxin test to the screening process. This would be beneficial to monitor the level of mycotoxins the neonates and preterm infant recipients of said breast milk and to use an already existing system to provide critical information on human mycotoxin exposure in two vulnerable populations. Research from human biomonitoring programs could help inform and encourage policy makers to change regulatory limits as necessary as well as educate the public so that they can make better consumer choices.

Efforts to educate the public about the chronic persistence of mycotoxins in daily diet as well as make mycotoxin occurrence data information available for consumer information would be beneficial. Simply stating that certain mycotoxins are present in the product on the product label would be a step forward. Gluten for example is on the product label and now the gluten free industry is projected to be worth over 7.5 billion USD world wide by 2020 [Masih, 2018]. In this way a combination of regulatory and dietary guidelines might help influence consumer choice, which may

help change our agricultural processes. Education gaps about mycotoxins among the agricultural industry and public could be bridged so that people are more aware of this chronic problem, and come up with helpful ways to mitigate mycotoxin occurrence and exposure. This could hopefully encourage mycotoxin detection levels to be made more public and updated for U.S. products.

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Appendix A

Supplementary Materials

A.1 Chapter 1 Supplementary Figures

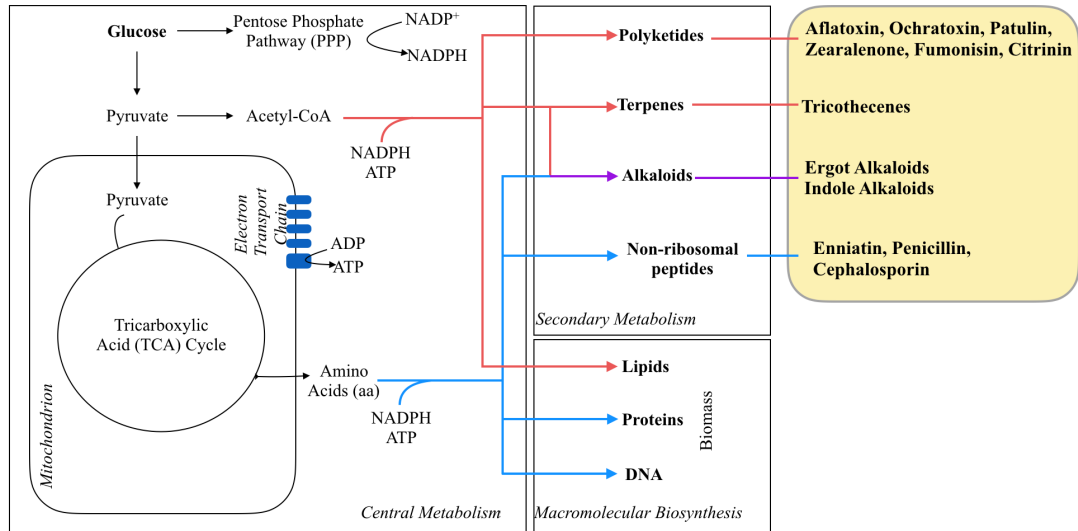


Figure A.1: Fungal cell metabolism chart showing secondary metabolism pathways and products (modified from [Nielsen and Nielsen, 2017]).

A.1 Mycotoxin Structure & Characteristics

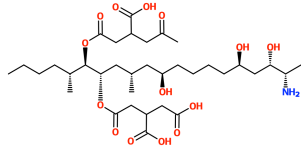
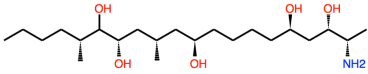
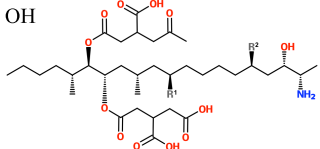
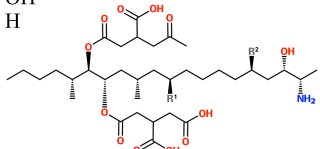
Fumonisin		MW(g/mol)
FB1		721.838
HFB1		
FB2	R1: H R2: OH 	705.839
FB3	R1: OH R2: H 	705.839

Figure A.2: Fumonisin derivatives and molecular weight.

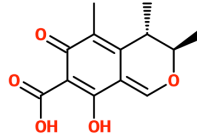
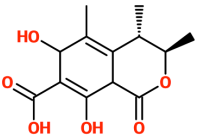
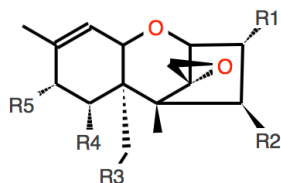
Citrinin		MW (g/mol)
CIT		250.25
HO-CIT		

Figure A.3: Citrinin derivatives and molecular weight.

Tricothecene



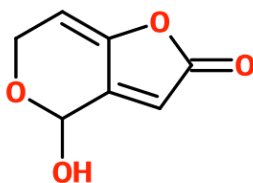
		R1	R2	R3	R4	R5	MW (g/mol)
A	T-2	OH	OAc	OAc	H	OCOCH ₂ CH(CH ₃) ₂	466.527
	HT-2	OH	OH	OAc	H	OCOCH ₂ CH(CH ₃) ₃	424.49
	DAS	OH	OAc	OAc	H	H	366.41
	NEO	OH	OAc	OAc	H	OH	382.16
B	DON	OH	H	OH	OH	O=	296.12
	3-ADON	OAc	H	OH	OH	O=	338.356
	15-ADON	OH	H	OAc	OH	O=	338.35
	NIV	OH	OH	OH	OH	O=	312.12
	FUS-X	OH	OAc	OH	OH	O=	354.13

Figure A.4: Tricothecene derivatives and molecular weight.

Patulin

MW (g/mol)

PAT



154.12

Figure A.5: Patulin derivatives and molecular weight.

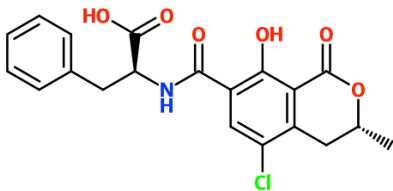
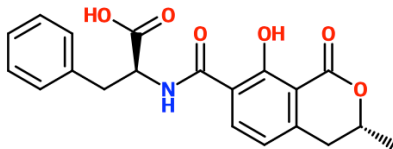
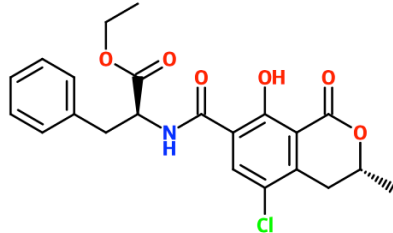
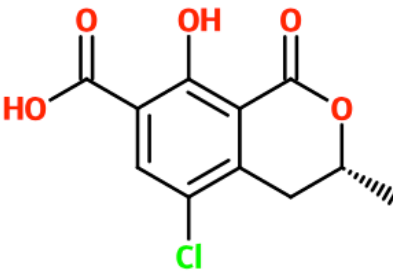
Ochratoxin		MW (g/mol)
OTA		403.81
OTB		369.373
OTC		431.869
OTα		256.638

Figure A.6: Ochratoxin derivatives and molecular weight.

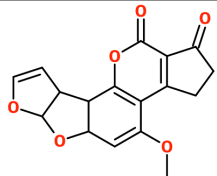
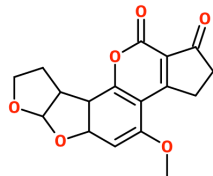
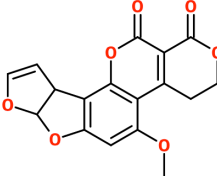
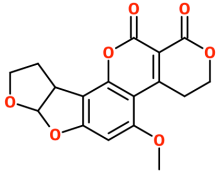
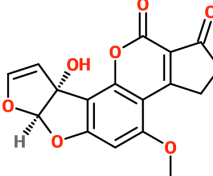
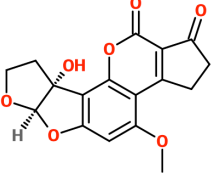
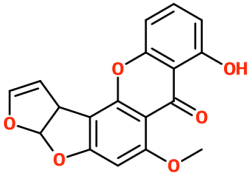
Aflatoxins		MW (g/mol)
AFB1		312.277
AFB2		314.293
AFG1		328.276
AFG2		330.292
AFM1		328.276
AFM2		330.292
STG		324.284

Figure A.7: Aflatoxin derivatives and molecular weight.

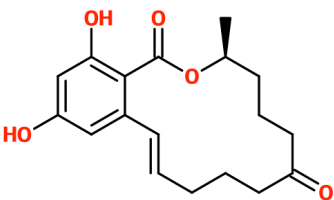
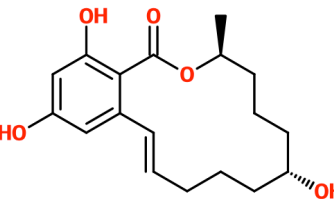
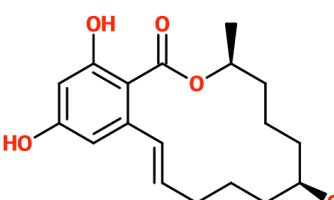
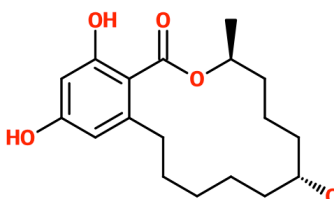
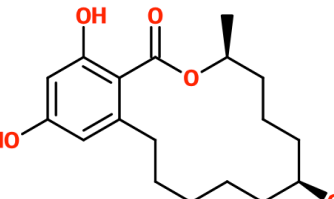
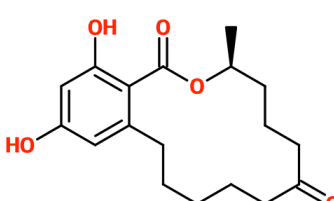
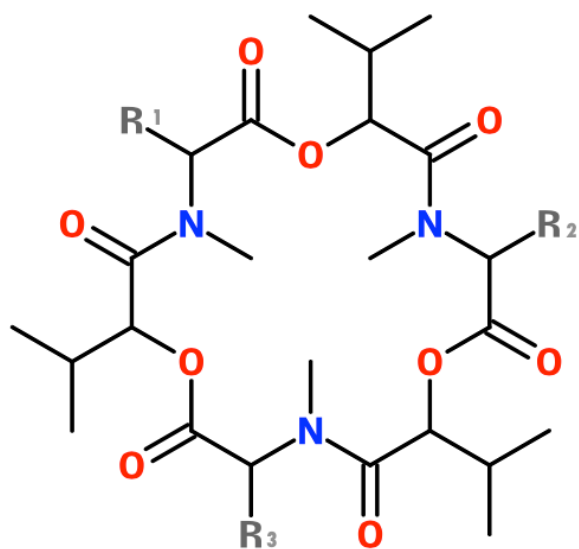
Zearalenone		MW (g/mol)
ZEA		318.3691
α -Zol/ zearalenol		320.385
β -Zol/ zearalenol		320.380
α -ZAL/ zearalanol		322.401
β -ZAL/ zearalanol		322.4
ZAN (Zearalanone)		320.39

Figure A.8: Zearalenone derivatives and molecular weight.



	R1	R2	R3	MW(g/mol)
ENA	sec-butyl	sec-butyl	sec-butyl	681.90
ENA1	isopropyl	sec-butyl	sec-butyl	667.87
ENB	isopropyl	isopropyl	isopropyl	639.82
ENB1	isopropyl	isopropyl	sec-butyl	653.85
BEA	benzyl	benzyl	benzyl	783.50

Figure A.9: Enniatins and beauvericin derivatives and molecular weight

A.2 Chapter 3 Supplementary Material

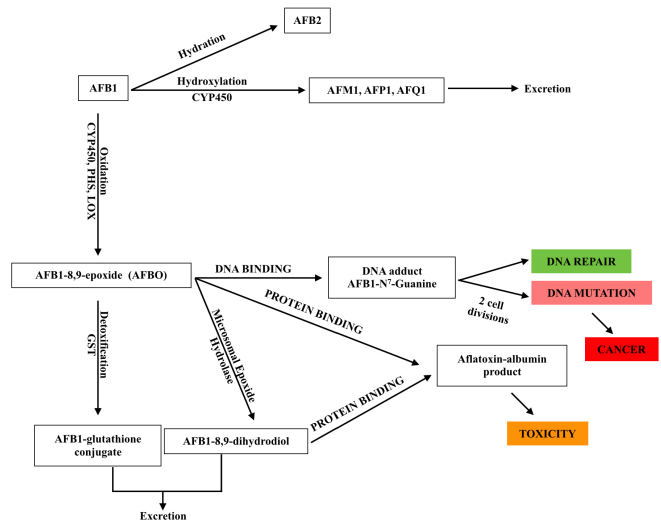


Figure A.10: Bioactivation of AFB1 (modified from [Bbosa et al., 2013])

A.3 Chapter 4 Supplementary Material

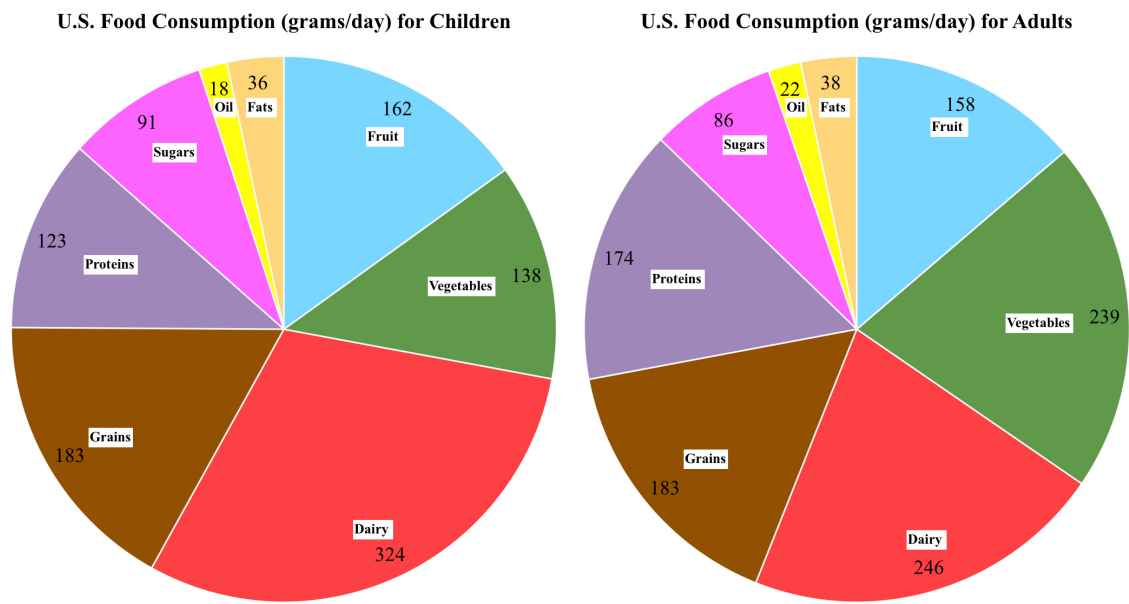


Figure A.11: Total foods consumed in g/day by children and adults. For total population consumption see figure 4.1. Source: 2007-10 National Health and Nutrition Examination Survey []

Table A.1: GEMS/Food cluster diets 2012 (modified from [World Health Organization, 2012]).

Cluster	Country
G01	Afghanistan, Algeria, Azerbaijan, Iraq, Jordan, Libya, Mauritania, Mongolia, Morocco, Occupied Palestinian Territory, Pakistan, Syrian Arab Republic, Tunisia, Turkmenistan, Uzbekistan, Yemen
G02	Albania, Bosnia & Herzegovina, Georgia, Kazakhstan, Kyrgyzstan, Montenegro, Republic of Moldova, Ukraine
G03	Angola, Benin, Burundi, Cameroon, Congo, Côte d’Ivoire, Democratic Republic of the Congo, Ghana, Guinea, Liberia, Madagascar, Mozambique, Paraguay, Togo, Zambia
G04	Antigua and Barbuda, Bahamas, Barbados, Brunei Darussalam, French Polynesia, Grenada, Israel, Jamaica, Kuwait, Netherlands Antilles, Saint Kitts and Nevis, Saint Lucia, Saint Vincent and the Grenadines, Saudi Arabia, United Arab Emirates
G05	Argentina, Bolivia, Brazil, Cape Verde, Chile, Colombia, Costa Rica, Djibouti, Dominican Republic, Ecuador, El Salvador, Guatemala, Guyana, Honduras, India, Malaysia, Maldives, Mauritius, Mexico, New Caledonia, Nicaragua, Panama, Peru, Seychelles, South Africa, Suriname, Tajikistan, The former Yugoslav Republic of Macedonia, Trinidad and Tobago, Bolivarian Republic of Venezuela
G06	Armenia, Cuba, Egypt, Greece, Islamic Republic of Iran, Lebanon, Turkey
G07	Australia, Bermuda, Finland, France, Iceland, Luxembourg, Norway, Switzerland, United Kingdom, Uruguay
G08	Austria, Germany, Poland, Spain
G09	Bangladesh, Cambodia, China, Democratic People’s Republic of Korea, Guinea Bissau, Indonesia, Lao People’s Democratic Republic, Myanmar, Nepal, Philippines, Sierra Leone, Thailand, Timor Leste, Vietnam
G10	Belarus, Bulgaria, Canada, Croatia, Cyprus, Estonia, Italy, Japan, Latvia, Malta, New Zealand, Republic of Korea, Russian Federation, United States of America
G11	Belgium, Netherlands
G12	Belize, Dominica
G13	Botswana, Burkina Faso, Central African Republic, Chad, Ethiopia PDR, Gambia, Haiti, Kenya, Malawi, Mali, Namibia, Niger, Nigeria, Senegal, Somalia, Sudan, Swaziland, United Republic of Tanzania, Zimbabwe
G14	Comoros, Fiji Islands, Papua New Guinea, Solomon Islands, Sri Lanka, Vanuatu
G15	Czech Republic, Denmark, Hungary, Ireland, Lithuania, Portugal, Romania, Serbia and Montenegro, Slovakia, Slovenia, Sweden
G16	Gabon, Rwanda, Uganda
G17	Samoa, Sao Tome and Principe

A.4 Chapter 6 Supplementary Material

Table A.2: Table of Combined Mycotoxin Effects (see Figure 6.1).

Mixture	Effect	Combined Effect	Ref.
OTA-FB1	Cytotoxicity	Synergistic	Creppy et al., 2004
OTA-CIT, OTA-PAT, CIT-PAT	Cytotoxicity	Synergistic (OTA-CIT), Antagonistic (OTA-PAT), Additive (CIT-PAT)	Bernhoft et al., 2004
OTA-CIT	Cytotoxicity, Genotoxicity	Synergistic	Bouslimi et al., 2008
AFB1-OTA	Cytotoxicity, Genotoxicity	Additive	Golli-Bennour et al., 2010
AFB1-ZEA, AFB1-DON	Cytotoxicity, Genotoxicity	Synergistic	Lei et al., 2013
AFB1-T-2	Cytotoxicity	Additive, Synergistic	McKean et al., 2006
DON-FB1	Cytotoxicity	Antagonistic	Ficheux et al., 2012
DON-T-2	Cytotoxicity	Additive, Synergistic	Ficheux et al., 2012
DON-ZEA	Cytotoxicity	Additive	Ficheux et al., 2012
T-2-ZEA	Cytotoxicity	Additive	Ficheux et al., 2012
FB1-ZEA	Cytotoxicity	Antagonistic	Kouadio et al., 2007

Vita

Adrianna Isobel Pribil was born in New Orleans, Louisiana but has lived in several places around the U.S. She has spent most of her time however on her family's farm in Houma, Louisiana. She received her Bachelor's degree in Biochemistry with minors in Chemistry and Disaster Science Management in 2015 from Louisiana State University. In August of 2016 she began her Masters degree in Environmental Science with a biophysical concentration. Isobel plans to graduate in August of 2018.