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Understanding the Influence of Changing Climate on Mycotoxin Contamination of Food and Indoor Fungi-Mediated Respiratory Illness

Mayomi Helen Omebeyinje

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UNDERSTANDING THE INFLUENCE OF CHANGING CLIMATE ON MYCOTOXIN
CONTAMINATION OF FOOD AND INDOOR FUNGI-MEDIATED RESPIRATORY
ILLNESS

by

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DEDICATION

Firstly, I dedicate this work to Almighty God for making this work a reality. To the memory of my late Mother, Mrs. Essin Mabel Omebeyinje, who, besides numerous sacrifices, believed in me and taught me to be independent as well as never to give up on my dreams.

To my dad, Mr. Samuel Koko Omebeyinje, who laid the foundation for my success and encouraged me even when I almost gave up. To my friends, Nefe Omofuma, Chigozie Nkwonta, and Bennie Ukhueduan, for being there in the darkest moments of my life. To my husband, Adewale, for leading me right and making my Ph.D. dreams come through. Finally, to my children, Tobiah and Atarah, for bringing the best out of me amidst struggle and challenges.

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ABSTRACT

Over the years, climate change has been a major core issue in public and environmental health. The World Health Organization (WHO) estimated that about 250,000 additional death per year would be attributed to climate change between 2030 and 2050 if this situation persists. Since our population depends on (but not limited to) the availability of clean water, good air quality, and exposure to environmental hazards/pathogens, the overall health impact caused by climate change is likely to be overwhelming in the nearest future. This dissertation explored the influence of climate change on fungi diseases and pathogenesis after severe weather conditions (hurricanes and flooding). Specifically, I investigated the severity and health implications of the fungal infestations in agriculture and residential environments.

One of my specific aims assessed the influence of hurricane and tidal flooding on indoor mold community structures and the respiratory health of residents. Using both experimental and NHANES database, my study explored fungal involvement in causing harm in the built environment after flooding events and explored the association between mold-induced sensitization in patients diagnosed with asthma and chronic bronchitis. My findings indicate that (1) certain fungi strains impacts health quality and indoor air quality of residential environments after extreme weather condition, (2) this dissertation also identified a statistically significant association between specific mold-induced sensitization in patients diagnosed with asthma conditions but not with chronic bronchitis. My second specific aim explored fungi secondary metabolite (mycotoxins) and its significance as a

threat to food safety, especially in countries in hot, humid regions. Using ELISA and other specific laboratory techniques, this study shows that children (especially those from developing countries) are at risk of contamination of food by certain fungi secondary metabolites.

In summary, this study investigated how fungi influence the environment and human health, and findings from this thesis suggest that fungal pathogens indoors and outdoors are of immediate concern to food safety and human health. As the current pandemic (Covid-19) continues to threaten immunity, it is likely diseases associated with exposures to opportunistic fungal pathogens, and their toxins will increase and lead to a significant burden to healthcare if not addressed and mitigated promptly.

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LIST OF ABBREVIATIONS

DON	Deoxynivalenol
DMPO	5,5- Dimethyl-1-pyrroline N-Oxide
EU	European Union
ROS	Reactive Oxygen Species
SOD	Superoxide Dismutase
SU-1	<i>Aspergillus parasiticus</i>
OR	Odds Ratio

CHAPTER 1

INTRODUCTION

1.1 General Overview of Climate Change and Health Financial

Implication

Recent reports from Butler., 2018, show a drastic change in climate over the last two decades (Butler, 2018). In the previous decades, the evidence of rapid climate change can be seen in sea-level rise, warm oceans, ocean acidification, glacial retreat, and global temperature rise (Allergy Centre, Hong Kong Sanatorium & Hospital, Happy Valley, Hong Kong, et al., 2018). It is predicted that by 2050, many cities within the United States (U.S.) will experience extreme heat days (Patz et al., 2014).

The adverse effect associated with climate-related physical risks – e.g., via natural disasters, can potentially lead to substantial financial loss, some of which are borne by insurance companies while others are uninsured. Damages due to climate-related natural disasters could not only affect the financial strength of individual financial institutions but also as other government organizations.

Apart from direct physical environmental damages, climate change impacts human health, and the cost associated with climate-related health challenges could be enormous. At least a total of six (6) health-related case studies on morbidity/mortality in the United States, from various climate events including ozone pollution, hurricanes, infectious disease outbreaks, flooding, and wildfire estimated that health care cost exceeded \$14

billion, of which about 95% of this cost is allocated to the value of lives lost due to climate event (Knowlton et al., 2011).

Though the association between climate change and health is complex and not fully understood, evidence indicates that climate is changing and its impact on health would be enormous in the future if it continues at this trend. Possible climate health-related consequences include morbidity and mortality due to repeated episodes of heatwaves, extreme weather events, mental stress, food and water shortages, respiratory diseases, poor air quality, and infectious diseases (Younger et al., 2008).

As such, this dissertation will focus on climate change concerning fungi induced allergic diseases and food toxicity. Firstly, it will discuss fungi epidemiology concerning health challenges after extreme weather events (emphasis on hurricane/flooding) by emphasizing indoor air quality. It will then highlight some peculiar diseases condition pathognomonic to fungi pathogen. Finally, It will include some of my studies on fungal toxins (mycotoxins) relevant to public health and food safety.

1.2 Fungi Epidemiology, Health, and Climate Change

Fungi (a eukaryotic microorganism) are essential to the functioning of the ecosystem. Climate conditions such as temperature and precipitation are significant environmental variables necessary for the growth of fungi. In other words, these climate variables influence biochemical processes essential for growth, survival, pathogenicity, and reproduction of fungi pathogen.

Understanding the mechanism by which climate initiates fungi-borne diseases provides knowledge on fungi disease epidemiology as well as being able to predict fungi disease outbreak, especially after extreme weather conditions (e.g., flooding).

Out of an estimated number of 1.5 million identified fungal species, only about 300 are reported to be harmful to humans (Garcia-Solache and Casadevall, 2010a). Fungal associated disease in humans could be mild or severe depending on host factor (immune status), fungal virulence, and environmental factors. Most fungal species thrive within a temperature range of 12°C to 30°C (Garcia-Solache and Casadevall, 2010a). However, few fungi species grow at a low temperature of -10°C or temperature as high as 65°C. With constant weather fluctuation, due to climate change, people (especially immunocompromised individuals) are at risk of fungal diseases.

The most implicated human fungal pathogens include; *Candida*, *Pneumocystis*, *Cryptococcus*, and *Aspergillus* (Sanglard, 2016). It is reported that these fungal species accounts for a total of about 1.4 million death globally (Brown et al., 2012). By ranking, infections caused by genus *Aspergillus* are the most researched fungal infections, accounting for about 30-50% mortality in patients diagnosed with invasive aspergillosis (Garcia-Solache and Casadevall, 2010a).

The mechanisms used by most pathogenic fungi cause harm to host cells and include primarily direct physical force during host cell invasion or escape or through secreted enzymes or molecules that lead to immune-suppression, dysfunction, and cell death (Brunke et al., 2016). All of these mechanisms are triggered by unique environmental factors.

1.3 Climate Change, Indoor Mold, and Air Quality

Air quality is one parameter used to determine the health of an environment. For decades, studies have explored the association between climate change and air quality with a significant focus on outdoor air environment. Hence, it is no surprise that environmental

law policy, such as the ‘Clean Air Act’ in the United States favors policies on outdoor than indoor environments.

Since most Americans spend about 90% of their time indoors, with 66% of total spending in residential homes (Kamaruzzaman et al., 2017), the significance of maintaining a healthy and safe indoor air quality cannot be overestimated. Poor indoor air environment has been implicated as likely cause of certain conditions involving the cardiovascular, immune as well as the respiratory system (Śmiełowska et al., 2017).

The extreme weather event presents an opportunity for indoor air hazards caused by molds. It is reported that fungal growth is visible in about 15% to 40% of buildings in Northern Europe and North America, respectively, after extreme weather conditions following storms and flooding (Andersen et al., 2011). Other studies have reported an increased likelihood of indoor misty smell and dampness after each episode of hurricane events (Grimsley et al., 2012).

Other studies carried out on people who participated in environmental cleanup post-Hurricane Sandy (New Jersey/2012), reported 34.4% of participants with lower respiratory tract symptoms (Poole et al., 2019).

Up until now, there still exists considerable uncertainty on the exact concentration or length of exposure required to produce specific mold health-related problems post-hurricane in humans. It is also reported that people react differently to mold exposure in the indoor environment. While some individuals may require several episodes of mold exposure before reporting symptoms, an already sensitized individual may show symptoms with just a little time of mold exposure.

In both scenarios, further studies are needed to estimate better the exact average indoor mold spore concentrations required to cause health problems after mold exposure post-hurricane.

1.4 Mold Allergy and Respiratory Health

Many molds implicated in causing allergic diseases proliferate in indoor environments. Exposure to molds could lead to immune-mediated inflammatory (Allergic) diseases. In sensitized patients, inhalation of fungi spores induces respiratory allergy symptoms in about 20-30% of individuals (Gioulekas et al., 2004). Prolonged exposure to a high concentration of mold could lead to an immune-mediated disease known as hypersensitivity pneumonitis (Bush et al., 2006). Medically, hypersensitivity pneumonitis is known by the variety of exposures that leads to disorder, including farmer's lung, woodworker's lung, and malt worker's lung (Riario Sforza and Marinou, 2017).

Allergic fungal rhinosinusitis (AFR), another type of allergic disease caused by fungi sensitization, affects ~ 20% of people at some point in their lifetime. It is estimated that 4% of the United States adults are affected by AFR annually (Chakrabarti and Kaur, 2016). AFR, which could be triggered as a response to diverse fungi communities (Schubert, 2009), causes nasal obstruction, polyps, and impairment in vision.

Also, colonization of the respiratory airways by some mold species is common in chronic lung diseases such as asthma and cystic fibrosis. In the U.S., approximately 5 million people have asthma due to the inhalation of mold spores and Volatile Organic Compounds (VOC) in indoor environments, leading to roughly \$3.5 billion in healthcare costs (Mudarri and Fisk, 2007).

Mold species implicated in causing allergic diseases include but are not limited to *Penicillium*, *Aspergillus*, *Alternaria*, and *Cladosporium* species (Bozek and Pyrkosz, 2017). The pathogenesis of the mold-induced allergic disease (refer to Figure3.1) results from immune-mediated inflammatory responses to fungal allergen. The fungal allergens induce hypersensitivity reactions includes type I Immunoglobulin E (IgE) mediated response. Together with other specific allergic reactions (type III IgG/IgM-mediated and (type IV delayed-type hypersensitivity), mold induces a cascade of different allergic diseases (Pfavayi et al., 2020). However, specific mold allergens responsible for developing allergic symptoms in humans are poorly understood.

The initial process of allergic sensitization begins by triggering specific immune responses, including allergen-specific T-helper 2 (Th2) cells response and Immunoglobulin E (IgE) production. IgE then binds to high-affinity IgE receptors located on the surface of mast cells. Following re-exposure to mold specific allergen, cross-linking of IgE occurs on the surface of the mast cell. This invariably leads to a cascade of reaction leading to mast cell response in secreting active mediators, including histamine (Pfavayi et al., 2020). The mechanistic pathway by which mold triggers or exaggerate asthmatic symptoms is not fully understood. However, it is reported that mold releases enzymes that induce inflammatory responses. These responses alter respiratory mucociliary, epithelial barrier, and activate the innate immune response, which leads to asthma development.

1.5 Case Study Associated with Mold Allergic Diseases

Allergic diseases commonly associated with mold allergens include; Asthma, allergic rhinitis, and allergic fungal sinusitis (Mohammed Saeed Tayeb and Family Medicine Department, Faculty of Medicine, University of Jeddah, Saudi Arabia, 2020).

Another rare diagnosed mold-induced allergic condition is allergic bronchopulmonary mycosis, a rare form of the hypersensitive disease caused by fungi (Pfavyi et al., 2020). It is reported that about 40% of people living in Europe and the United States suffer from allergic rhinitis condition.

A retrospective mold sensitization study carried out in fifty-one (51) participants living in Saudi Arabia, shows that 69% were positive for *Aspergillus fumigatus*, 63% positive for *Alternaria*, 57% positive for *Cladosporium* and 18% participants were positive for *Penicillium*. This study also shows that mold sensitive patients were at risk of developing allergic rhinitis, allergic fungal sinusitis, and asthma (Mohammed Saeed Tayeb and Family Medicine Department, Faculty of Medicine, University of Jeddah, Saudi Arabia, 2020).

While some studies have reported mold sensitization as a causal link in asthma symptoms severity (Gabriel et al., 2016), other reports show no association between mold sensitization and asthma severity. For example, a case-control study carried out by Vincent et al., (2018) on diagnosed asthma patients with or without mold sensitization showed that mold exposure was not associated with asthma symptoms severity (Vincent et al., 2018).

1.6 Fungal Secondary Metabolite and Food Security

Apart from humans, fungal diseases also threaten global food security. Mycotoxins, a secondary metabolite produced by most fungi species, contaminate food and food produce. Aflatoxin B1, a potent carcinogenic metabolite (Gummadidala et al., 2016), is common and found in larger quantities and concentrations than other naturally occurring forms of fungal metabolites globally. Foods such as maize, peanuts, and peanut products, cottonseed and its extractions, and, to some extent, chilies, peppers, and pistachio nuts are

not spared from aflatoxin B1 contamination. It is estimated that about one-quarter of food produced globally is lost due to mycotoxin contamination (Cinar and Onbaşı, 2019). Also, aflatoxin B1 has been implicated as a potent liver carcinogen (Vincent et al., 2018), capable of causing cancer in humans.

Aflatoxin M1, a metabolite of aflatoxin B1, contaminates milk and its products when obtained from livestock that has ingested aflatoxin B1 toxic feed. Even though aflatoxin M1 contamination incidence has been under-reported in many parts of the world (especially in developing countries), it still poses a severe public health issue.

1.7 Public Health Significance of Climate Change in Fungi diseases

Some fungi diseases attributed to climate change are directly connected to environmental factors. For example, the association between thunderstorms and the severity of asthma symptoms has been well documented in different studies. A study carried out by Dales et al. on an asthma patient, shows that there was an increased hospital visit during thunderstorm days compared to days without thunderstorm events (Dales et al., 2003).

Also, the unexplained severe cough was associated with fungi pathogens amongst healthy medical personnel after the Great East Japan Earthquake that occurred in 2011. Culture from sputum samples from 6 medical staff identified *Aspergillus fumigatus*, *Aspergillus flavus*, and basidiomycetous fungi (Benedict and Park, 2014).

Furthermore, studies carried out on immunocompromised individual post-hurricane Katrina, and Harvey revealed that these populations were at risk of mold infection after each weather event (Benedict and Park, 2014; Chow et al., 2019).

Apart from allergy and infections, filamentous fungi produce secondary metabolites (mycotoxin) that are harmful to both animals and humans (Table 1). Under certain environmental conditions, fungi produce toxin and ingestion of these toxins have been implicated in chronic diseases, including liver cancer (Cary et al., 2017). As such, strategies to reduce fungal infections associated with extreme weather events should be taken into consideration with a focus on sustainable risk reduction linked to fungi pathogen.

1.8 Conclusion

In this chapter, I have discussed, to a great extent, the impact of changing climate on fungi biology. Specifically, I started by explaining how hurricane and tidal flooding impacts indoor air quality through the production of visible mold and the production of their volatile organic compounds. I then went on to discuss how molds induce specific allergic diseases by colonizing the respiratory tract of certain sensitized individuals. Finally, I concluded by discussing the public health significance of certain harmful fungi secondary metabolites on food safety and human health.

The main objective of this dissertation is to gain insight into how the ongoing changes in climate and the increase in frequency and severity of weather events potentially influence fungi biology in the built and natural environments and, as a consequence, impacts human health. I hypothesize that extreme weather conditions will alter the fungi ecosystem there, leading to an increased prevalence of fungi pathogens and diseases in the inbuilt environment. Based on these goals, this dissertation was classified into two broad specific aims, from which four studies were explored (see figure 1.1).

1.9 Figures

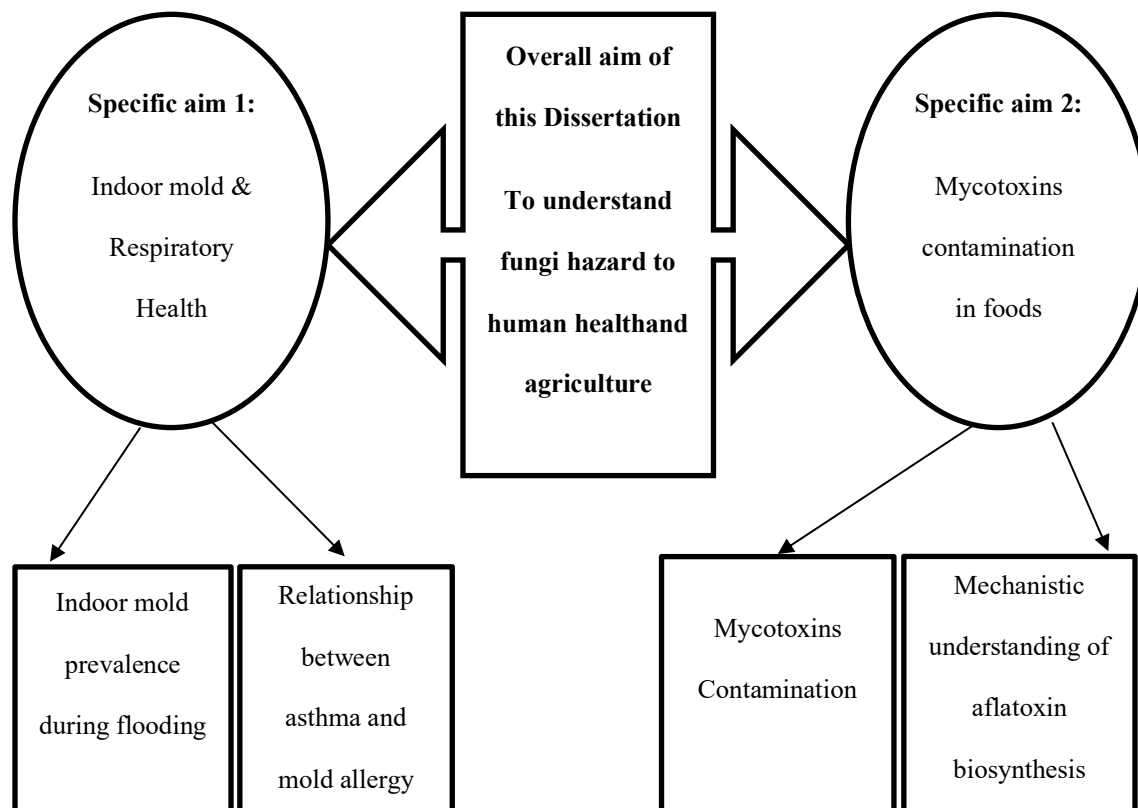


Figure 1.1. The central idea of this dissertation

1.10 Tables

Table 1.1 Mold producing mycotoxins and their impacts on human and animal health

Mold Species	Mycotoxins	Major Food	Impacts on human & animals health
<i>Aspergillus parasiticus</i> , <i>A. Flavus</i>	Aflatoxins	All cereal grains	Acutely toxic, immunosuppressive, mutagenic, teratogenic, carcinogenic
<i>Alternaria</i>	Alternaria toxins	All cereal grains	Esophageal cancer
<i>Fusarium graminearum</i>	Deoxynivalenol	All cereal grains	Vomiting and feed Refusal
<i>Fusarium</i>	Trichothecenes	All cereal grains	Alimentary toxic leukemia, nausea, vomiting & decrease in the immune response
<i>Fusarium Moniliforme</i>	Fumonisin	Maize, Rice	Possible carcinogenic in humans, edema in pigs & esophageal carcinoma
<i>Penicillium verrucosum</i>	Ochratoxin	All Cereal grains	Nephrotoxic, Immunosuppressive, Carcinogenic, teratogenic
<i>Penicillium Rhizopus</i> , <i>Aspergillus</i> , <i>Claviceps purpurea</i>	Ergot alkaloids	Rye, Wheat	Neurotoxic, gangrenous, edema causing severe pains & paresthesias

CHAPTER 2

**FLOODING AS A POTENTIAL DETERMINANT IN THE
SELECTION OF *ASPERGILLUS* AND *PENICILLIUM* SPP. IN
INDOOR AIR: A COMPARATIVE STUDY OF FLOOD-IMPACTED
VERSUS NON-FLOODED MOLDY HOMES**

2.1 Abstract

Dampness and the moldy infested indoor environment caused by flooding has raised public health concern regarding human exposure to pathogenic fungi that are of public health significance. To determine if the indoor environment is contaminated by harmful fungi pathogen, this study assessed residential homes after three (3) severe weather events in the United States.

Mold samples that were collected from three flood-impacted and three non-flood impacted residential homes after the “100-year flood” and Hurricane Matthe were analyzed for mold species diversity post-hurricane. The selection of residential homes used in this study was based on easy access, the extent of flood damage, and the presence of visible mold growth within selected buildings.

Using DNA extraction, Internal Transcribed Spacer (ITS), and Phylogenetic analysis, we identified that mold taxa *Aspergillus* and *Penicillium* were specific to the indoor environment from flood-impacted residential homes compared to non-flooded homes. Results suggest that indoor flooding selected for pathogenic molds that belonged to the genera *Aspergillus* and *Penicillium* implicating that moldy homes caused by flooding have more detrimental effects on health than moldy homes in which the mold infestations were caused by factors other than indoor flooding. Understanding how flooded residential buildings selectively enhance the growth of some *Aspergilli* and *Penicillia* and impact the health of residents are of paramount importance, especially during the ongoing pandemic that has threatened the respiratory health and immunity of millions of residents working primarily from homes.

2.2 Introduction

Over the years, with the different repeated episodes of flooding, millions of Americans, especially those living in coastal areas have been impacted severely (Balica et al., 2012; Hummel et al., 2018). It is projected that more lives will be affected in the next 30-40 years, as sea level continues to rise (Ray et al., 2011). Since August 2005, three out of ten unprecedented floodings associated with hurricanes, Harvey, Katrina, and Rita have been recorded in the United States. These extreme weather events favor mold growth in the indoor environment. With prolonged exposure and stay in a mold-contaminated environment, residents (especially immunocompromised individuals) are prone to mold induced diseases .

Molds cause serious health problems through the release of spores that are airborne and inhaled. In the event of natural disasters, large-scale disruption of the fungal ecosystem could lead to clusters of respiratory and cutaneous fungal related illnesses (Benedict and Park, 2014). In June 2006, reports by the Centers for Disease Control and Prevention (CDC), “*Mold Prevention Strategies and Possible Health Effects in the Aftermath of Hurricanes and Major Floods,*” stated that susceptible individuals could suffer from mold-related adverse health effect regardless of the type of mold species (Brandt et al., 2006).

Molds, especially those in the genus *Aspergillus* and *Penicillium*, produce poisonous toxins (Mycotoxins) and hazardous volatile organic compounds (VOCs) that can weaken the immune system and make residents (especially those with underlying illness) very sick and vulnerable to infectious and other non- communicable diseases.

Ambient mold spore exposures are also associated with repeated childhood asthma attacks even in a neighborhood where mold spore concentrations were relatively low

(12~hr daytime mean spore concentration of ~4,000 spores/m³(Solomon et al., 2006)). Besides, mold associated misty smell was correlated with about two (2) fold increase in asthma in children between 1 to 7 years old (Jaakkola et al., 2005).

Overall, the wellbeing associated with mold exposure causes serious public health concerns for children and other vulnerable populations. Besides health challenges, mold contamination, and its associated disease burden create enormous economic impact in the health care system. It is estimated that health care and financial cost related to indoor mold contamination totals ~ \$US 3.5 billion annually (Centers for Disease Control and Prevention (CDC), 2006; Mudarri and Fisk, 2007).

Since Americans spend about 90% of their time indoors (Bajc et al., 2016), it is only expected that residents impacted after extreme weather event reports of illnesses directly or indirectly linked to mold contamination. This indoor time is likely higher with the ongoing Coronavirus pandemic. As such, there is a need to understand the prevalence of mold species in homes, especially in areas impacted by repeated flooding, and their implications to respiratory health and the vulnerability of the residents to coronavirus infections.

The selection, abundance, and distribution of mold species in the indoor environment after each extreme weather event is not fully understood. Various studies have reported the influence of indoor environmental factors, including; temperature, moisture, and humidity, in the growth and survival of fungi organisms. For example, *Penicillium marneffei*, an indoor specific mold is more prevalent during the rainy months while *Aspergillus*, another indoor associated mold, correlates with seasons or weather patterns in the different geographic areas (Solomon et al., 2006).

The genus *Aspergillus* has also been implicated as an agent that caused tsunami lung after an earthquake disaster that occurred in Great East Japan in 2011. During its occurrence, a previously healthy near-drowning victim who later died was found to have pneumonia caused by Aspergillosis (Kawakami et al., 2012). Other identified fungi species post-hurricane include; *Cladosporium sp* and *Penicillium sp*, both identified after Hurricane Katrina event in 2005 (Bloom et al., 2009; Rao et al., 2007). Besides, other studies (Jayaprakash et al., 2017; Meadow et al., 2014) have suggested that an increase of molds in indoor air severely influences airborne microbial dynamics in damp homes leading to quantitative and qualitative changes in fungal exposures.

Southeastern coastal areas in recent years have experienced repeated flooding in the past years that have led to moldy homes in several communities. However, there is a gap in knowledge of what indoor mold species dominate in the homes impacted by flooding. To address this gap, we conducted a mini-survey to compare indoor molds within homes that were flooded versus the moldy homes that were not flooded after two different weather events: 100-year flood in Columbia, South Carolina in 2015 and Hurricane Matthew in 2016 2015; North Carolina). Finally, we have also investigated the homes in New Jersey that were once flooded and mold-infested after Hurricane Sandy. Mold remediations were done in those homes but were mold-infested again after five years (in 2017). We tried to understand if prevalent mold genera in those New Jersey homes displayed any similarity to the homes that were flooded and mold-infested in the Carolinas.

Given that flooding brings with it several natural nutrients that support the growth of indoor molds, we hypothesized that the nutrients would select for the more robust

species of molds such as those within the genus *Aspergillus* and *Penicillium*. Presented in this chapter are the results of this study.

2.3. Material/Methods

Selection of Homes. Homes were randomly selected based on accessibility (consent from homeowners to enter the building), the extent of damage (only for flood-impacted home), and the presence of visible mold contamination (both for flood and non-flooded impacted buildings). Samples were collected from 17 households in total. Three (3) Flooded and moldy homes and three (3) non-flooded but moldy homes in Columbia, SC after the 100-year flood), three (3) flooded and moldy homes in Charleston, SC, and three (3) non-flooded but moldy homes in Jacksonville, NC, where both groups of homes received impacts from Hurricane Mathew, and five (5) moldy homes in New Jersey that was mold-infested and then remediated after Hurricane Sandy flooding in 2012 but showed mold infestations again in 2017 due to condensations that were not due to flooding. We hypothesize that flooded homes will display less diversity in community structure as compared to non-flooded homes.

Molds were collected from residential homes by settle plate techniques. This method collects both live and dead fungi cells that settle by gravity. The process of the settle plate technique begins by exposing potato dextrose agar (PDA) to the atmosphere to collect fungi particles. These plates were securely sealed, labeled with time, dates of collection, and transported to the laboratory for analysis. Plates were incubated at a temperature of 30°C, and 80% relative humidity for about 4 to 5 days. When visible colonies were noticed, we sub-cultured again onto new PDA plates and incubate at the same temperature until there uniform fungi cultures. One fungi colony was picked from a

population of fungi community and used for morphological characterization and identification of fungi species. Laboratory techniques that were used for fungi identification include; DNA extraction, ITS sequencing, and Phylogenetic analysis, and indoor air quality standard was conducted using known fungus species, following similar analysis carried out by Hong and Linz, 2008.

Molecular Identification. Speciation of the mold colonies was done using ITS sequencing, as established previously in our laboratory by Hopkins(Hopkins, n.d.). Briefly, DNA was extracted from the pure colonies using a methodology described previously with minor modifications (Hong and Linz, 2008). Quickly, mycelia were ground into a fine powder using liquid nitrogen. DNA was extracted from 100 mg of the mycelial powder with DNA extraction buffer (1.2 M NaCl;5 mM EDTA;0.2 M Tris HCl;20 μ L Proteinase K) then incubated for 18h in a 50 C water bath. The DNA containing fraction was isolated from the extraction mix using a phenol, chloroform, and isoamyl alcohol (25:24:1) solution. The DNA containing solution was purified using RNase (10 mg/mL) and precipitated with ethanol (200 proof). DNA pellets were then dried and dissolved in T.E. buffer.

The nuclear rDNA internal transcribed spacer barcodes (ITS; ITS1-5.8S-ITS2) were amplified using previously described primers (Cheng et al., 2014; Gardes and Bruns, 1993; White et al., 1990). Amplification was conducted according to the following previously described PCR conditions (Luque et al., 2012): denaturation 94 C, 5 minutes; annealing, 95 C, 30 s, 57 C, 40s, 72 C, 1 min, 33 cycles; extension, 72 C, 5 min. PCR amplicons were sequenced using an Applied Biosystems 3730 DNA Analyzer (Life Technologies, Carlsbad, California) following manufacturer's protocols. For phylogenetic

analyses, multiple sequence alignment was performed using the Basic Local Alignment Search Tool (BLAST) from NCBI's Genbank database. Phylogenetic analyses were conducted by the previously described neighbor-joining (N.J.) method (Davolos et al., 2012; Tamura et al., 2007).

2.4. Results

Comparison of flooded versus non-flooded moldy homes after the South Carolina 100-year flood.

Mold spore concentrations differed significantly within and between locations of residential homes compared. The most identified mold genera isolated from indoor samples collected from bedrooms, bathrooms, and living room in both flood and non-flood impacted homes, were *Aspergillus/Penicillium*, *Cladosporium*, and *Trichoderma*. Apart from *Trichoderma* species, the mean indoor concentration of these mold species differs between flood-impacted homes and non-flooded homes. Flooded homes demonstrated approximately twice the level of *Aspergillus/Penicillium* species compared to non-flooded impacted homes.

Columbia and forest acres (both cities located in South Carolina) are neighboring cities about 5.5 miles apart and had visible mold growth in indoor residential buildings post-hurricane (Figure 2.1a). The mean spore concentration isolated from residential homes within both cities varied from one home to the other.

The isolated mean spore concentration of *Aspergillus/Penicillium* and *Cladosporium* species from the indoor flooded environment in Columbia is 62% and 26%, respectively, compared to non-flood impacted homes in Forest Acres (27% and 66%) (Figure 2.1b).

Comparison of flooded versus non-flooded moldy homes after Hurricane Matthew (2016)

Post-hurricane Matthew (2016), predominant mold species isolated from the indoor environment in flooded home (Charleston, SC) and non-flood impacted homes (Jacksonville, NC) were *Aspergillus /Penicillium* and *Cladosporium species* at a mean mold concentration of 89% (*Aspergillus /penicillium*) in flood-impacted homes. However, in non-flood impacted homes, about 10% mean concentration of molds were attributed to *Aspergillus/Penicillium*, and 55% mean concentration of mold spores was identified as *Cladosporium species* (Figure 2.2). Unlike the 100-years flooding event, there was no association between *Cladosporium species* and flooded homes.

Comparison of flood-impacted homes after remediation in New Jersey

Lastly, five (5) flood-impacted New Jersey residential homes after mold remediation showed the same pattern of mold diversification in the indoor environment after Hurricane sandy event. We isolated a mean concentration of 96% *Aspergillus/Penicillium* and 4% of *Cladosporium*, respectively (Figure2.3).

Overall, in total, *Aspergillus/Penicillium* genera accounted for between 60% and 90% of all mold spores in indoor air samples collected from flood-impacted homes while mold belonging to *Cladosporium* genera, accounts for about 90% identified mold species from non-flood impacted homes. Due to the morphological similarity between *Aspergillus* and *Penicillium* spores, we were unable to categorize them differently. We were also unable to determine the specific species present within these mold genera. Other fungi that significantly contributed to the total spore count at one or more locations include *Trichoderma species*.

2.5. Discussion

There have been several reports about molds issues within and around the in-build environment post-hurricane (Bloom et al., 2009; Rao et al., 2007; Riggs et al., 2008). In this study, one key finding peculiar to all flood-impacted homes post-hurricane is that molds genera belonging to taxa *Aspergillus*, *Cladosporium*, and *Penicillium* were prevalent. Our result is in line with a similar report by Barbeau et al., 2010, who investigated mold contamination in four parish areas after Hurricane Katrina and Hurricane Rita. In this study, 112 homes across four parish areas were assessed for mold hazards in the indoor and outdoor air environment, and results showed that mold species diversity was the same in the flooded impacted indoor environment (Barbeau et al., 2010).

Bloom et al., 2009 also reported 23 mold species in the air sample associated with Hurricane Katrina. Amongst these samples, *Aspergillus*, *Penicillium*, and *Cladosporium* were the most identified mold species, with a 40% prevalence rate of *Stachybotrys* species (Bloom et al., 2009). However, our study did not identify *Stachybotrys* species from samples collected from all three-hurricane event.

To the best of our knowledge, there has been no published data on mold spore concentration as well as mold species diversity in indoor environment after any hurricane event in South Carolina. Our study is the first to report an association between *Aspergillus/Penicillium* species in an indoor flooded environment in South Carolina. According to Shelton et al, there are presently no acceptable standard for indoor fungi concentrations in indoor environment (Shelton et al., 2002). However, studies have reported high spores concentration between 11,000 to 645,000 spores/m³ in indoor and 21,000 to 102,000 spores/m³ in outdoor environment after flooding events (Solomon et al.,

2006). Although our study did not investigate mold contamination in the outdoor environment post-hurricane, there seems to be a commonality of fungi species diversity in an indoor environment from most researched studies carried out in the flood-impacted home. The harm caused by these fungi species does not only pose a threat to public health but could account for a substantial economic burden attributed to combat this situation. These mold species are known to cause allergy, fatal respiratory tract infections, and environmental contamination that contain carcinogenic and immune-suppressive mycotoxins. Hence, there is a need to input specific public health measures in place targeting mold contamination post-hurricane.

The findings in this study have at least three limitations. Firstly, few numbers of buildings were sampled, and participants were not examined for direct health impact associated with mold exposure caused by hurricane aftermath. Even though the measurement and identification of certain pathogenic mold species confirm the chances of high exposure of participants, our findings cannot be extrapolated to all residential homes and residents living in South Carolina, North Carolina, and New Jersey.

Secondly, although it was ascertained that the three fungi species (most of which is implicated in causing allergic disease) were prevalent in flood-impacted homes, no data was collected on mold sensitization and underlying respiratory condition from participants/residents from which homes mold sample were analyzed. As such, it was not possible to ascertain the overall wellbeing of participants and its association with mold exposure after each hurricane episode.

Finally, information and assessment of residential buildings for mold infestation before each hurricane disaster was not collected. For example, we did not investigate from

participants questions pertinent to indoor air quality as regards musty smell, visible mold growth exposure, and other leakages within the indoor environment before each hurricane event. Mold contamination associated with flooding might be prominent even before the start of each hurricane episode, invariably influencing total microbial diversity post-hurricane.

Another possibility is that due to the small sample size and no participant investigation, our study might have underreported actual mold contamination incidence associated with each hurricane event in this study.

2.6. Figures

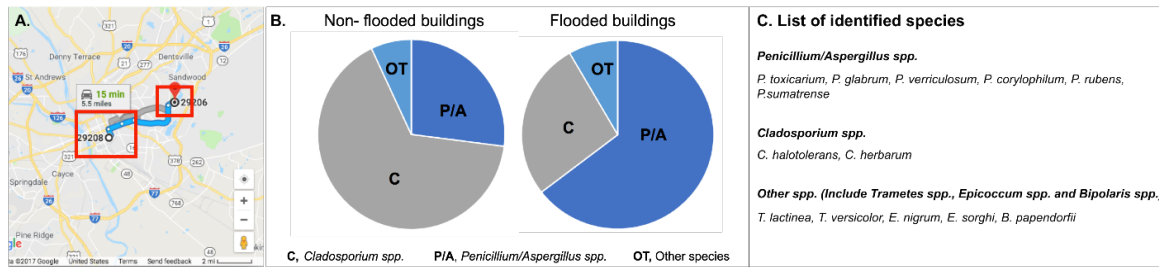


Figure 2.1. Comparison of predominant mold genera collected from in indoor environment of flooded and non-flooded fungi impacted buildings in two adjoining cities. Fungi samples in indoor air of non-residential fungi impacted buildings analyzed after South Carolina’s 100-year flood of 2015. **A.** A total of 3 flooded building and 3 non-flooded buildings that were experiencing fungi problems within Columbia and Forest Acres (spanning 6 miles) were sampled after the weather event. **B.** Pie charts demonstrating average relative abundance of *Penicillium* and/or *Aspergillus* (P/A), *Cladosporium* (C) and other species (OT) in the non-flooded and flooded buildings are shown. The actual percentage values (\pm SD) were as follows: Non-flooded buildings [P/A (27 ± 8), C (66 ± 9) and OT (7 ± 3)] and flooded buildings [P/A (62 ± 5), C (26 ± 3) and OT (8 ± 4)] **C.** Species of the identified fungi genera.

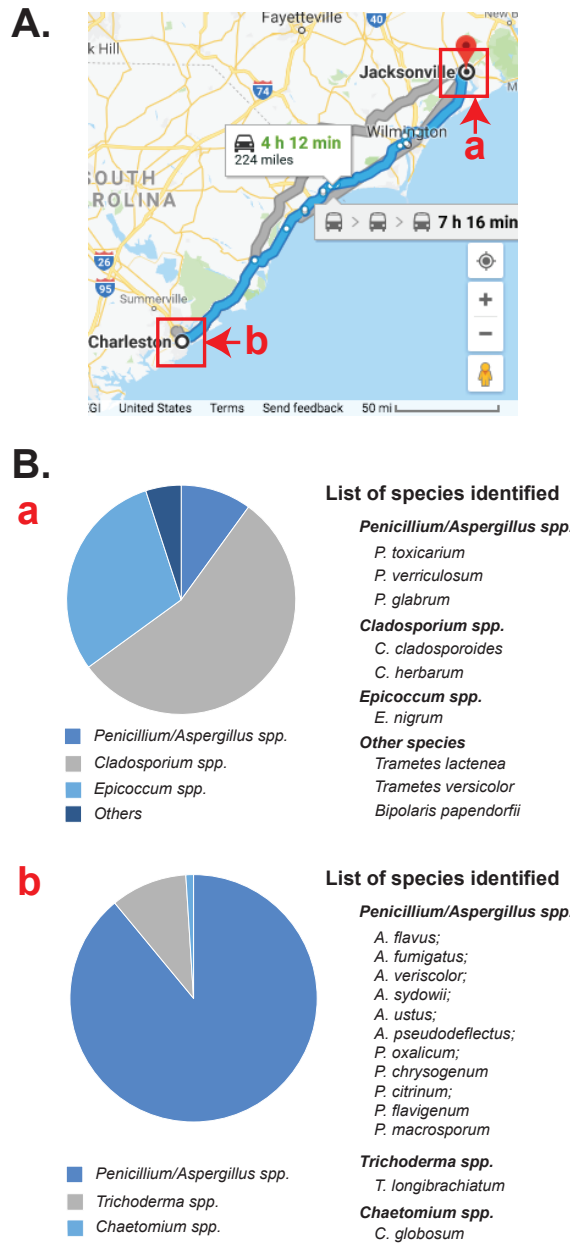
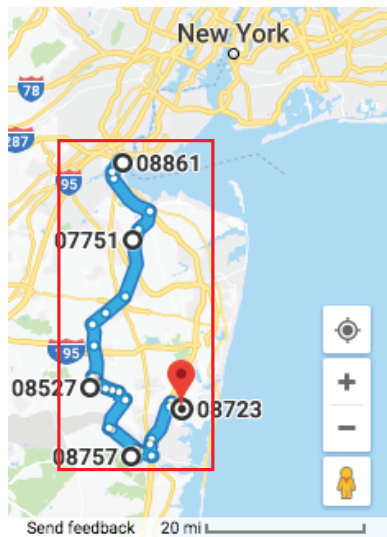
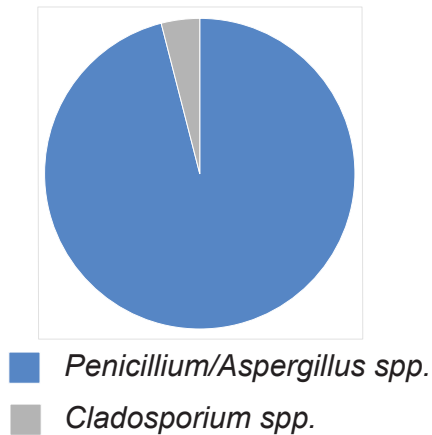


Figure 2.2. Comparison of predominant mold genera collected from the indoor environment of flooded and non-flooded mold impacted buildings after hurricane Matthew in two cities ~720 miles apart. A. Mold samples in indoor air of 3 residential mold impacted non-flooded buildings in Jacksonville, NC (**location a**) were compared with 3 residential mold impacted flooded buildings in Charleston, SC (**location b**) after Hurricane Matthew in 2016. The cities are 724 miles apart on the south-eastern coast of the United States. **B.** Pie charts demonstrating average relative abundance of mold genera in the buildings are shown. The actual percentage values (\pm SD) were as follows: Non-flooded buildings [Penicillium/Aspergillus (10 ± 3), Cladosporium (55 ± 8), Epicoccum (30 ± 6) and Others (5 ± 2)] and flooded buildings [Penicillium/Aspergillus (89 ± 9), Trichoderma (10 ± 3) and Others (≤ 2)]. The species of the identified mold genera are listed beside the charts.

A



B



C. List of species identified

Penicillium/Aspergillus spp.

P. rubens;
P. glabrum
P. crustosum;

Cladosporium spp.

C. cladosporoides

Figure 2.3. Comparison of predominant mold genera collected from indoor environment of mold impacted buildings that were flooded during hurricane Sandy. A. Mold samples in indoor air of 5 residential buildings in New Jersey spanning ~40 miles six years after the buildings were flooded during Hurricane Sandy in 2012. **B.** Pie charts demonstrating average relative abundance of mold genera in the buildings are shown. The actual percentage values (\pm SD) were as follows: *Penicillium/Aspergillus* (96 ± 3), *Cladosporium* (4 ± 1). The species of the identified mold genera are listed beside the charts.

CHAPTER 3

**THE PREVALENCE OF MOLD SPECIFIC ALLERGY IN PRE-
EXISTING ASTHMATIC AND PRE-EXISTING CHRONIC
BRONCHITIS PATIENT USING NHANES 2005-2006 DATABASE**

3.1 Abstract

Mold allergens have been associated with the induction and exacerbation of respiratory symptoms. How and why some specific mold species pose a potential risk factor for specific respiratory diseases of asthma and chronic bronchitis in diagnosed patients is not entirely understood. The portion of mold allergens responsible for sensitization in both asthma and chronic bronchitis individuals has not been thoroughly investigated. As such, this study contributes to this knowledge gap by exploring the association between specific mold pathogens implicated in eliciting sensitization in asthma and chronic bronchitis patient. Multivariate logistic regression was used to examine the association between asthma, chronic bronchitis, and specific mold sensitivity. Data on asthma and specific mold allergen were assessed from NHANES 2005-2006 database, and participants (N=4459) were our overall analytical sample size. From this number, we stratified our data to get clean asthma and chronic bronchitis population. Our results show that *Alternaria alternata*, as well as *Aspergillus fumigatus* induced IgE levels, were associated in the pre-existing population (*A. alternata*: OR=3.58., CI 2.36-5.44 and *A. fumigatus*: OR=2.91, CI 1.69-5.04) and did not find any association between mold-induced allergic response in pre-existing chronic bronchitis patients. Our findings suggest that there is an urgent need to explore the role each mold specific allergens (including their allergenic component) plays in inducing an allergic reaction in asthma patients.

3.2. Introduction

Over the past 50 years, allergic disease and asthma have been a significant disease burden in the United States (Meng et al., 2016). As of 2016, an estimated number of 26 million people living in America had asthma conditions (Perry et al., 2019). Demographic,

sex distribution in the United States shows that the prevalence and morbidity of asthma severity is 6.3% among males and 9.0% among females (Ebell et al., 2019). Also, Thakur et al., 2019 also reported a prevalence rate of 36.5% (Puerto Ricans), 21.8% (Cubans), 15.4% (Dominican Americans), and 7.5% among Mexican Americans. It is projected that by 2025, more than 100 million individuals will be living with asthma conditions globally (Nunes et al., 2017).

Asthma, a chronic inflammatory disease (Wang et al., 2019), is characterized by inflammation and constriction of the airways. Various studies have shown that airway hyperresponsiveness and inflammation occur in individuals whose asthma condition has been persistent for many years (Yaghoubi et al., 2019). As such, the need to put an effective mechanistic contemporary preventive strategy in managing asthma cases as well as reducing the risk associated with asthma symptoms exacerbation cannot be overemphasized. Evidence-based studies have associated the risk of asthma attacks and symptoms to a range of different environmental factors including; dust mites, mold, rodents, pets, scents, tobacco smoke, chemical particulate matter and other outdoor allergens (Breysse et al., 2010; Gruber et al., 2016; Krieger et al., 2011; Vincent et al., 2018).

Mold allergy (one out of many risk factors associated with asthma) is a global problem (Mari et al., 2003), especially in countries with hot, humid weather. In order to avoid mold-induced health problems, it has been recommended that mold-sensitive individuals reduce indoor humidity at or below 60% as well as avert outdoor environments when mold contamination is highest (Hayes Jr et al., 2013).

The precise prevalence of mold sensitization within the general population is not known. However, it is estimated that about 3% to 10% of the population is impacted by mold sensitization (Twaroch et al., 2015). In the United States, close to 12.9% of the general population between 6 to 59 years tested positive to mold (*Alternaria alternata*) sensitization during routine medical examination (Twaroch et al., 2015).

Molds produce allergenic proteins in copious amounts (Frew, 2004), and a single mold species can produce up to 40 different proteins that stimulate IgE production in humans. In total, about 107 allergens from 28 fungal genera have been approved by the international Nomenclature Sub-committee and the four (4) most implicated mold genera associated with eliciting allergic response are *Alternaria*, *Cladosporium*, *Penicillium*, and *Aspergillus* (Mari et al., 2003; Twaroch et al., 2015). Mold exposure occurs through inhalation, ingestion, or via cutaneous route. Inhalation route exposure is considered the most common means of exposure, and respiratory symptoms characterize it. The shape and size of mold spores vary in different diameters and can penetrate the lower respiratory airways. Mold spore size ranges between 2 and 10 μm in size and can persist in ambient air for a long time (Peden and Reed, 2010). However, the exact spore concentration necessary to elicit mold-allergic symptoms in sensitized individuals is not known and differs between mold species. Averagely, it is estimated that fungi release between 60 to 60,000 spores per day, depending on whether such homes are mold-infested or not (Pringle, 2013). It is also reported that *Alternaria* spore concentrations greater or equal to 100 spores/ m^3 can evoke allergic symptoms (Twaroch et al., 2015), while *Cladosporium* has an estimated cut off point of about 3,000 spores/ m^3 to elicit reactions in sensitized individuals. Apart from spore induced allergic reaction, some laboratory technique (such

as Halogen Immunoassay), have detected and identified a considerable number of allergens in mold fragments/hyphae and its extracellular structures(Cho et al., 2005), and argued that spore concentration alone is not a perfect fit to estimate mold exposure in a sensitized individual. As such, there is a need to explore the entire mold biological process in inducing/eliciting allergic response in sensitized individuals to mitigate mold-induced allergic health problems.

The process of mold-induced allergic disease (Figure 3.1) begins from an immune-mediated inflammatory response to mold allergen. This molds allergen induce hypersensitivity reactions, including type I Immunoglobulin E (IgE), mediated response. Together with other specific allergic response (type III IgG/IgM-mediated) and type IV delayed hypersensitivity, mold induces a cascade of different allergic diseases (Pfavayi et al., 2020). Other immune cells triggered by mold-allergic response include; allergen-specific T-helper 2 (Th2) cells response and Immunoglobulin E (IgE) production. IgE then binds to high-affinity IgE receptors located on the surface of mast cells. Following another episode of re-exposure to mold specific allergen, cross-linking of IgE occurs on the surface of the mast cell. This invariably leads to a cascade of reaction and the release of some active mediators compound, including histamine (Pfavayi et al., 2020). The mechanistic pathway by which mold triggers or exaggerate asthmatic symptoms is not fully understood. Various reports from the evidence-based study show that mold releases enzymes that induce inflammatory responses. These responses alter respiratory mucociliary action, epithelial barriers, and activates innate immune response, which leads to asthma development. Since Americans spend about 90% of their time indoors (Kader et al., 2018), inhalation of contaminated air with mold spores or fragments, volatile organic compounds

(VOCs), and mycotoxins remains one of the biggest health challenges causing approximately 4.6 million new cases of asthma. As such, this study explored the relationship between pre-existing asthma and mold-specific allergy using the National Health and Nutritional Examination Survey (NHANES) 2005-2006 database. My definition of pre-existing asthma is; participants that have responded 'YES' to the question; ever been told they have asthma?. The main aim of this study is to evaluate the association and test the hypothesis between mold-specific allergic response and pre-existing asthma and chronic bronchitis. Specifically, I have accomplished the following primary objectives; Determined the prevalence of pre-existing asthma and chronic bronchitis in my study population.

- 1) Tested the hypothesis that the odds of identifying detectable mold IgE antibodies are higher among asthma cases relative to controls without asthma when exposed to the same mold specific allergen (Figure 3.2).
- 2) Tested the hypothesis that the odds of identifying detectable mold IgE antibodies are higher among chronic bronchitis cases relative to controls without chronic bronchitis when exposed to the same mold specific allergen.

Specifically, to assess mold IgE levels I have analyzed both *Aspergillus fumigatus* and *Alternaria alternata* IgE levels. These were the only available mold IgE values available within the NHANNES 2005-2006 database.

3.3 Methods

Study Design

A cross-sectional study was conducted using the National Health and Nutritional Examination Survey (NHANES) 2005-2006 allergy component database. NHANES is a

cross-sectional study that combines both interview and physical examination to discern information on dietary, medical, physiological, demographical, and biochemical components concerning non-institutionalized United States (U.S) population within a 2-years increment (Burch et al., 2015). NHANES 2005-2006 study population is a complex, stratified, multistage probability sample that oversampled low-income participants, people above 60 years of age, adolescents, African and Mexican Americans to get an accurate sample size (Wells et al., 2014).

The main aim of the NHANES 2005-2006 allergy component is to assess the impact of common indoor allergens on allergic sensitization within the U.S population. The structural design of this component comprises; measuring allergy exposure, sensitization, and allergic symptoms amongst people who participated in this study. It consists of three (3) main parts;

- 1) Questionnaires data collecting self-reported information on allergic diseases from participants.
- 2) Household dust data, where dust extracts were collected and analyzed for indoors allergens and endotoxins.
- 3) Blood samples data, where the blood of participants was drawn at the site of the NHANES mobile examination site, measuring both total and allergen-specific immunoglobulin E (IgE).

For allergen-specific IgE (focus of this study) analysis, blood samples were evaluated using Pharmacia Diagnostics ImmunoCAP 1000 System. This is a procedure where specific allergen of interest binds to ImmunoCap™ cellulose carrier (sponge) and reacts with allergen-specific IgE in the serum sample of participants. A full description of

the laboratory procedure used for allergen-specific immunoglobulin can be found on the NHANES 2005-2006 data page (https://wwwn.cdc.gov/Nchs/Nhanes/2005-2006/AL_IGE_D.htm).

Between participants, analyzed allergic-specific IgE tests varied in number and types. Due to low blood volume, participants less than six years of age were tested for only nine allergens specific IgE (including mold *Alternaria*). In comparison, children six years plus were tested for 19 allergens specific IgE (Including mold *Alternaria* and *Aspergillus*) (Visness et al., 2009). In this study, the detection level for mold specific allergy is defined as being positive to both *Aspergillus* and *Alternaria* molds at a lower detection limit of 0.35kU/L or upper detection limits of 1000 kU/L of elicited IgE responses.

Study Population

The schematic representation of the participants used in this study is well described in figure 3.3. Participants, less than 12 years of age were excluded from this study because of the possibility that children might outgrow asthma conditions as they grow older and because children less than six years of age did not have information on *Aspergillus* induced allergic response on NHANES 2005-2006 database. Also excluded from this study are participants diagnosed with emphysema. As such, my final working analytical sample comprised of 4459 participants used for both *Alternaria* and *Aspergillus* mold-induced IgE allergy study. Health-related information of interest was obtained from 4459 participants through a questionnaire. Specifically, questions such as ‘Ever been told you have asthma,’ ‘Ever been told you have chronic bronchitis,’ ‘Ever been told you have allergies,’ ‘Ever been told you have stoke,’ were used to determine the significance of mold-induced allergy in specific health conditions. From the list of all listed health

conditions, asthma and chronic bronchitis became the primary outcome of interest-based on calculated p-values (<0.0001 asthma) and p-values (0.20 vs. 0.96 chronic bronchitis) from detectable and Non-detectable IgE level of both *Aspergillus fumigatus* and *Alternaria alternata* used in this study(see table 3.1). In this study, we used both *Aspergillus fumigatus* and *Alternaria alternata* species in answering our research questions, which states that; 1) Does pre-existing asthma increases the vulnerability of mold allergy, and 2) Does pre-existing bronchitis increases the vulnerability of mold allergy?

To get a sample size for asthma and chronic bronchitis analysis, participants from mold allergy study (N=4459) was stratified into two (2) categories;

- 1) Asthma Analytical sample size (N=4144): Amongst the analytical sample size for asthma study, 439 (11%) participants had been told at some point in their lives that they had asthma disease (Cases). In comparison, 3705 (89%) participants have no asthma history(Controls)(Table 3.2).
- 2) Chronic Bronchitis Analytical sample size (N=3830): On the other hand, 125 out of 3830 participants have been diagnosed with chronic bronchitis, and 3705 does not have a history of Chronic bronchitis(Table3.2).

Assessment of Mold Specific Allergic Response

All statistical analyses were conducted using SAS statistical software. The enrollment of participants into the NHANES study is by the clustered sample design. In order to get accurate estimates of population parameters, NHANES design variables, and 2-year weights (WTMEC2YR) were created for the analyses using standard procedure. The strata (SDMVSTRA) and cluster (SDMVPSU) variables were included in all statistical analyses to account for population stratification and cluster sampling procedures used

during enrollment. A Chi-square test was used to compare demographic characteristics between cases and control. Association between pre-existing asthma and mold specific allergy was performed by multivariate logistic regression (Proc Survey Logistic).

Firstly, the crude analysis was done to explore the association between each mold-induced IgE level and pre-existing asthma/chronic bronchitis condition from participant's asthma and chronic bronchitis analytical datasets. Then, all models were adjusted for potential confounders. Using a manual selection procedure, covariates were included in multivariate logistic analyses. Potential confounders included: age, sex, race, education, marital status, smoking, cigarette used last five days, other allergies, and family history on asthma condition. Each variable was entered in the model only if they had a significant P-value of less than 0.2 (case vs. control). In the final model, all covariate that was either significant or met the 10% rule was retained for analysis.

3.4 Result

For both mold allergy study, participants were more of females than males (52% vs. 48%), non-Hispanic white ethnicity(50%), married or living with a partner (63%) and has less than a college degree (51% vs. 49%) (see table 3.1). Overall, the characteristic demographic distribution among participants used for both asthma and chronic bronchitis analytical study is the same(Table 3.2). There were approximately 52% female and 48% male representatives in each study. The average age of participants in the asthma analytical study is 46years, while 47years is the average age of participants in chronic bronchitis study(Data not showed). However, in both studies, the age range of participants is between 20 years to 85 years. A vast number of participants in this study were of the non-Hispanic

white race (49%), had less than a college degree (approximately 53%), and are married or living with a partner (64%).

The prevalence cases of *Aspergillus fumigatus* induced allergic IgE response in asthma and chronic bronchitis was 14% versus 6%, respectively, while then the prevalence of *Alternaria alternata* induced allergic IgE response in asthma and chronic bronchitis is 19% versus 2%. Amongst *Aspergillus fumigatus* sensitized asthma participants, 64% were females, with the majority originating from non-Hispanic black ethnicity (43%), married (55%), and had some college degree (42%) (Data not shown).

The crude association between mold allergy (caused by both mold species) and each respiratory condition was investigated. Participants who were diagnosed with asthma conditions had a higher statistically significant *Alternaria alternata* detectable IgE (OR =3.58, 95%CI=2.36-5.44,P=<0.0001) compared to *Aspergillus fumigatus* detectable IgE concentration(OR=2.91, 95%CI=1.69-5.04,P=0.0009). On the other hand, participants diagnosed with *chronic bronchitis* have no association with both molds-induced allergic response used in this study OR=1.01,95% CI= 0.317-3.185, P=0.99 (*Aspergillus fumigatus*) and OR= 0.16, 95%CI=0.031-0.79, P=0.03(*Alternaria alternata*). In the comparison of both mold-induced sensitivities, *Alternaria alternata* showed a significantly higher odds than *Aspergillus fumigatus* in asthma condition than in chronic bronchitis.

After adjusting for numerous variables, the association between pre-existing asthma and mold specific IgE response still had higher significant values of mold detectable IgE level. Specifically, *Aspergillus fumigatus* had an OR=2.44(1.36-4.40), and *Alternaria alternata* had an OR=2.9(1.79-4.69) in pre-existing asthma cases, On the contrary the association between chronic bronchitis and each mold specific IgE allergic

response were found not significant: OR=1.05(0.31-3.51) for *Aspergillus fumigatus* IgE and OR=0.03(0.003-0.236) for *Alternaria alternata* IgE(Table3.3).

3.5 Discussion

Although mold-induced allergy has been studied extensively in asthma disease, no study to date has examined the influence of mold-induced sensitization in chronic bronchitis diseases. Our results show no association between mold-induced sensitization caused by both *Aspergillus fumigatus* and *Alternaria alternata* in participants diagnosed with Chronic bronchitis condition (See table 3.4). However, the odds of *Alternaria alternata* induced IgE increased with Chronic bronchitis after adjusting for the different covariate. Since the number of chronic bronchitis participants showing *Alternaria alternata* induced IgE were small, there is an obvious limitation in results interpretation. However, studies have shown that mold exposure is associated with Chronic bronchitis(Fisk et al., 2010); as such, there is a need for further studies on mold-induced sensitization amongst bronchitis individuals in general.

More so, to the best of our knowledge, no research has thoroughly explored what portion of mold specific allergen is associated with asthma conditions using adult population characteristics. Salo et al., 2011, explored the significance of both total and specific allergen risk factors (including mold) and its association with allergic diseases within the United States population using the NHANES 2005-2006 database. Their results showed that mold specific allergen was consistently associated with allergic disease outcomes. Also, their study identified that the prevalence of sensitization induced by mold specific IgE was higher in children compared to adults living in the United States(Salo et al., 2011). Another study carried out by Stevenson et al. on the influence of

sociodemographic disparity and allergen sensitivity using the NHANES database showed that African American children between ages 6 to 16 years had a significantly higher odds of sensitivity to *Alternaria alternata* allergen (Stevenson et al., 2001).

The results of this present study show that pre-existing asthma patients have higher exposure to mold allergy sensitization compared to patients diagnosed with Chronic bronchitis. Precisely, this study has estimated that pre-existing asthma has a significantly higher crude odd to *Alternaria alternata* (OR 3.35, CI 2.36-5.44) compared to *Aspergillus fumigatus* mold-induced sensitization (OR 2.91, CI 1.69-5.04). These results provide evidence that adult patient that was diagnosed with the asthmatic condition are susceptible to specific mold-induced sensitization, particularly those allergies caused by *Alternaria alternata*. Importantly, this study reveals that specific mold sensitivity is not associated with chronic bronchitis.

Because the prevalence of each of these mold allergens (*Aspergillus fumigatus* and *Alternaria alternata*) have been associated with flood-impacted homes post-hurricane (Bloom et al., 2009; Chew et al., 2006; Dumon et al., 2009; Rao et al., 2007) and asthma morbidity ((Agarwal and Gupta, 2011; O'Driscoll et al., 2005; Stevenson et al., 2001), this study is of utmost significance as informed decision could be taken in putting the effective measure in place for controlling specific mold allergens associated with the indoor and outdoor environment.

Findings from this study are also similar to other studies showing that the prevalence of mold allergen *Alternaria alternata* (8.6%) is higher than *Aspergillus fumigatus* (2.4%) allergen in the atopic population living in the United States (Gergen et al., 2009). The consistency of these findings identifying *Alternaria alternata* mold allergen

exposure amongst asthma patient raises questions on how individuals get exposed to specific mold allergens since *Alternaria alternata* is an outdoor allergen, and most Americans spend about 90% of their time indoors(Kader et al., 2018).

Alternaria alternata, a typical outdoor allergen, has been reported to be associated with asthma(Gabriel et al., 2016). Other studies have also associated asthma severity, frequent asthma hospitalization, and Asthma-related death to *Alternaria alternata* allergen(Black et al., 2000; Neukirch et al., 1999; Zureik, 2002). Although other contributing risk factors associated with sensitization and asthma exist, it seems unlikely that the difference between each mold-induced sensitivity observed in this study is influenced by different potential Asthma associated risk factors, mainly because we modeled for the same covariate (allergies, being overweight, family history and educational background) in both molds induced cases of asthma disease

Other risk factors associated with asthma diseases and severity, including sex, race, and cigarette smoking(Eisner and Iribarren, 2007; Gwynn, 2004; Kim and Camargo, 2003), were also included in our model analysis. However, this study did not find any significant association between those factors and asthma induced by mold sensitivity. Consequently, after adjusting for those variables, pre-existing asthma still has a strong association with *Alternaria alternata* allergen compared to *Aspergillus fumigatus* allergen.

The strength of this study lies in the fact that the NHANES database was used to explore the association between pre-existing asthma and mold specific induced allergy. NHANES database is comprehensive and does not select participants based on disease condition, but instead, participants were selected to reflect the civilian non-institutionalized

U.S. population. All data collected were also processed in a consistent and standardized way.

One major limitation of using the NHANES survey is that participants reported self-asthma conditions without confirmatory measures of asthma symptoms (Gergen et al., 2009). Also, NHANES is a cross-sectional study. Thus, the cause and effect between exposure and response cannot be determined. Finally, although the standard value of specific IgE used by NHANES 2005-2006 is the same for the general U.S. population, this might be a difference between the individual or people living in a particular region. For example, the detectable value of specific mold allergy was considered positive at a lower value of 0.35kU/L. This value might actually vary between individuals as some may be way higher than what is reported in the NHANES database.

Figures 3.6

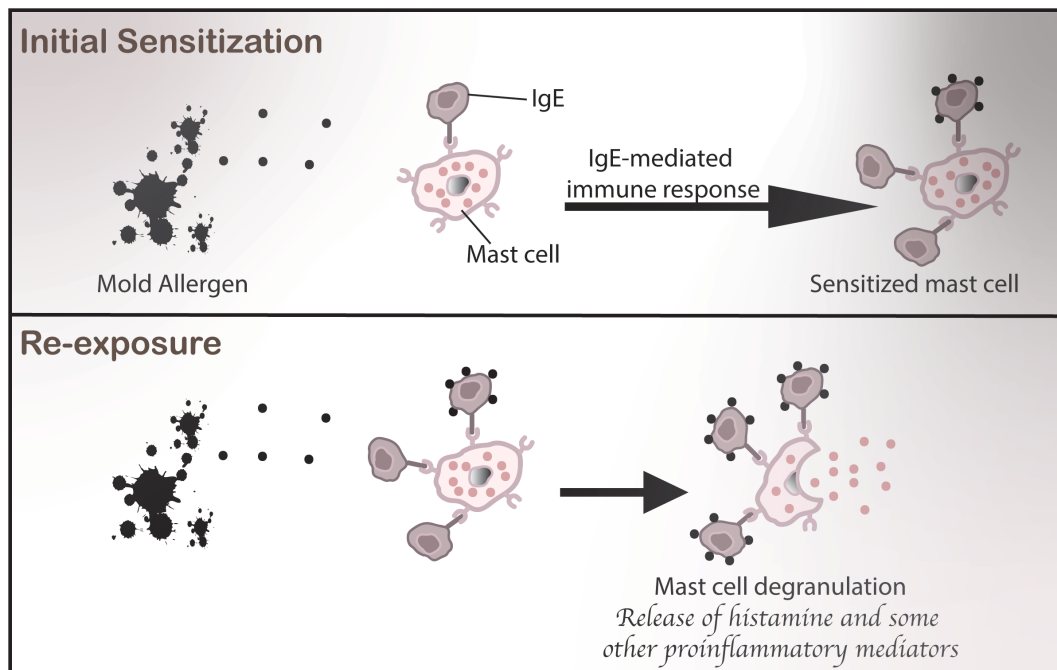


Figure 3.1. Activation of mast cell by mold allergen

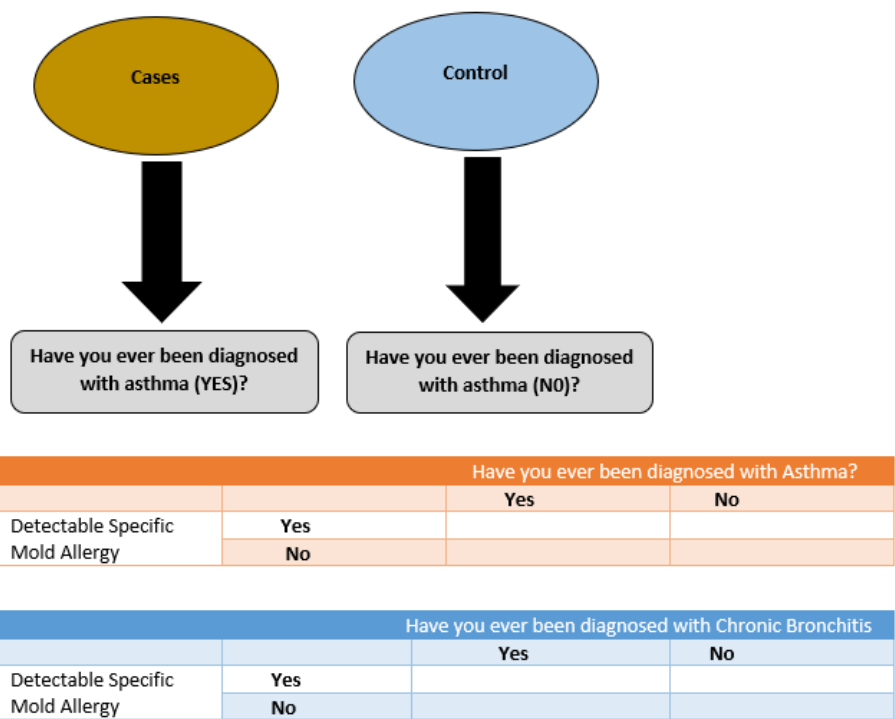
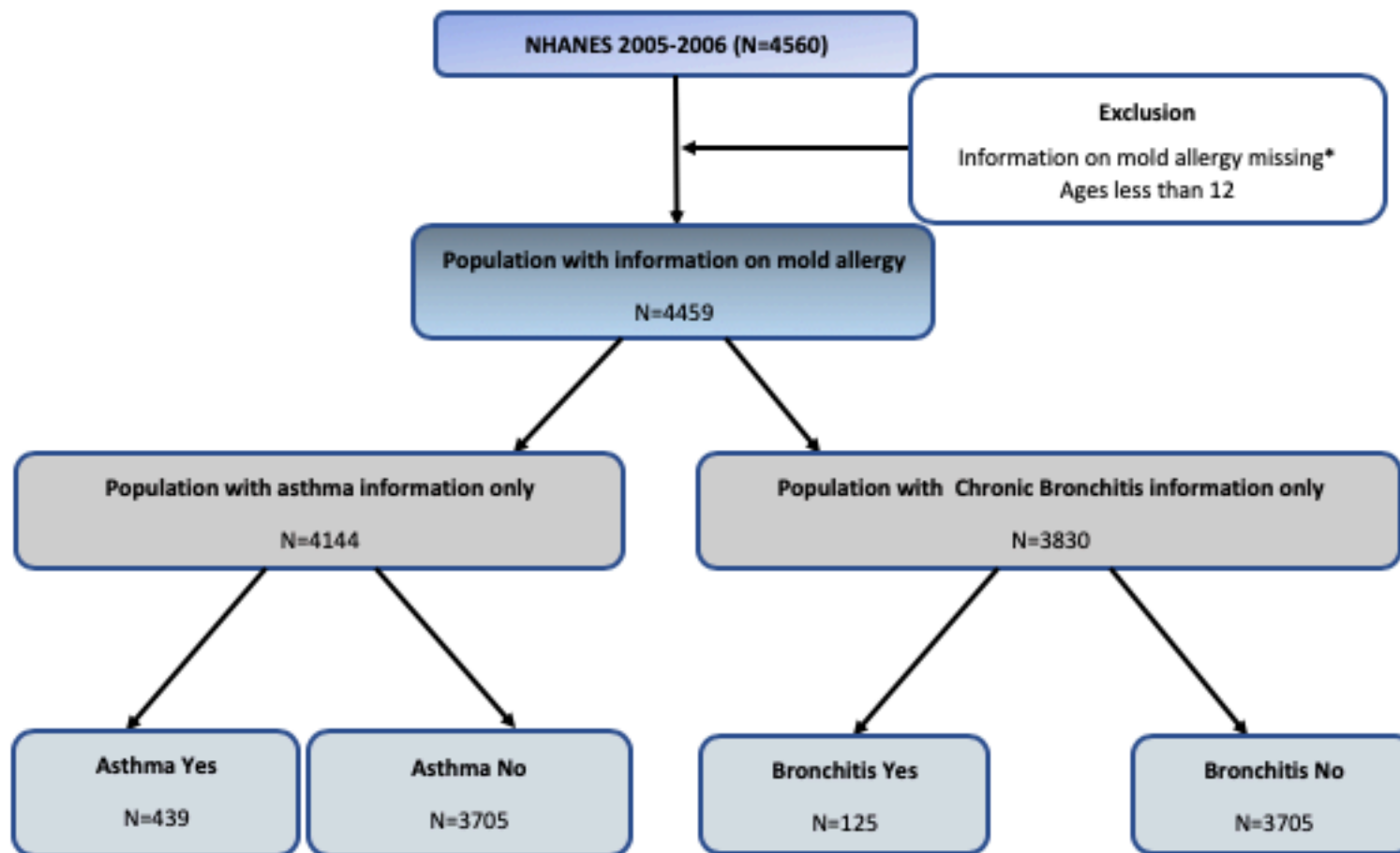


Figure 3.2. Study Design for Objective 1



***In this Study, we looked at *Aspergillus Fumigatus* and *Alternaria alternata* Species**

Figure 3.3: NHANES 2005-2006 Analytical Sample (Pre-existing asthma is associated with mold specific allergy)

3.7 Tables

Table 3.1. Demographic Characteristics of Study Population used for *Aspergillus Fumigatus* and *Alternaria Alternata* IgE Allergy Study

Analytical Sample for <i>Aspergillus Fumigatus</i> IgE Allergy Study				
Variable ^a	Total Sample size (n=4459) n (%)	Undetectable IgE (n=4166) (93)	Detectable IgE (n=293) (7)	Detectable vs Undetectable P-value ^b
Sex				
Male	2137 (48)	1952 (47)	185 (63)	<0.0001
Female	2322 (52)	2214 (53)	108 (37)	
Race/Ethnicity				
Latino	1216 (27)	1140 (27)	76 (26)	<0.0001
Non-Hispanic White	2251 (50)	2146 (52)	105 (36)	
Non-Hispanic Black	992 (22)	880 (21)	112 (38)	
Marital Status				
Married/Living with Partner	2823 (63)	2658 (64)	165 (56)	<0.0001
Widowed	389 (9)	376 (9)	13 (4)	
Divorced/Separated	560 (13)	523 (13)	37 (13)	
Never Married	684 (15)	606 (15)	78 (27)	
Education Level				
Less than college degree	2289 (51)	2137 (51)	152 (52)	0.14
Some collage /graduate degree	2165 (49)	2024 (49)	141 (48)	
Veteran/Military Status				
Yes	641 (14)	585 (14)	56 (19)	0.06
No	3817 (86)	3580 (86)	237 (81)	

Tobacco/Nicotine used last 5 days				
Yes	1086 (26)	1004 (24)	82 (28)	0.08
No	3063 (74)	3166 (69)	210 (72)	
General Health Condition				
Very Good	1668 (37)	1558 (37)	110 (38)	0.82
Good	1600 (39)	1496 (36)	104 (35)	
Fair	761 (18)	715 (17)	46 (16)	
Poor	127 (3)	120 (3)	7 (2)	
Specific Health Conditions				
Overweight				
Yes	1383 (31)	1288 (31)	95 (32)	0.15
No	3073 (69)	2876 (69)	197 (67)	
Asthma				
Yes	575 (13)	496 (12)	79 (27)	<0.0001
No	3884 (87)	3670 (88)	214 (73)	
Chronic bronchitis				
Yes	259 (6)	237 (6)	22 (8)	0.20
No	4200 (94)	3929 (94)	271 (92)	
Emphysema				
Yes	85 (2)	78 (2)	7 (2)	0.64
No	4367 (98)	4081 (98)	286 (98)	
Coronary Heart Disease				
Yes	177 (3)	167 (4)	10 (3)	0.73
No	4262 (96)	3981 (96)	281 (96)	
Stroke				
Yes	171 (4)	164 (4)	7 (2)	0.30
No	4281 (96)	3996 (96)	285 (97)	
Close Relative Had Asthma				
Yes	913 (20)	836 (20)	77 (26)	0.02
No	3449 (77)	3242 (78)	207 (71)	

Allergies				
Yes	1386 (31)	1271 (31)	115 (39)	0.006
No	3063 (69)	2885 (69)	178 (61)	
Eczema				
Yes	285 (6)	261 (6)	24 (8)	0.34
No	4167 (93)	3898 (94)	269 (92)	
Analytical sample for <i>Alternaria Alternata</i> IgE Allergy Study				
Variable^a	Total Sample Size (n=4459) n (%)	Undetectable IgE (n=4128) (93)	Detectable IgE (n=331) (7)	Detectable vs. Undetectable P-value^b
Sex				0.004
Male	2137 (48)	1953 (47)	184 (56)	
Female	2322 (52)	2175 (53)	147 (44)	
Race/Ethnicity				0.0001
Latino	1216 (27)	1167 (28)	49 (15)	
Non-Hispanic White	2251 (50)	2094 (51)	157 (47)	
Non-Hispanic Black	992 (22)	867 (21)	125 (38)	
Marital Status				<0.0001
Married/Living with Partner	2823(63)	2635 (64)	188 (57)	
Widowed	389 (9)	381 (9)	8 (2)	
Divorced/Separated	560 (13)	525 (13)	35 (11)	
Never Married	684 (15)	584 (14)	100 (30)	
Education Level				<0.0001
Less than college degree	2289 (51)	2167 (52)	122(37)	
Some collage/ graduate degree	2165 (49)	1956 (47)	209(63)	
Veteran/Military Status				0.73
Yes	641 (14)	598 (14)	43 (13)	
No	3817 (86)	3529 (85)	288 (87)	

Tobacco/Nicotine used last 5 days Yes No	1086 (26) 3063 (74)	996 (24) 2846 (69)	90 (21) 217 (66)	0.19
General Health Condition Very Good Good Fair or Poor	1668 (37) 1600 (39) 761 (18) 127 (3)	1524 (37) 1480 (36) 721 (17) 123 (3)	144 (44) 120 (36) 40 (12) 4 (1)	0.004
Specific Health Conditions Overweight Yes No	1383 (32) 3073 (68)	1278 (31) 2847 (69)	105 (32) 226 (68)	0.85
Asthma Yes No	575 (13) 3884 (87)	475 (12) 3653 (88)	100 (30) 231 (70)	<0.0001
Chronic bronchitis Yes No	259 (6) 4200 (94)	240 (6) 3888 (94)	19 (6) 312 (94)	0.96
Emphysema Yes No	85 (2) 4367 (98)	84 (2) 4037 (98)	1 (0) 330 (100)	0.06
Coronary Heart Disease Yes No	177 (4) 4262 (96)	169 (4) 3939 (95)	8 (2) 323 (98)	0.14
Stroke Yes No	171 (4) 4281 (96)	164 (4) 3957 (96)	7 (2) 324 (98)	0.18

Close Relative Had Asthma Yes No	913 (20) 3449 (77)	820 (20) 3224 (78)	93 (28) 225 (68)	<0.0001
Allergies Yes No	1386 (31) 3063 (69)	1225 (30) 2893 (70)	161 (49) 170 (51)	<0.0001
Eczema Yes No	285 (6) 4167 (93)	251 (6) 3870 (94)	34 (10) 297 (90)	0.008

^a Column percentage may not add up to 100% due to rounding or missing data,

^b Chi-squared test used for computing difference in proportion between cases and controls

Table 3.2. Demographics Population Characteristics Used for Studying Association between Asthma, Chronic Bronchitis, and Mold Allergy

The analytical sample used for Asthma Analysis				
Variable ^a	Total Sample Size (n=4144) n (%)	Controls (n=3705) n (89)	Cases (n=439) n (11)	Cases vs Controls P-value ^b
Sex Male Female	2002 (48) 2142 (52)	1804 (49) 1901 (51)	198 (45) 241 (55)	0.15
Race/Ethnicity Latino Non-Hispanic White Non-Hispanic Black	1177 ((28) 2042 (49) 926 (22)	1089 (29) 1808 (49) 808 (22)	88 (20) 233 (53) 118 (27)	0.0005

Marital Status				
Married/living with partner	2653 (64)	2401 (65)	252 (57)	0.0004
Divorced/separated	843 (20)	743 (20)	100(23)	
Never Married	645 (16)	558 (15)	87 (20)	
Veteran/Military Status				
Yes	573 (14)	525 (14)	48 (11)	0.17
No	3570 (86)	3179 (86)	391 (89)	
Education Level				
Less than College degree	2136 (52)	1953 (53)	183 (42)	<0.0001
Some college/graduate degree	2004 (48)	1748 (47)	256 (58)	
Tobacco/Nicotine used last 5 days				
Yes	960 (25)	846 (23)	114 (33)	0.15
No	2891 (75)	2595 (70)	296 (67)	
Do you now smoke Cigarettes?				
Yes	870 (21)	769 (21)	101 (23)	0.32
No	1020 (25)	918 (25)	102 (23)	
Smoked at least 100 cigarettes in life				
Yes	1890 (46)	1687 (46)	203 (47)	0.80
No	2254 (54)	2018 (54)	236 (54)	
Secondhand Smoke at workplace				
Yes	498 (12)	436 (12)	62 (12)	0.48
No	1976 (48)	1766 (48)	210 (48)	
Doctor said you were overweight				
Yes	1253 (30)	1077 (29)	176 (40)	<0.0001
No	2888 (70)	2625 (71)	263 (71)	
General Health Condition				
Very Good	1594 (38)	1436 (39)	158 (36)	0.53
Good	1491 (36)	1327 (36)	164 (37)	
Fair/Poor	772 (19)	684 (18)	88 (20)	

Close Relative Had Asthma				
Yes	821 (20)	641 (17)	180 (41)	<0.0001
No/Refused	3323 (80)	3064 (83)	259 (59)	
Allergies				
Yes	1217 (29)	957 (26)	260 (59)	<0.0001
No/Refused	2927 (71)	2748 (74)	179 (41)	

Analytical Sample Used for Chronic Bronchitis Analysis				
Variable^a	Total Sample Size (n=3830) n (%)	Controls (n=3705) n (97)	Cases (n=125) n (3)	Cases vs Controls P-value^b
Sex				
Male	1852 (48)	1804 (49)	48 (38)	0.02
Female	1978 (52)	1901 (51)	77 (62)	
Race/Ethnicity				
Latino	1105 (29)	1089 (29)	16 (13)	0.0007
Non-Hispanic White	1890 (49)	1808 (49)	82 (66)	
Non-Hispanic Black	835 (22)	808 (22)	27 (22)	
Marital Status				
Married/living with partner	2465 (64)	2401 (65)	64 (51)	0.01
Divorced/separated	786 (21)	743 (20)	43 (34)	
Never Married	576 (15)	558 (15)	18 (14)	
Veteran/Military Status				
Yes	545 (14)	525 (14)	20 (16)	0.83
No	3284 (86)	3179 (86)	105 (84)	

Education Level				
Less than a college degree	2016 (53)	1953 (53)	63 (50)	0.02
Some college/ graduate degree	1809 (47)	1748 (47)	61 (49)	
Tobacco/Nicotine used last 5 days				
Yes	898 (25)	846 (23)	52 (42)	<0.0001
No	2662 (75)	2595 (70)	67 (54)	
Do you now smoke Cigarettes?				
Yes	816 (21)	769 (21)	47 (38)	0.007
No	952 (25)	918 (25)	34 (27)	
Smoked at least 100 cigarettes in life				
Yes	1768 (46)	1687 (46)	81 (65)	0.0001
No	2062 (54)	2018 (54)	44 (35)	
Secondhand Smoke at workplace				
Yes	452 (12)	436 (12)	16 (13)	0.30
No	1807 (47)	1766 (48)	41 (33)	
Doctor said you were overweight				
Yes	1125 (29)	1077 (29)	48 (38)	0.08
No	2702 (71)	2625 (71)	77 (62)	
General Health Condition				
Very Good	1470 (38)	1436 (39)	34 (27)	0.01
Good	1378 (36)	1327 (36)	51 (41)	
Fair/Poor	718 (19)	684 (18)	34 (27)	
Close Relative Had Asthma				
Yes	667 (17)	641 (17)	26 (21)	0.02
No/Refused	3163 (83)	3064 (83)	99 (79)	
Allergies				
Yes	1009 (26)	957 (26)	52 (42)	<0.001
No/Refused	2821 (74)	2748 (74)	73 (58)	

^a Column percentage may not add up to 100% due to rounding or missing data,

Table 3.3. Pre-Existing Asthma Status by Specific Mold Allergy

Allergy	Asthma							
	Case (n=439) (n=11%)	Control (n=3705) (n=89%)	Crude OR	95% CI	P-value	Adjusted OR ^a	95% CI	P-value
<i>Aspergillus Fumigatus</i>			2.91	1.69-5.04	0.0009	2.44	1.36-4.40	0.0056
Detectable IgE	63 (14)	203 (5)						
Non-Detectable IgE	376 (86)	3502 (94)						
<i>Alternaria alternata</i>			3.58	2.36-5.44	<0.0001	2.9	1.79-4.69	0.0003
Detectable IgE	83 (19)	228 (6)						
Non-Detectable IgE	356 (81)	3477 (94)						

^aAdjusted for Allergies, Family history, Overweight, Education Level, Marital status, Race, Nicotine Used ,Sex and Veteran

Table 3.4. Chronic Bronchitis Status by Specific Mold Allergy

Allergy	Chronic Bronchitis							
	Case (n=125) (n=3%)	Control (n=3705) (n=97%)	Crude OR	95% CI	P-value	Adjusted OR ^a	95% CI	P-value
<i>Aspergillus Fumigatus</i>			1.01	0.317-3.185	0.99	1.05	0.31-3.51	0.9363
Detectable IgE	7 (6)	203 (5)						
Non-Detectable IgE	118 (94)	3502 (95)						
<i>Alternaria alternata</i>			0.16	0.031-0.79	0.03	0.03	0.003-0.236	0.028
Detectable IgE	3 (2)	228 (6)						
Non-Detectable IgE	122 (98)	3477 (94)						

^aAdjusted for Allergies, Family history, Overweight, Education Level, Marital status, Race, Nicotine Used ,Sex ,Veteran Status, General

CHAPTER 4

ARE CHILDREN IN DEVELOPING NATIONS BEING EXPOSED TO MULTIPLE MYCOTOXINS THROUGH COMPLEMENTARY FEEDING?

1

^{1 1} Gummadidala PM, ¹Omebeyinje MH, Burch JA, Chakraborty P, Biswas PK, Banerjee K, Wang Q, Jesmin R, Mitra C, Moeller PDR, Scott G, Chanda A. Published in Food and Chemical, 2018.

4.1 Abstract

Contamination of food by molds and its metabolite is a global public health issue. Molds contaminated feed can put Indian infants and toddlers at risk of simultaneous exposures to deoxynivalenol and aflatoxin M1, a fungi secondary metabolite produced mostly in the region with hot and humid condition. While mycotoxin contamination of baby foods has been reported earlier, there is little or no knowledge of the presence of multiple mycotoxin contamination in baby food products produced and manufactured in India. As such, our studies explored the presence of mycotoxins produced by *Aspergillus* (Aflatoxin B1, B2, G1, G2 & M1) and *Fusarium* (Deoxynivalenol) in baby food products manufactured and produced in Kolkata, Indian.

Twenty-nine (29) commercially produced baby food samples, manufactured by twenty-one (21) different manufacturers were obtained and analyzed for this study. Using a previously established direct competitive enzyme-linked Immunoassay (ELISA), we analyzed 50g of each baby food samples for the content of either aflatoxin (B1, B2, G1, G2&M1) and 10g of baby food sample was analyzed for deoxynivalenol (DON).

Results showed that 100% of each analyzed baby food sample was contaminated with aflatoxin M1 at a level exceeding the recommended European Union level of 25ng kg⁻¹. Two of these samples (6.9%) also demonstrated the occurrence of DON at levels higher than EU guidelines for baby food products (200 µg/ Kg), and 19 others (66%) demonstrated the presence of DON at concentrations ranging from 14 - 107 µg/ Kg. Lastly, none of the samples contained aflatoxins B1, B2, G1, and G2.

4.2 Introduction

Food contamination by fungi secondary metabolite (mycotoxin) is a global public health issue. The prevalence of fungi-contaminated food is high and most common in regions where environmental conditions (optimum temperature and humidity) favors the growth and secondary metabolism of mycotoxin producing fungi (Marroquín-Cardona et al., 2014; Wambacq et al., 2016). India and the neighboring Southeastern Asian countries, for example, face enormous mycotoxin contamination problems when humidity exceeds 13% and the temperature ranges between 25 °C and 35 °C (Ali et al., 2015; Hesseltine, 1976; Mudili et al., 2014; Priyanka et al., 2014; Reddy et al., 2009; Ruadrew et al., 2013; Vasanthi and Bhat, 1998; Waenlor and Wiwanitkit, 2003). Indian standing crops and food grains are fragile and venerable to fungal invasion and mycotoxin contamination during tidal floods coupled with tropical temperatures during the monsoon season (Bilgrami and Choudhary, 1998). Some of the most economically valuable food grains of these regions (including rice, maize, wheat, gram, sorghum) are contaminated by fungi during pre- and post-harvest procedures. Aflatoxins and deoxynivalenol (DON), two of the most common foodborne mycotoxins in Indian food products, have significant impacts on the health of humans and animals (Kumar et al., 2017; Payros et al., 2016; Shephard, 2016). With ongoing climate change (including high temperature and flash floods), mycotoxin occurrence in foods will increase as fungi eradication will become more difficult (Paterson and Lima, 2010).

Aflatoxins are synthesized as secondary metabolites by some fungi species within the genus *Aspergillus* (such as *A. flavus*, *A. parasiticus*, *A. nomius*) and are responsible for about 0.025- 0.15 million cases of hepatocellular carcinoma globally (Liu and Wu, 2010).

It is reported that Aflatoxin B₁ is the most potent naturally occurring carcinogen known (Gummadidala et al., 2016). Aflatoxin M₁, a hydroxylated metabolite of aflatoxin B₁, contaminates milk from livestock fed with feed contaminated with Aflatoxin B₁. Aflatoxin M₁ is a milk-based food product and is also classified as a Group 2 carcinogen by the International Agency for Research on Cancer (Hsieh et al., 1985). Although it is less potent than AFB₁ in cytotoxicity, it is still an active cytotoxin and a genotoxin. For example, it displays toxicity in primary cultures of rat hepatocytes at 0.6 µg / culture as compared to 0.05-0.1 µg / culture for AFB₁. Since baby foods are predominantly milk-based, the risk of aflatoxin M₁ occurrence in baby foods manufactured in India (including infant powdered milk and other milk-based foods and drinks for toddlers) is high. Several previous reports on aflatoxin M₁ monitoring in Indian baby foods (Kanungo and Bhand, 2014; Rastogi et al., 2004; Siddappa et al., 2012) indicated presence of aflatoxin M₁ exceeding the recommended EU level of 0.025 µg/ Kg, suggesting that children in India are vulnerable to the risk of high dietary intake aflatoxin M₁.

DON, a trichothecene, also known as vomitoxin, is synthesized by some fungi species within the genus *Fusarium*, such as *F. graminearum* and *F. culmorum* (Drochner, 1989; Magan et al., 2010; Wegulo, 2012). Health effects of DON include; dysregulation in immune function (immune suppression), gastrointestinal epithelial atrophy, and inflammation (Pestka, 2010; Rotter, 1996). Based on the toxicity of DON, EU's recommended maximum permissible limit for DON in baby foods is 200 µg/Kg. They were additionally based on toxicokinetic data on DON, the joint FAO/WHO expert committee on Food Additives (JECFA) has established a provisional maximum tolerable daily intake (PMTDI) of 1 µg/Kg body weight (JECFA, 2002). India is the second-largest producer of

wheat in the world, and frequent occurrence of DON in food has been implied through multiple studies (Bhat et al., 1989; Mishra et al., 2013). Although studies showing the presence of DON in Indian baby foods are rare, the baby foods comprising a combination of milk and cereal products are likely to contain DON (as well as other *Fusarium* toxins) in addition to aflatoxin M1. Although the Indian Food Safety and Standard Regulation (FSSR) has a recommended maximum level of 1mg/Kg of DON in wheat (Mishra et al., 2013), there are currently no recommendations for DON in baby foods.

Hence, the goals of this study were: (1) to evaluate whether a combined dietary intake of aflatoxins and DON can occur in Indian children (ages 0-2 years) through ingestion of commercially available baby food products; and (2) to assess whether there is a need for more focused surveillance of mycotoxin profiles in baby foods. To address these goals, we conducted a convenience sampling of the most popular commercially available baby food products in India that were purchased from the local markets in the city of Kolkata. Specifically, we investigated if these samples contained aflatoxins B₁, B₂, G₁ and G₂, aflatoxin M1 and DON at levels that either exceeded the European Union (EU) recommended levels (van Egmond et al., 2007) or could result in exposure greater than accepted tolerable daily intakes (TDI) recommended by the EU (van Egmond et al., 2007). Reported here are the results of this survey.

4.3 Materials and Methods

Samples. A total of 29 baby food samples were obtained randomly from different retail stores in Kolkata and stored at -20 °C before the opening of the food packages for analysis. Food samples were broadly classified into five different types;

(1) Nutritional Drinks (5 samples) comprising both milk and grain products.

- (2) milk and grain-based breakfast foods (7 samples).
- (3) essential food ingredients (6 samples) that are commonly utilized to prepare baby foods
- (4) snacks (6 samples) and
- (5) infant milk powder (5 samples).

Mycotoxin extraction and analysis. A total of 50g from each sample was used for quantitative analysis of aflatoxins B₁, B₂, G₁ and G₂, and aflatoxin M₁, while 10g of the sample was used for DON analysis. Mycotoxins in the samples were extracted using a chloroform: methanol extraction in which samples were initially vigorously mixed with 15 mL of chloroform in a 50 mL conical tube. The chloroform extract was transferred to a glass vial and air-dried. Finally, 5 mL of 70% methanol was added to the dried extract and shaken vigorously. The methanol extract was subsequently centrifuged at 10,000g for 5 min before analysis. Quantitative analysis of DON and aflatoxins B₁, B₂, G₁, G₂, and M₁ were performed using competitive enzyme-linked immunoassay method using three separates highly sensitive veratox kits (Neogen, Lansing, MI) for measuring: (a) total aflatoxins, B₁, B₂, G₁, and G₂ (cat. 8035), (b) DON (cat. 8332), and (c) aflatoxin M₁ (cat. 8019). The assays were performed using the manufacturer's instructions. For positive control- a sample of food was spiked with all seven mycotoxins with known concentrations- This was used to conduct an initial validity test corresponding to each kit used in this study. Each positive control sample was measured in triplicates to obtain an estimation of the average standard errors in our mycotoxin measurements. This validity test was an essential quality assurance measure of our mycotoxin analyses.

Exposure assessment. Exposure to mycotoxins DON and aflatoxin M₁ were calculated based on previously published ("CSFII Analysis of Food Intake Distributions,"

n.d.) average estimated daily intake of grain and milk-based baby foods during 0-2 years. The estimated daily intake (EDI) of the mycotoxins were calculated according to the formula $EDI = (\text{mean mycotoxin concentration in the samples} \times \text{estimated daily intake of the food sample}) / \text{body weight}$. Finally, the EDI of the mycotoxins per kg body weight were then compared with the provisional maximum tolerable daily intake (PMTDI) advised by the joint FAO/WHO expert committee on Food Additives (Joint FAO/WHO Expert Committee on Food Additives et al., 2011). Exposure risk was inferred if the EDI exceeded the PMTDI.

4.4 Results

At a detection limit of 5 parts per trillion (ppt), our analyses reveal that aflatoxin M1 dominated in all the food samples and ranged between 0.8 µg/ Kg to 3.2 µg/ Kg, which were an order of magnitude higher than the EU recommended levels of 0.025 µg/ Kg. The aflatoxin M1 range detected, with a 2% standard error in measurements, for the different food categories, is shown in Table 4.1. Alongside aflatoxin M1, we conducted a quantitative analysis of aflatoxins B₁, B₂, G₁, and G₂, which indicated non-detectable levels of these mycotoxins in the baby foods analyzed (data not shown). At a detection limit of 5 ppb, DON was detected in 19 out of 29 samples (66%). The concentration levels of the DON detected in the samples ranged from 10 µg/ Kg to 258 µg/ Kg (Table 4.2). In two of these samples (6.9%), the concentrations exceeded the EU recommended 200 µg/ Kg maximum limit for DON in baby foods. Both products were wheat-based foods and belonged to the ‘snacks’ category. Infant milk powder demonstrated the lowest presence of DON; one out of the four samples tested (25%) contained 14 µg/ Kg of DON. The DON concentration for other categories displayed a higher variance relative to the snacks and

infant milk powder samples (ranging between 0 -77 $\mu\text{g}/\text{Kg}$), with higher values observed for wheat-based food products.

Next, using these preliminary values of DON and aflatoxin M1 levels, we estimated a possible daily dietary intake of DON and aflatoxin M1 in children with 0-2 years in Kolkata (Figure 4. 1). Aflatoxin M1 is an IARC classified carcinogen (Ostry et al., 2017), but there is currently no recommended PMTDI value for aflatoxin M1 in baby food. However, based on animal studies, an EDI of 0.2 ng/Kg body weight has been proposed as a “safe dose” (Kuiper-Goodman, 1990). As suggested in Table 4.3, infants (age 0 -6 months) may exceed the proposed safe dose for aflatoxin M1 with EDIs \approx 1,000 fold higher than the recommended safe dose. This suggests that infants are at a high risk of aflatoxin M1 induced health hazards. The EDI for aflatoxin M1 drops by more than 3- fold at age \geq six months, possibly due to reduced intake of infant powdered milk. However, at ages higher than six months, the introduction of grain-based food along with milk-based products potentially adds additional mycotoxin (DON) exposure. As suggested by Tables 4.1 and 4.2, although >90% of the samples analyzed contained DON at concentrations below the recommended level of 200 $\mu\text{g}/\text{Kg}$, the EDI values suggest that in addition to aflatoxin M1, children between ages 6 – 24 months are exposed to \approx 4 – 5 μg DON / Kg body weight, which is 4 – 5 fold greater than the PMTDI recommended for DON (Joint FAO/WHO Expert Committee on Food Additives et al., 2011).

4.5 Discussion

In this survey, we demonstrate that Indian baby food contamination with multiple mycotoxins does occur, with EDI levels that exceed the PMTDI for the two mycotoxins (aflatoxin M1 and DON) detected. Although none of the samples analyzed indicated the

presence of aflatoxins B₁, B₂, G₁, and G₂, all of these samples contained aflatoxin M1 at levels 30- 120 fold higher than the EU recommended levels and ~ 15-60 fold higher than the recommended levels (0.05ng/Kg) in India (FSSAI, 2011). Interestingly, aflatoxin M1 range of concentrations detected in our samples were higher than previous reports on aflatoxin occurrence in baby foods in India (Kanungo and Bhand, 2014; Rastogi et al., 2004; Siddappa et al., 2012). This suggests an upward trend in the EDI values of aflatoxin M1 in children. The high (~1000 fold) EDI of aflatoxin M1 relative to the proposed safety level of exposure indicates that baby foods are a health hazard to Indian children. The increasing trends of aflatoxin M1 occurrence emphasize the need to impose stricter mycotoxin surveillance and enforce regulations on aflatoxin M1 levels in food. In addition to aflatoxin M1, most (66%) of the analyzed food samples contained DON that has the potential to result in an EDI significantly higher than PMTDI recommended by JECFA. This demonstrates the feasibility of simultaneous exposure to aflatoxin M1 and DON from the same commercially available baby food in Indian markets. The likelihood of DON exposure in children is higher at ages > 6 months if exposure to grain-based foods occurs.

An essential limitation of this study was the limitation of the knowledge of per-capita consumption of commercially available baby foods in India. The EDI values have been calculated with the assumption that the per capita intake of baby foods in India is similar to a previously published US EPA report. With the changing lifestyles and dietary patterns (increased dependence on commercially available baby foods) in Indian children, it is possible that the actual EDI values are higher than the values obtained from our calculations. Secondly, all the available recommended PMTDI levels for mycotoxins are based on toxicity studies conducted using single mycotoxins. It is possible that multiple

mycotoxin exposures may occur, which may cause additive and/or synergistic effects resulting in significantly higher toxicity than individual mycotoxin exposures. Studies to investigate the facts of co-exposure to multiple mycotoxins are, therefore, essential at this point and is a crucial focus of our ongoing research. Finally, this was a study with a limited sample size, which was a fundamental limitation for the determination of the true prevalence of exposure.

We emphasize here that although this study provides the first evidence to the best of our knowledge, of multiple mycotoxin occurrence in baby foods in India, similar baby food contaminations have been reported in other countries. For example, the presence of numerous mycotoxins was observed in baby foods commercially obtained in Italy (Juan et al., 2014), Canada (Lombaert et al., 2003), and Turkey (Baydar et al., 2007). Since the manufacturers of several of the baby foods analyzed in our study have global markets, it is possible that that baby food harboring multiple mycotoxins can impact the health of children globally. Chronic high daily intake of aflatoxin M1 and DON may have severe impacts on growth, metabolic pathways, development of tissues and organs and immune function of children (Gong et al., 2003; Ishikawa et al., 2016; Magoha et al., 2016) which may result in increasing immune comprised populations, that may be more prone to infectious diseases. Hence more research with a larger sample size is needed to accurately quantify the impacts of mycotoxin exposure in children. Such a global food monitoring initiative for regulation and prevention of mycotoxins in baby foods is critical to prevent the detrimental effects of the foodborne mycotoxins on the health of children worldwide.

4.6 Figures

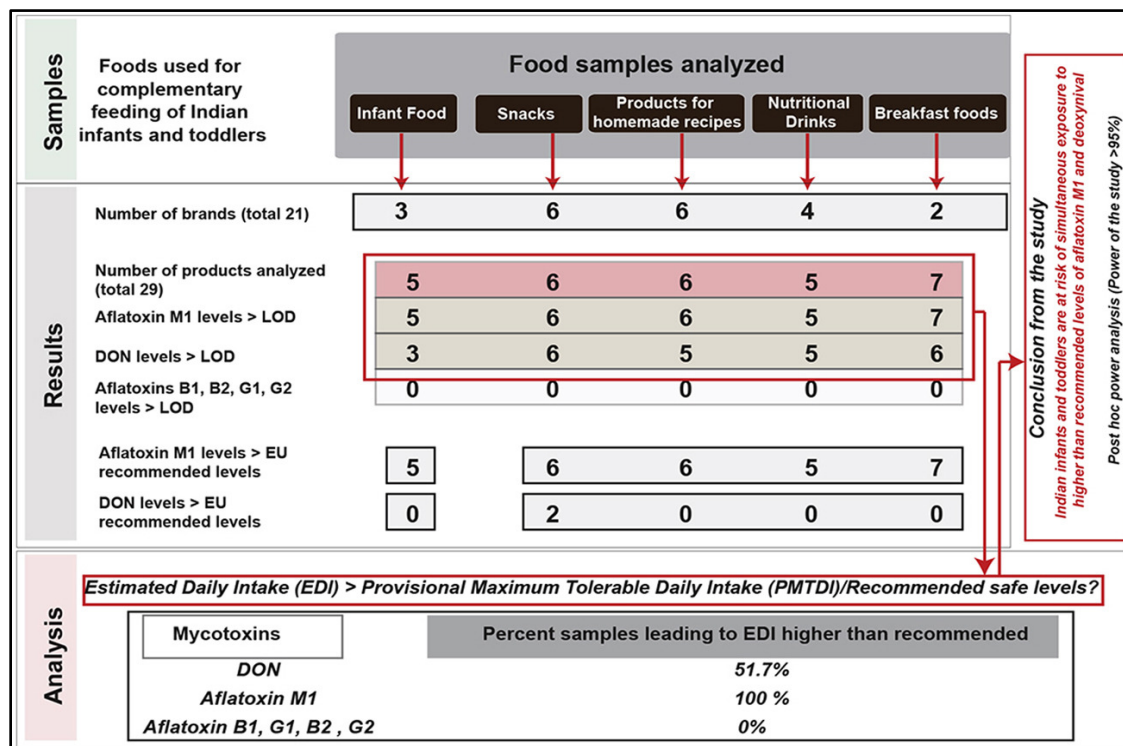


Figure 4.1. Estimated daily dietary intake of DON and Aflatoxin M1 in Children 0-2 years in Kolkata, India

4.7 Tables

Table 4.1. Aflatoxin M1 in analyzed sample of baby food available in Kolkata India, for ages 0-2 yrs

<i>Aflatoxin M1</i>			
<i>Food Type</i>	Number of samples	Mean Concentration range ($\mu\text{g kg}^{-1}$) from triplicate readings (\pm mean SE in the readings)	% of samples exceeding EU recommended levels ($0.025 \mu\text{g kg}^{-1}$)
<i>Infants foods</i>	5	1.0-3.2 (± 0.01)	100
<i>Snacks</i>	6	0.8-2.6 (± 0.01)	100
<i>Food ingredients</i>	6	1.0-3.3 (± 0.02)	100
<i>Nutritional drinks</i>	5	1.7-2.5 (± 0.02)	100
<i>Breakfast foods</i>	7	1.1-2.4 (± 0.01)	100

Table 4.2. DON in analyzed sample of baby food available in Kolkata India for ages 0-2yrs

DON Levels			
<i>Food Type</i>	Number of samples	Mean Concentration range ($\mu\text{g kg}^{-1}$) from triplicate readings (\pm mean SE in the readings)	% of samples exceeding EU recommended levels ($0.025 \mu\text{g kg}^{-1}$)
<i>Infants foods</i>	5	0-14 (± 0.01)	0
<i>Snacks</i>	6	1-228 (± 0.03)	33
<i>Food ingredients</i>	6	0-77 (± 0.02)	0
<i>Nutritional drinks</i>	5	3-76 (± 0.02)	0
<i>Breakfast foods</i>	7	0-49 (± 0.01)	0

CHAPTER 5

MAKING AFLATOXINS TO REDUCE TOTAL ROS: A CASE

STUDY IN *ASPERGILLUS PARASITICUS*.

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5.1 Abstract

The machinery for secondary metabolite synthesis has been preserved in fungi during evolution, which allowed them to survive against adverse environmental conditions. Although several studies have explored the crosstalk between secondary metabolism and response to oxidative stress in filamentous fungi, an aspect of secondary metabolism that remains unclear in fungal physiology is its relationship with the cellular management of reactive oxygen species [ROS]. This study conducts a comparative study of the total ROS production in the wild-type strain (SU-1) of the plant pathogen and aflatoxin producer, *Aspergillus parasiticus*, and its mutant strain, AFS10, in which the aflatoxin biosynthesis pathway is blocked by disruption of its pathway regulator, *aflR*. Total ROS was measured in both strains spectrophotometrically using 2',7'-dichlorofluorescein diacetate (DCFH-DA). Results from this study show that SU-1 demonstrates a significantly faster decrease in total ROS than AFS10 between 24h to 48h, a time window within which aflatoxin synthesis is activated and reaches its peak levels in SU-1. The impact of aflatoxin synthesis in the alleviation of ROS correlates well with transcriptional activation of five superoxide dismutase [SOD], a group of enzymes that protects the cell from elevated levels of a class of ROS, the superoxide radicals (O_2^-). Also, aflatoxin uptake in AFS10 resulted in a significant reduction of total ROS only in 24h cultures without other significant changes in SOD gene expression. In conclusion, findings from this study show that activation of aflatoxin biosynthesis in *A. parasiticus* alleviates ROS generation, which in turn, can be both *aflR* dependent and aflatoxin dependent.

5.2 Introduction

Filamentous fungi synthesize and release a diverse array of secondary metabolites into their environment, many of which have profound impacts on agriculture, industry, environmental sustainability, and human health (Keller et al., 2005). Many compounds are used as medicines, including statins, penicillin, and other antibiotics. Many others, like aflatoxins and fumonisins, can be life-threatening to humans and animals. Aflatoxin B₁ (AFB₁), a highly carcinogenic secondary metabolite synthesized by a group of *Aspergilli*, is a life-threatening toxin causing significant morbidity and mortality worldwide, as well as billions of dollars in annual economic losses (Magnussen, 2013). Due to the considerable human and agricultural impacts of aflatoxin (AF), its biosynthetic pathway is one of the most characterized and widely studied models for understanding fungal secondary metabolism (Roze et al., 2011b).

The aflatoxin biosynthesis process is activated by several environmental cues and orchestrated by a complex regulatory network of more than 25 genes and 17 enzymatic steps (Brakhage, 2013; Chanda et al., 2009; Yin and Keller, 2011; Yu et al., 2004). The operation of this network is governed by the interactions of a set of global transcription factors, including LaeA and VeA (Bayram et al., 2008; Brakhage, 2013; Calvo, 2008; Calvo et al., 2004; Kale et al., 2008). Upon receiving signals from cell surface receptors, these global transcription factors communicate with pathway-specific transcription factors (examples include AflR (Cary et al., 2006) and GliZ (Bok et al., 2006; Scharf et al., 2012)) to activate specific aflatoxin biosynthesis genes. Many of the enzymes synthesized by this pathway then localize to distinct vesicles known as toxisomes (Chanda et al., 2010, 2009; Lim and Keller, 2014; Menke et al., 2013; Roze et al., 2011a), which provide a platform

for the completion of biosynthesis, sequestration, and export of aflatoxin to the environment. To manipulate secondary metabolism in fungi for the benefit of public and environmental health, it is essential to understand the motivation for a fungal cell to preserve such an energy-consuming metabolic process with the enormously complex molecular and cellular organization throughout evolution. One of the most commonly hypothesized functions of fungal secondary metabolites is a defense against other organisms in the same ecological niche. Antibacterial properties of secondary metabolites like penicillin and other beta-lactam antibiotics are well established in the literature (van Krimpen et al., 1987). Beyond antibacterial properties, reports from Rohlf et al. (Rohlf et al., 2007) suggest that aflatoxin and sterigmatocystin protect fungal cells from pests and insects. These studies all indicate that secondary metabolism provides fungi with a survival mechanism in nature. Several recent studies suggest that secondary metabolism is integrated with primary metabolism and its associated cellular mechanisms (Chanda et al., 2009; Linz et al., 2012; Roze et al., 2011b, 2010), which implies that secondary metabolism may have a regulatory impact on other fungal cellular processes as well. One cellular process that appears to be associated with secondary metabolism in fungi is an oxidative stress response. Recently, several basic leucine zipper (bZIP) transcription factors in filamentous fungi have been reported in the literature that not only regulate antioxidant genes participating in oxidative stress response but are also associated with the regulation of secondary metabolism (Baidya et al., 2014; Hong et al., 2013b, 2013a; Montibus et al., 2015, 2013, 2013; Reverberi et al., 2012, 2007; Yin et al., 2013). These reports are in line with previous reports (Jayashree and Subramanyam, 2000; Narasaiah et al., 2006; Reverberi et al., 2008, 2006) suggesting that oxidative stress induces aflatoxin synthesis in

Aspergillus parasiticus. While these lines of evidence collectively demonstrate that the two cellular processes (aflatoxin biosynthesis and intracellular oxidative stress management) communicate at different regulatory nodes and are co-regulated, the effect of aflatoxin on oxidative stress remains unclear. In this study we address this knowledge gap through a comparative study of total reactive oxygen species (ROS) output between the wild-type *A. parasiticus* and its mutant, AFS10, in which the aflatoxin pathway regulator gene, *aflR*, is disrupted (Cary et al., 2002; Ehrlich et al., 1999). In addition to measuring ROS, we also conducted a comparative assessment of superoxide dismutase (SOD) gene expression. SODs are conserved in eukaryotes and are synthesized in response to intracellular (O_2^-) radicals (a type of ROS) generated as a byproduct of primary cellular functions (Fridovich, 1995). To differentiate the aflatoxin-dependent effect on ROS generation from the possible genetic effects (of *aflR* disruption), we also conducted aflatoxin supplementation studies on AFS10. The results of this work provide direct evidence in support of the regulatory role of aflatoxin synthesis on total ROS output and explain the rationale for the co-regulation of oxidative stress with aflatoxin synthesis.

5.3 Materials and Method

5.3.1. Strains, Media, and Growth Conditions

Aspergillus parasiticus wild type strain SU-1 (ATCC56775) and the *aflR* disrupted mutant, AFS10 (Ehrlich et al., 1999; Roze et al., 2007), were used for this study. Yeast extract sucrose (YES) (2% yeast extract, 6% sucrose; pH 5.8) was used as the liquid growth medium for the entire study for both strains. Fungal cells were grown for 24 h and 48 h by inoculating 10^7 spores per 100 mL of growth medium and incubating the cells at 29 °C in a dark orbital shaker at 150 rpm.

5.3.2. Quantification of ROS

Comparison of ROS concentrations between SU-1 and AFS10 was conducted spectrophotometrically using 2',7'-dichlorofluorescein diacetate (DCFH-DA) based on a previously described protocol (Chang et al., 2011). Equal weight (0.5 g) of mycelia from a 24 and 48 h culture was placed into 1 mL of freshly made one μ M DCFH-DA in phosphate-buffered saline (PBS). After four h of incubation in the dark at room temperature (25 °C), the fluorescent yield of the DCFH-DA oxidation product, dichlorofluorescein (DCF), was measured using a Victor™ X3 2030 Multilabel Reader (PerkinElmer, Waltham, MA, USA) with an excitation/emission wavelength of 490/525 nm.

5.3.3. Identification of Superoxide Dismutase Genes

Since the functional characterization of the SOD genes in *A. parasiticus* has not yet been completed, bioinformatics analysis was performed to identify SOD gene sequences to allow for a comparative expression analyses to address our hypothesis. The SOD genes analyzed in this study were identified by searching for “superoxide dismutase” in the accessible genome database (Yu et al., 2004) of *A. flavus*, a close relative of *A. parasiticus* that exhibits ~98–100% amino-acid sequence identity with *A. parasiticus* proteins that have been sequenced (Roze et al., 2011b). The search rendered five annotated amino-acid sequences which were then queried in the PROSITE database (Sigrist et al., 2012) against the 390 available SOD genes to investigate whether they contained (a) the conserved functional domains typical of SODs, or (b) motifs with a high probability of occurrence that are commonly present in the SOD genes.

5.3.4. Aflatoxin Supplementation Experiments

For aflatoxin supplementation studies, 0.5 g of AFS10 mycelia were collected from YES media at 24 and 48 h, and each placed in 12-well trays containing 1 mL of their culture

media. Total aflatoxin (in 70% methanol solution) isolated from a SU-1 culture using our standard chloroform-methanol isolation procedure (Gummadidala et al., 2017) was added to each sample well at a final concentration of 50 ppm. The control mycelia were supplemented with an equal volume of 70% methanol solution. After a four h incubation, mycelia were transferred to 1 mL of 1 μ M DCFH-DA in PBS substrate for an additional 1-hour incubation in the dark before being measured (in triplicate) for DCF fluorescence. Aflatoxin uptake into the mycelia during the incubation period was quantified by measuring the total percent removal of aflatoxin from the medium every hour until four hours and by measuring the total accumulation of aflatoxin in the mycelium in parallel, after four hours. Percent removal of aflatoxin from the medium was calculated as follows: Percent removal = ((Initial total aflatoxin in the supplementation medium – total aflatoxin in the medium at a time point)/Initial total aflatoxin in the supplementation medium) \times 100.

Aflatoxin accumulation in the mycelium was measured after washing three times with PBS buffer followed by extracting aflatoxin from the mycelium using a chloroform: methanol procedure as described previously (Roze et al., 2007). Aflatoxin in the extract was then measured using an enzyme-linked immunosorbent assay (ELISA). Dead cells of AFS10 obtained upon autoclaving at 121 $^{\circ}$ C for 15 min were used in the uptake experiments as controls for free diffusion systems. The loss of viability in these cells was confirmed before experimentation by confirming their inability to grow in the fresh growth medium.

5.3.5. Aflatoxin Quantification

Qualitative comparisons of aflatoxin accumulation in the growth media were performed using thin-layer chromatography (TLC) as described previously (Hong and Linz,

2008). Quantification of aflatoxin for the aflatoxin uptake experiments was performed using a Veratox for Aflatoxin ELISA kit (Neogen Food Safety, Lansing, MI, USA) and measured on a Stat Fax 4700 Microstrip Reader (Awareness Technologies, Palm City, FL, USA).

5.3.6. Statistical Analyses

Statistical analyses for this study were conducted using the GraphPad Prism Software (GraphPad, CA, USA). The statistical significance of two-tailed p -values was determined using an unpaired t -test, using $n = 3$ and $p < 0.05$. For the gene expression studies, a two-fold increase or decrease in transcript level was our cutoff for comparing expressions between two genes.

5.4. Results

5.4.1. SU-1 Demonstrates a Significantly Larger Decrease in Total ROS Compared to AFS10 between 24 h and 48 h

Aflatoxin biosynthesis is activated in SU-1 at 24 h under our culture conditions and reaches peak levels by the start of the stationary phase at 48 h (Chanda et al., 2009; Skory et al., 1993). Under these conditions, aflatoxin biosynthesis is not activated in AFS10. As shown in Figure 5.1, during the 24 h–48 h-time window, aflatoxin accumulation in the growth medium was observed, and aflatoxin genes were activated in SU-1 but not in AFS10. The genes *nor-1* and *ver-1* were chosen as representative aflatoxin genes that demonstrated drastic increases in expression similar to previously reported semi-quantitative analysis of transcript and protein analysis (Roze et al., 2007). Quantitative comparison of total ROS (Dichlorodihydrofluorescein [DCF] fluorescence measurements shown in Figure(5.1b) shows that at 24 h, both strains demonstrate similar levels of total

ROS, but by 48 h the total ROS decreased at a significantly higher rate in SU-1 than in AFS10. This demonstrated an association between the activation of aflatoxin biosynthesis and a decrease in total ROS, which may be attributable to either the presence of aflatoxin or the regulatory role of *aflR*.

5.4.2. Higher Total ROS in AFS10 Compared to SU-1 at 48 h Associates with Significant Differences in SOD Gene Expression

Since SOD genes are synthesized in eukaryotes in response to intracellular O_2^- radicals (a type of ROS) generated as a byproduct of primary cellular functions (Fridovich, 1995), we investigated whether higher ROS at 48 h in AFS10 is correlated with the transcriptional activation of SOD genes. As a first step to do so, we initiated a search for SOD genes within the available genome database of a closely related species, *A. flavus* (Yu† et al., 2008), and identified five amino acid sequences. Out of these five sequences, two different sequences of copper-zinc SOD genes are annotated in the database as *CuZnsod1*, and cytosolic *CuZnsod*, two sequences of iron SOD are annotated as *Fesod* and *FesodA*, and one manganese SOD is annotated as *Mnsod*. These five sequences were queried against the PROSITE database (Sigrist et al., 2012) to verify whether they contained any of the conserved functional domains or patterns that are present in the well-characterized SODs within the database. The gene expression of all five SODs were examined in both SU-1 and AFS10 at 24 h and 48 h post-inoculation in yeast extract sucrose (YES). Quantitative comparison of the transcript levels between 24 h and 48 h, with levels normalized to 24 h (raw expression data relative to 18s rRNA) and the list of primers used are mentioned as shown under table 5.1. The data suggest that the SOD expression profile in this fungus is growth phase-dependent. Hence, while the expressions

of *Fesod* and *CuZnsod1* are higher in 24 h cultures (corresponding to the exponential growth phase), the *Mnsod* expression is significantly higher in the 48 h cultures (corresponding to the stationary growth phase). As seen in Figure 5.2, AFS10 displayed a substantially more significant increase in *Mnsod* expression from 24 h to 48 h (~70-fold increase in AFS10 versus a ~40-fold increase in SU-1). Additionally, *CuZnsod* expression that remained constant in SU-1 showed a significant increase from 24 to 48 h in AFS10. No significant difference was observed between SU-1 and AFS10 for genes *Fesod* and *CuZnsod1*. Our results, therefore, demonstrate an association between higher ROS levels in AFS10 (compared to SU-1) and absence of aflatoxin biosynthesis during the 24 h–48 h time window in AFS10 with the significantly larger increases (compared to SU-1) in *Mnsod* and *CuZnsod* transcripts from 24 h to 48 h.

5.4.3. Aflatoxin Supplementation to AFS10 Growth Medium Changes Total ROS Output without Changing the SOD Transcript Levels

The significantly larger decrease in total ROS in SU-1 compared to AFS10 could either be aflatoxin dependent, *aflR* dependent, or both. To examine if total ROS production is in-part aflatoxin dependent, we investigated whether aflatoxin supplementation to AFS10 impacts the total ROS levels. The results of this experiment are shown in Figure 5.3. A 4 h supplementation of 24 h mycelia with total aflatoxin isolated from a SU-1 growth medium resulted in a significant decrease of total ROS (Figure 5.3a). In contrast, the four h aflatoxin supplementation to 48 h AFS10 mycelia significantly increased the total ROS. To understand this differential effect of aflatoxin supplementation on the 24 h and 48 h AFS10 cultures, we conducted an examination of aflatoxin uptake by the mycelium during the four h time-period. As shown in Figure 5.3b, the percentage removal of aflatoxin per

unit mass of mycelium by the end of 4 h was significantly higher for 48 h cultures than 24 h cultures. This data also agreed with the aflatoxin accumulation in the mycelia, which demonstrated a significantly higher accumulation of aflatoxin in 48 h cultures than in 24 h cultures. To examine whether the aflatoxin accumulation was a free diffusion versus an active uptake mechanism by the mycelium, we conducted a similar experiment with equal masses of dead AFS10 cultures obtained upon autoclaving the cultures. Our results demonstrate that while the free diffusion of aflatoxin from the medium to the immersed dead cells resulted in faster removal of aflatoxin from the medium, the aflatoxin could not be retained in the dead mycelia, unlike the live cells when taken out of the medium and washed. Collectively the gradual increase in aflatoxin removal from the medium (unlike the dead cells) and the ability to retain the aflatoxin in the mycelium suggests an active uptake mechanism of aflatoxin by the cells. The significantly higher uptake of aflatoxin in 48 h cultures than the 24 h cultures indicates that the differential effects of the aflatoxin supplementation on total ROS in the 24 h versus 48 h cultures are associated with the differential levels of aflatoxin uptake by the mycelia of these ages. Finally, we also examined whether aflatoxin supplementation resulted in changes in the expression levels of the SOD genes either in 24 h or 48 h cultures. Contrary to the total ROS readings, there were no significant changes in SOD expression that were attributable to AF supplementation (Figure 5.3c), thereby suggesting the possibility that aflatoxin supplementation induced changes in the total ROS are acute biochemical effects.

5.5 Discussion

This study provides the first direct demonstration of the regulatory role of a secondary metabolite on a cellular process of the producer's oxidative stress management.

It also can now explain the previous reports on the crosstalk between oxidative stress and secondary metabolism (Jayashree and Subramanyam, 2000; Narasaiah et al., 2006; Reverberi et al., 2008, 2006). Based on our current findings and previously published literature, we propose here a ROS management model for aflatoxin producers (illustrated in Figure 5.4). According to this model, aflatoxin biosynthesis protects cells against ROS accumulation from at least three different sources: (a) primary metabolic processes, (b) secondary ROS generated from aflatoxin biosynthesis, as proposed previously by Roze et al. (Roze et al., 2011b), and (c) ROS made upon aflatoxin uptake by cells during the stationary phase of growth (aflatoxin supplementation data from 48 h AFS10 cultures in the current study). The aflatoxin-dependent protection occurs in one or a combination of the following ways: (a) utilization of ROS in the biochemical steps of the biosynthesis pathway (Narasaiah et al., 2006), (b) aflatoxin-dependent reduction of ROS in cells at exponential growth phase (aflatoxin supplementation data from 24 h AFS10 cultures in the current study) and (c) *aflR*-dependent reduction of ROS (present research) possibly through its gene regulatory impacts outside the aflatoxin pathway gene cluster (Price et al., 2006; Yin et al., 2012). Our data support the likelihood that disruption of *aflR* blocks all the three modes of aflatoxin-dependent protection, leading to a higher accumulation of superoxide radicals in AFS10 compared to SU-1. This can explain the increased demand for SOD activation and the higher SOD transcript levels in AFS10 than in SU-1.

To address the direct effect of aflatoxin on total ROS, we designed a four h supplementation experiment to compare the personal impact of the supplementation on the 24 h and the 48 h AFS10 cultures. We understand based on previous literature (Banerjee et al., 2014; Chanda et al., 2009; Roze et al., 2007) that 24 h cultures and 48 h cultures (under

our standard growth conditions) are very different physiological systems; 24 h cultures demonstrate no secondary metabolite synthesis, and in 48 h cultures secondary metabolite synthesis occurs at peak levels. The four h time was optimized from initial uptake experiments in which we noticed no significant increase in the growth of the mycelia until four h under the given experimental conditions (data not shown). We reasoned that supplementation beyond 4 h would result in the adaptation of fungal cells, and that would not allow us to observe the acute effects as described in this study. It is speculated that fungal toxisomes, which are sites for the synthesis and compartmentalization of secondary metabolites (Chanda et al., 2009; Roze et al., 2011a), receive input from peroxisomes and mitochondria as well as from the secretory and Cvt vesicle transport pathways (Roze et al., 2011a). A significant increase in the mitochondrial SOD, MnSOD, at 48 h suggests that it is primarily responsible for dismutating the superoxides during the stationary phase. Previous proteomic data on fungal toxisomes in *A. parasiticus* (Linz et al., 2012) demonstrated enrichment of superoxide dismutases, especially MnSOD, within the toxisomes as well. Catalases also present in the toxisomes then convert the hydrogen peroxide product of the dismutation reactions into oxygen and water. The data shown here correspond increased MnSOD with ROS levels after the initiation of aflatoxin biosynthesis support the possibility that superoxides are compartmentalized into fungal toxisomes in addition to the mitochondria, and become available for incorporation into secondary metabolite biosynthetic pathways, including aflatoxin synthesis, in addition to dismutation by SODs. We emphasize here that while the SOD expression profiles are closely and independently associated with total ROS and the activation of aflatoxin biosynthesis, our data (Figure 5.6c) do not support aflatoxin as a direct regulator of SOD gene transcription,

thereby suggesting that additional regulator(s) work in concert with AflR to regulate SOD gene expression. An example of such a regulator is the bZIP transcription factor AtfB (Roze et al., 2011b; Wee et al., 2017), which is in part one regulator of the SODs and the cellular response to intracellular oxidative stress (Hong et al., 2013b, 2013a; Wee et al., 2017) that binds to *aflR* gene promoter and physically interacts with the AflR (Miller et al., 2005; Roze et al., 2004).

One limitation of this study is the lack of an appropriate methodology for clean biochemical measurements specific for superoxide radicals (O_2^-) within *Aspergillus* cells. Commercially available small molecules like DMPO that can successfully trap O_2^- within mammalian and yeast cells have conventionally been used for such O_2^- quantifications. However, these small molecules fail to enter *Aspergillus* cells (data not shown). Within the cell, toxisomes are very dynamic systems that are continuously exporting protein and metabolite contents to the extracellular environment (Chanda et al., 2010). At this time, any present superoxide radicals would be detectable by molecules such as DMPO. Therefore, unless the extremely unstable O_2^- radicals are incorporated into the location of aflatoxin synthesis within toxisomes, as in case of SU-1 (but not in AFS10), commercial cellular stains like MitoSOX or CellROX cannot provide a true overall quantification of the total O_2^- radicals or total ROS through cellular imaging experiments as done for many mammalian cells and will lead to inaccurate interpretations. The protocol used in these experiments is based on a methodology previously established by Chang et al. (Chang et al., 2011). The method allows the substrate DCFH-DA to react with the total ROS generated within mycelia and form the fluorescent marker DCF that can then be quantified spectrophotometrically. While we acknowledge the technical limitations of the DCFH-DA

probe in providing accurate quantification of superoxides and total ROS(Kalyanaraman et al., 2012), we reason that our experimental design, being dependent of relative ROS levels rather than accurate ROS quantifications, was able to circumvent these challenges and therefore our interpretations on relative ROS levels were not impacted.

In conclusion, our findings establish the foundation for a long-term study that will investigate the molecular, cellular, and biochemical mechanisms underlying the differential effects of aflatoxin on ROS accumulation in cells that are in an exponential growth phase versus those in a stationary phase. We hypothesize based on these findings that secondary metabolites have a regulatory role in the cellular coordination of secondary metabolism and oxidative stress response in filamentous fungi. Our future studies will shed more light on revealing the complexity of such coordination and thereby help identify novel targets for the manipulation of secondary metabolism.

5.6 Figures

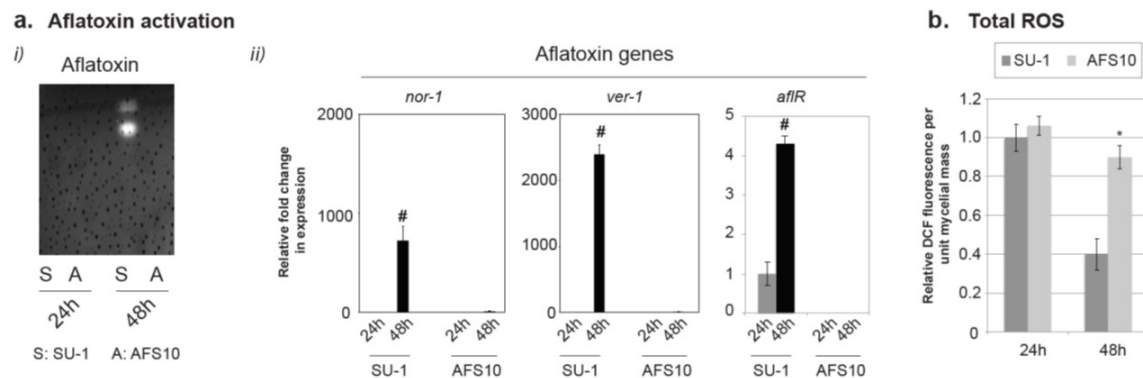


Figure 5.1. Decrease of total ROS during activation of aflatoxin biosynthesis. (a) Comparison of (i) aflatoxin accumulation and (ii) Gene expression levels were relative to 24 h of three aflatoxin pathway genes in SU-1 and AFS10. (b) Comparison of total ROS at 24 h and 48 h. The error bars represent the standard error of the mean. The two-tailed P-value was determined using an unpaired t-test (GraphPad statistical software). #, Significant difference of transcript levels between 24 h and 48 h (p -value < 0.05, $n = 3$); * Significant difference of total ROS between SU-1 and AFS10 (p -value < 0.05, $n = 3$)

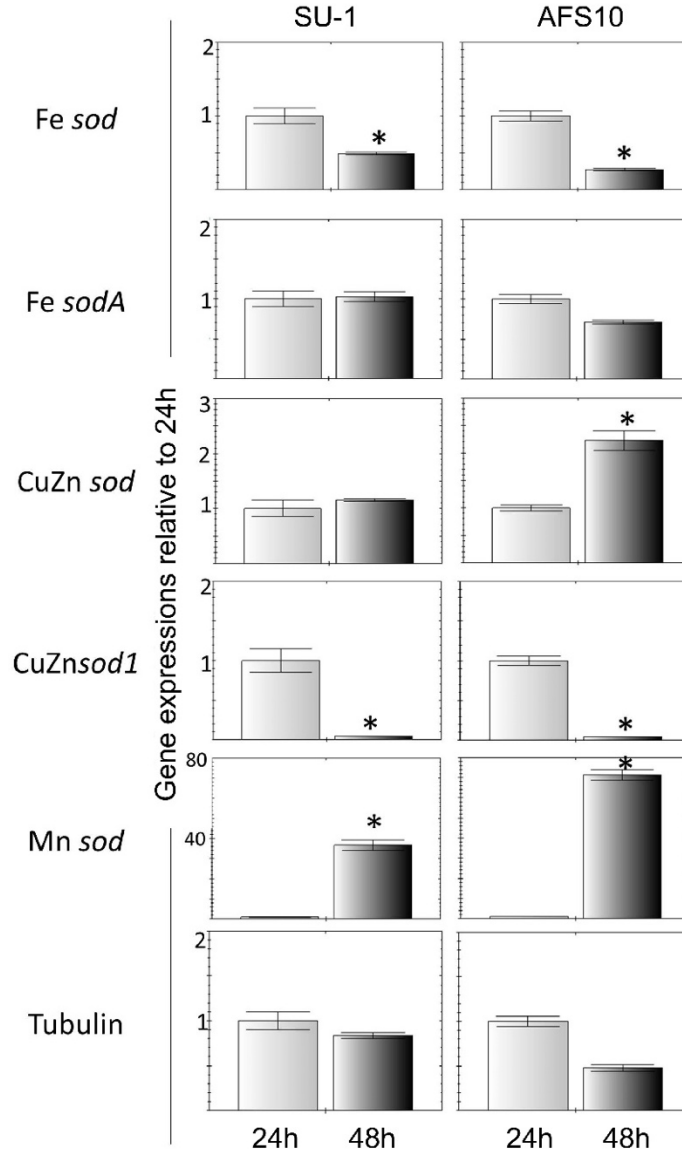


Figure 5.2. Comparison of SOD gene expression in SU-1 and AFS10. Quantitative PCR (qPCR) comparison of SOD gene expression in the two strains at 24 and 48 h of culture growth. All expression quantifications were conducted in triplicate. For each gene, the expression value was normalized against the 18s rRNA reference gene and compared to a β -tubulin control. The expression values for each target gene at the early stationary phase (48 h) were expressed as the fold change relative to the 24 h time point. Fold changes ≥ 2.0 were considered up- or down-regulated. All data and statistical analysis (Student's *t*-test) were performed using CFX Manager software (Bio-Rad Laboratories). Compared to 24 h gene expression, *Fesod* showed a significant decrease in both the wild-type (2.1-fold; $p = 0.003$) and AFS10 (3.9-fold; $p < 0.001$); *FesodA* showed no significant change for either strain; *CuZnsod* expression did not change in the WT, but showed a 2.1-fold increase ($p = 0.003$) in AFS10; *CuZnsod1* showed a large, significant decrease in expression for both the WT (22.4-fold; $p = 0.001$) and AFS10 (26.4-fold; $p < 0.001$); *Mnsod* had a dramatically significant 36.2-fold increase in gene expression in the WT ($p < 0.001$), and an even greater 69.8-fold increase in AFS10 ($p < 0.001$) compared 24 h expression.

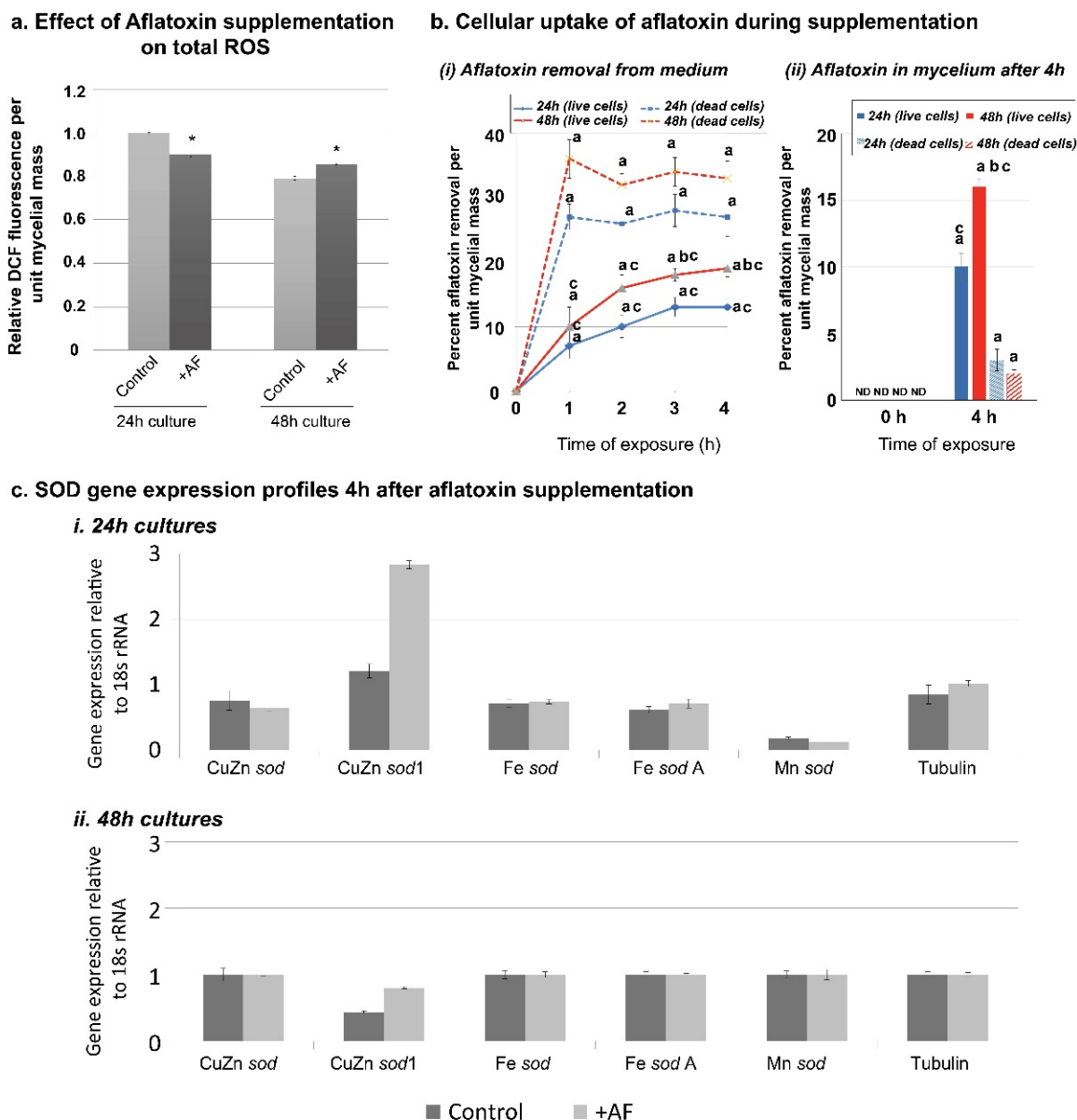


Figure 5.3. Aflatoxin supplementation to AFS10. (a) Effect on total ROS. A quantitative comparison of ROS in AFS10 supplemented with 50 ppm aflatoxin (in 70% methanol), and a 70% methanol control was conducted. Total ROS was quantified at 24 h, and 48 h of growth + 4 h of incubation in 1 μ M 2',7'-dichlorofluorescein diacetate (DCFH-DA) in phosphate-buffered saline (PBS) substrate with the corresponding AF concentration. Error bars represent SEM. (*) denotes statistically significant difference ($p < 0.05$; $n = 3$) in ROS compared to the 70% methanol control for the corresponding growth time. **(b)** Cellular uptake of aflatoxin during aflatoxin supplementation. (i) Percent removal of aflatoxin from the supplementation medium in live cells of 24 h and 48 h AFS10. The percent removal was calculated at every hour until four h to compare the aflatoxin removal pattern by live cells with the dead cells that allow free diffusion from the medium into the cells. (ii) Percent aflatoxin accumulation in the mycelium of 24 h and 48 h cultures. Aflatoxin in the mycelia of live cells was compared to the dead cells. Error bars represent SEM. a, statistically significant difference ($p < 0.05$; $n = 3$) in aflatoxin levels with 0 h, b, statistically significant difference ($p < 0.05$; $n = 3$) in aflatoxin levels between 24 h and 48 h cultures, c,

statistically significant difference ($p < 0.05$; $n = 3$) in aflatoxin levels between live and dead cells at a particular time-point. (c) Comparison of SOD gene expression in aflatoxin supplemented and control AFS10. qPCR comparison of SOD gene expression in the control and four h aflatoxin supplemented cells. The gene expression values were normalized against the 18s rRNA reference gene. Fold changes ≥ 2.0 were considered up- or down-regulated. All data and statistical analysis (Student's t -test) were performed using CFX Manager software (Bio-Rad Laboratories).

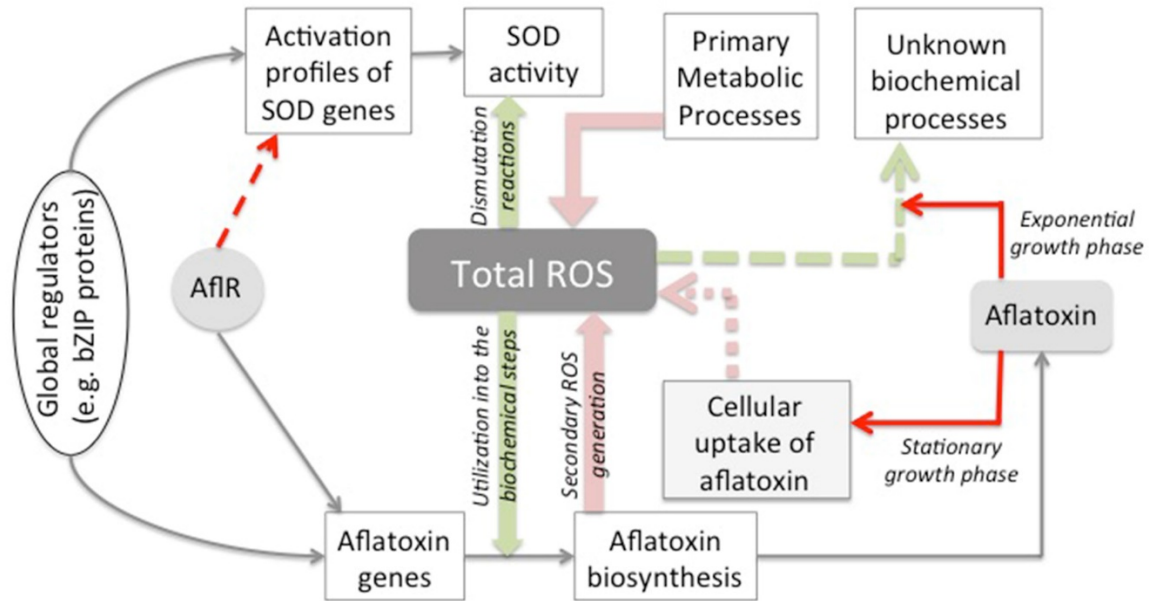


Figure 5.4. Proposed model for total ROS management in *A. parasiticus*. Based on our current findings and previous reports we propose that aflatoxin-dependent protection occurs in one or a combination of the following ways: (a) utilization of ROS in the biochemical steps of the biosynthesis pathway (Narasaiah et al., 2006), (b) aflatoxin-dependent reduction of ROS in cells at exponential growth phase (current study) and (c) *aflR*-dependent reduction of ROS (present research) possibly through its gene regulatory impacts outside the aflatoxin pathway gene cluster (Price et al., 2006; Yin et al., 2012). Aflatoxin-dependent biochemical processes that sequester ROS remain uncharacterized (green dashed arrow). Pink arrows indicate the sources of ROS accumulation. These include ROS generation from primary metabolic processes, secondary ROS generated from aflatoxin biosynthesis (Roze et al., 2015), and ROS generated upon aflatoxin uptake by cells during the stationary phase of growth (based on aflatoxin supplementation data from 48 h AFS10 cultures in the current study). The mechanisms that result in ROS accumulation upon cellular uptake of aflatoxin remain uncharacterized (pink dashed arrow). The model can now explain the physiological need of the cells to co-regulate secondary metabolism (in this case, aflatoxin biosynthesis) and oxidative stress response through the bZIP protein (Baidya et al., 2014; Roze et al., 2011b; Yin et al., 2013). Red arrows indicate the contributions of the current study. The molecular mechanism of *aflR*-mediated regulation of SOD genes remains uncharacterized (red dashed arrow) and will be investigated in our follow up studies.

5.7 Tables

Table 5.1. List of primers used in the study

Genes	Primer Sequences
1. <i>nor-1</i>	F 5'-CACTTAGCCAGCACGATCAA-3' R 5'-ATGATCATCCGACTGCCTTC-3'
2. <i>ver-1</i>	F 5'-AACACTCGTGGCCAGTTCTT-3' R 5'-ATATACTCCCGCGACACAGC-3'
3. <i>β-tubulin</i>	F 5'- TCTCCAAGATCCGTGAGGAG-3' R 5'- TTCAGGTCACCGTAAGAGGG- 3'
4. FeSOD	F 5'-GAGATGGCCTCCGTATTCAA-3' R 5'- CATCAATCCTTCCCTCTCCA-3'
5. FeSOD A	F 5'-CCAAGGAGGACATCACCCT-3' R 5'-GCATAGGTCTTGATGCCGAT-3'
6. CuZnSOD1	F 5'-CACCAGTTCGGTGACAACAC-3' R 5'- GTGTTCACCTACGGCCAAGGT-3'
7. CuZnSOD cytosolic	F 5'-CCTCCTTGCAATACAACCGT-3' R 5'-GTCTTCCTTCGCCTCTTCCT-3'
8. MnSOD	F 5'-CCACATCAACCACTCCCTCT-3' R 5'- TCCTGATCCTTCGTCGAAAC-3'

CHAPTER 6

CONCLUSION

Overall, the incidence of fungi disease due to climate change is on the rise (Garcia-Solache and Casadevall, 2010b) and the effect of fungal pathogens goes well beyond the ability of fungi to impact human health negatively. It is estimated that fungi are responsible for about a third destruction of food crops each year (Fisher et al., 2012). This does have a substantial economic impact, mostly in developing countries where the economy relies on agricultural production. Diseases caused by fungi pathogens are often challenging to manage. Though the fungal associated illness is reported in healthy individuals, immunocompromised patients, people with underlying illness (Kohler et al., 2015), and socially economically deprived groups share a significant burden of fungi disease.

As such, to tackle devastating challenges posed by fungi pathogens, it is essential to understand fungi biology and its pathogenesis with emphasis on its diseases on human health and agriculture. As such, this chapter will highlight the critical aspect of this study and recommends the advancement of each project.

6.1 Chapter 2 Summary

In this chapter, we investigated mold species diversity after three extreme weather events, including; 100-years flood, Hurricane Matthew, and Hurricane Sandy. This study was also the first to report mold species diversity associated with South Carolina buildings after

extreme weather events. Our findings from this study show that mold species that belong to the genus *Penicillium* and *Aspergillus* (allergens that could impact human health) are peculiar to the indoor environment in flood-affected homes compared to homes that were not flooded. Given that these findings are not different from other studies despite the difference in methodology. It shows that there may likely be some peculiarity associated with the indoor environment after flooding events. As such, there is a need to investigate microbial profiling of the indoor environment and its impact on human health after extreme weather. Also, inventing a proper universal methodology for fungi species identification will allow for adequate monitoring of fungal spore concentration in an indoor environment with less variation in results.

6.2 Chapter 3 Summary

Based on our findings in chapter 2, we extended our studies further to explore the association of mold-induced sensitization and its impact on human health. Specifically, we investigated the association between pre-existing asthma and pre-existing chronic bronchitis condition with specific mold-induced sensitivity. Using NHANES 2005-2006 allergen component database, we were able to answer our two (2) research question, which states that; 1) is mold allergy associated with pre-existing asthma and 2) is mold allergy associated with pre-existing chronic bronchitis?

Our findings from this study show that pre-existing asthma is highly associated with *Alternaria alternata* induced sensitivity compared to *Aspergillus fumigatus* induced sensitivity. Contrary to this finding, this study did not find any association between mold-induced allergic response and pre-existing chronic bronchitis. With these findings, and also since close to half of the United States population have one or several forms of allergic

diseases, there should be frequent monitoring of individuals for mold-induced sensitization, especially in cases of asthma conditions. The last allergic surveillance carried out in the United States, was in 2005-2006. With the rise in climate change, there is a possibility of change in mold species diversities capable of inducing various forms of an allergic reaction.

Also, further research should be channeled on exploring the association between fungi biology(including their spores and hyphae structures) and its contributory effect in eliciting symptoms in individuals that are not necessarily immune-mediated. This will give a full understanding of fungi pathogenesis and practical measures that can be put in place.

6.3 Chapter 4 Summary

Apart from humans, the fungal disease is one of the biggest threats to global food security. This situation is even worse in regions where environmental condition favors fungi growth and metabolism. Using India as a case study, we investigated 29 baby food samples from 21 manufacturers for Aflatoxin B1, B2, G1 G2, M1, and DON contamination. Results show that M1 contamination dominated all baby food samples at a range between 0.8 µg/kg to 3.2 µg/kg, a concentration higher than the EU recommendation of 0.025 µg/kg. No detection level of aflatoxin B1, B2, G1 &G2 was identified in baby food sampled analyzed.

DON was detected in 66% of the total food sample. In other words, DON was identified in 19 out of 29 baby food samples at a concentration between 10 µg/kg to 258 µg/kg. Two examples demonstrated a higher concentration of DON that exceeded 200 µg/kg maximum limit. The most exciting finding of this study is detecting Aflatoxin M1 in non-milk-based food samples while DON was detected in milk-based food samples. As

such, further studies are recommended to track the sources of mycotoxin contamination along the food chain. Also, there is a need to investigate the effect of co-exposure to multiple mycotoxin contamination to ascertain its impact on human health. Lastly, understanding how different environmental factors influence fungi metabolism will put effective eradication measures in place towards fungal diseases during the phase of ongoing climate change.

6.4 Chapter 5 Summary

To manipulate the fungi's biological process for the benefit of both public and environmental health, it is paramount we understand why fungi behave in a certain way under stressful environmental conditions. As such, this chapter investigated the mechanism of aflatoxin (fungi secondary metabolite) production between SU-1 and AFS10 strain (a mutant strain of SU-1) in the presence of ROS, a bi-product released from primary fungi cellular process.

To further explain fungi cellular metabolism, we explored SOD (gene produced as a result of ROS) genes between SU-1 and AFS10 strain. We set up an experiment to explain the process by which fungi cells take up its secondary metabolite. From all our findings, we concluded that the activation of aflatoxin biosynthesis in SU-1 alleviates ROS production, a situation that is different from AFS10. These findings could be dependent on the transcriptional gene responsible for aflatoxin biosynthesis or aflatoxin production by itself.

Overall, the importance of climate change in fungal diseases and contamination cannot be overestimated. Different weather conditions and environmental factors such as temperature, humidity, moisture, and flooding have been implicated in fungi growth and

possible harm it causes to both humans and animals. While we admit that not all fungi pathogens are harmful, other fungi belonging to the genus *Aspergillus*, *fusarium*, *Cladosporium* to mention, but a few have been implicated as likely cause of some infectious diseases. With ongoing climate change, fungi diseases and contamination may become difficult to control or eradicate. As such, there is a need for timely and constant surveillance of fungi diseases, especially within the immunocompromised population, and putting public health preventive measures in place to curb mold infestation resulting from extreme weather conditions such as flooding and drought.

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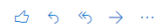
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