USING ATMOSPHERIC COLD PLASMA TO DECONTAMINATE *A. FLAVUS* IN COTTONSEED MEAL

A Thesis

by

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ABSTRACT

Due to the continuous increase in global population, there is a demand for innovative and sustainable technology to improve food and nutritional security. Contamination, spoilage, and safety issues are caused by pathogenic microbes including bacteria, yeast, and mold. *Aspergillus flavus* is an opportunistic fungus that colonizes various field crops including legumes, cereal grains, tree nuts, and oilseeds. Infection by this fungus can occur pre-harvest or during post-harvest operations and storage of cottonseed and cottonseed meal. In addition to infecting of the cottonseed there is the subsequent production of mycotoxins which are toxic secondary metabolites, such as aflatoxins. Under ideal environmental conditions, warm temperatures and high humidity, *A. flavus* can produce large amounts of aflatoxins. Aflatoxins are not typically destroyed by the post-harvest processes of contaminated cottonseed. Different methods have been used to remove aflatoxins from cottonseed meal, including biological controls, electromagnetic radiation, ozone fumigation, chemical control agents, and thermal treatments. However, many of these treatments negatively affect the nutritional content, flavor, color, texture. There is a need for alternative removal methods agricultural to better preserve quality and nutritional content of cottonseed meal.

Through this work we were able to identify physical properties of fuzzy cottonseed inoculated with *Aspergillus flavus* and understand the effects of microbial load and moisture content. This can lead to future sort measures for cottonseed during the post-harvest process. The results showed that sorting cottonseed based on physical appearance, dimensions, projected area, surface area, and density can help identify potentially infected

seeds. This work also measured the increase in aflatoxin levels throughout the post-harvest process of whole seed to cottonseed meal. The results showed that the difference in acid-delinting and the use of the mechanical dehuller can affect the microbial load on cottonseed meal. The higher microbial load was associated with lower protein content and higher fat acidity levels. Lastly, the research identifies treatment parameters to maintain nutritional components of cottonseed meal. The use of Atmospheric Cold Plasma (ACP) to treat *Aspergillus flavus* infected cottonseed meal which maintained the cottonseed fat acidity levels, protein levels, and water activity levels. Overall, this research can be used in the post-harvest process for sorting, measuring aflatoxin levels, and possible treatment using ACP.

DEDICATION

I would like to dedicate this thesis to my Lord and Savior Jesus Christ, without His love and grace I would have never made it this far. I would also like to dedicate this to my fiancé, Kristoffer Bridges, because throughout this process you continuously made me smile and encouraged me the entire time. Lastly, I would like to dedicate this thesis to all of my mentors including Josette, Karen, Kerra, Dr. McCoy, and especially Dr. Moore. Dr. Moore instilled in me that I am an engineer first and I can do anything and everything I put my mind too.

“Failure is an important part of your growth and developing resilience. Don’t be afraid to fail" - Michelle Obama.

CONTRIBUTORS AND FUNDING SOURCES

Contributors

This work was supervised by a thesis committee consisting of Dr. Janie Moore and Dr. Robert Hardin of the Department of Biological and Agricultural Engineering and Dr. Mian Riaz of the Department of Nutrition and Food Science. All work conducted for the thesis was completed by the student independently.

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CHAPTER I

INTRODUCTION AND OBJECTIVES

Introduction

Atmospheric cold plasma (ACP) is a promising technology that can reduce fungal growth, mycotoxin production, and pesticide residues in crops or harvested grains. ACP methods can have an advantage in agriculture due to low temperature operations and short processing time without inducing damage to crops, seeds, humans, and the environment (Ohta, 2016). (N. N. Misra, Yepez, Xu, & Keener, 2019). Fungal infection poses a threat to our food security due to toxin production, discoloration, off-flavor development, and spoilage of agricultural commodities (Filtenborg, Frisvad, & Thrane, 1996; Fisher et al., 2012; Gamba et al., 2015). ACP is gaining interest in the food processing industry because its attractive qualities include rapid decontamination at ambient temperature and pressure conditions resulting in little changes to nutritional composition and food quality. Plasma is an ionized gas that has reactive gas species (RGS) such as atoms, molecules, electrons, ions which are then exposed to a metastable condition with a roughly zero charge (N. Misra, Yadav, Roopesh, & Jo, 2019). In-package plasma treatment is the localization of the aforementioned RGS wherein they contact with the product to be disinfected. Using the in-package cold-plasma technology allows for prevention of post-process plasma treating contamination (N. N. Misra, Pankaj, Segat, & Ishikawa, 2016). With the knowledge of the beneficial effects of post-harvest treatment systems, ACP has the potential to reduce cost, time, and labor of treatment of contaminated cottonseed and cottonseed meal. This project focuses on determining the effect of ACP on aflatoxin

infected cottonseed meal. Chapter two will serve as a review of the literature involving the production, utilization, spoilage of cottonseed and cottonseed meal along with mechanisms of the reactions associated with ACP. Chapter three, four, five covers the research and provides results that promote good post-harvest practices of cottonseed, understanding of aflatoxin levels during cottonseed processing, and the usage of a novel non-thermal treatment method. This thesis research can be followed by an upscaled ACP treatment system in order to treat mass quantities of cottonseed meal.

Objectives

The overall goal of this thesis research project was to identify improvements in the post-harvest process of A. *flavus* inoculated cottonseed including sorting methods, effect of processing on aflatoxin levels, and using ACP as a nonthermal treatment method. This was accomplished by the following objectives:

1. Identify the differences between dimensions, projected area, sphericity, mass, volume, particle density, and surface area distributions of healthy and aflatoxin-inoculated cottonseed.
2. Quantify the increase in aflatoxin levels throughout post-harvest processing by analyzing the whole seed and the ending cottonseed meal.
3. Develop atmospheric cold plasma treatment parameters and quantify microbial load reduction in cottonseed meal.

Objective one, the identification of infected cottonseed by physical properties by determining the effects of microbial load, moisture content, and aflatoxin levels. The physical properties can be used for sorting practices during the postharvest process of

cottonseed. Objective two, the measurement of concentration increases in aflatoxin levels due to processing in cottonseed meal. This objective's results can help with understanding when the best time to decontaminate infected cottonseed with a novel treatment system. Lastly, objective three, the treatment of cottonseed meal using ACP for decontamination.

CHAPTER II

LITERATURE REVIEW

Aspergillus flavus and Aflatoxin Contamination

Aspergillus section *Flavi* is a group of saprophytic filamentous fungi that possess the ability to produce toxigenic secondary metabolites that can be harmful to human health (Carvajal-Campos et al., 2017). Aspergillus section *Flavi* is composed of 33 fungal species that are often found in soil and is capable of propagating asexually, sexually, and parasexually (Frisvad et al., 2019; Horn, Moore, & Carbone, 2009). *Aspergillus flavus* is an economically important fungal pathogen due to its production of aflatoxins in agricultural commodities (Ojiambo, Battilani, Cary, Blum, & Carbone, 2018). *A. flavus* is active between 10℃ and 45℃, and all stages of the infection cycle, from sporulation to host infection, can take place in this range, typically resulting in the production of mycotoxins known as aflatoxins (Sanchis & Cranfield, 2004). The growth of the opportunistic fungus, *A. flavus*, is optimal at high temperatures and at high relative humidity (above 85% rH) (Al-Shikli, A.Abdulrasool, & Al-Hiti, 2010). In addition to relative humidity, there are other factors that influence aflatoxin production such as growth stage, physiology, and grain composition (Ojiambo et al., 2018). *A. flavus* can produce aflatoxin B1 (AFB1), B2 (AFB2), G1 (AFG1), G2 (AFG2). AFB2 and AFG2 are not toxic, but AFB1 is the best-known naturally made carcinogen (Poor et al., 2017).

Since aflatoxin is a naturally-occurring potent carcinogen, it is commonly known as an “unavoidable contaminant” and is difficult to completely eliminate from feeds such as cottonseed meal (Park & Stoloff, 1989). Chronic exposure to aflatoxin-contaminated

feed sources increase the risk of liver cancer and can suppress the immune system (Rapisarda et al., 2016). About 5 billion people globally are at risk of chronic exposure to aflatoxin due to the absence of regulatory limits in order to enforce established limits, or due to the lack of resources and technology (Strosnider et al., 2006). Aflatoxin action levels have been set in the U.S. by the Food and Drug Administration, based on the type of animal consuming the product and the use of the feeding animal (Mitchell, Bowers, Hurburgh, & Wu, 2016). For beef cattle, swine, and poultry the action level for aflatoxin is 300 parts per billion (ppb). However, dairy animals are held to a 20-ppb action level for feed products due to the probability of aflatoxins being transferred into the milk. (Kotinagu, Mohanamba, & Kumari, 2015). Cottonseed industry is a common feed source for dairy cows; therefore, aflatoxin contamination affects cotton farmers. The dairy industry being hindered because of rejected milk containing high aflatoxin levels and cows being quarantined due to aflatoxicosis is negatively affecting cottonseed farmers (Wu, Liu, & Bhatnagar, 2008).

Many oilseed crops including legumes, peanuts, maize and cottonseed are associated with active *A. flavus* growth (Klich, 2007). Specifically, cottonseed contains several components that are fungal nutrients, including lipids, saccharides, and storage proteins (Mellon, Cotty, & Dowd, 2007). Higher aflatoxin levels increase during the pre- and post-harvest processing due to improper handling, processing, and storage. There are several pathways of fungal entrance into the seed. Cottonseed typically develops within a thick-walled boll (carpel), usually divided into four or five compartments (locules) (Figure 1). *A. flavus* can infect the nectaries (plant glands) of flowers, or insects can damage the

carpel walls of cottonseed, allowing fungal entrance (Goynes & Lee, 1989; Klich & Chimielewski, 1985).

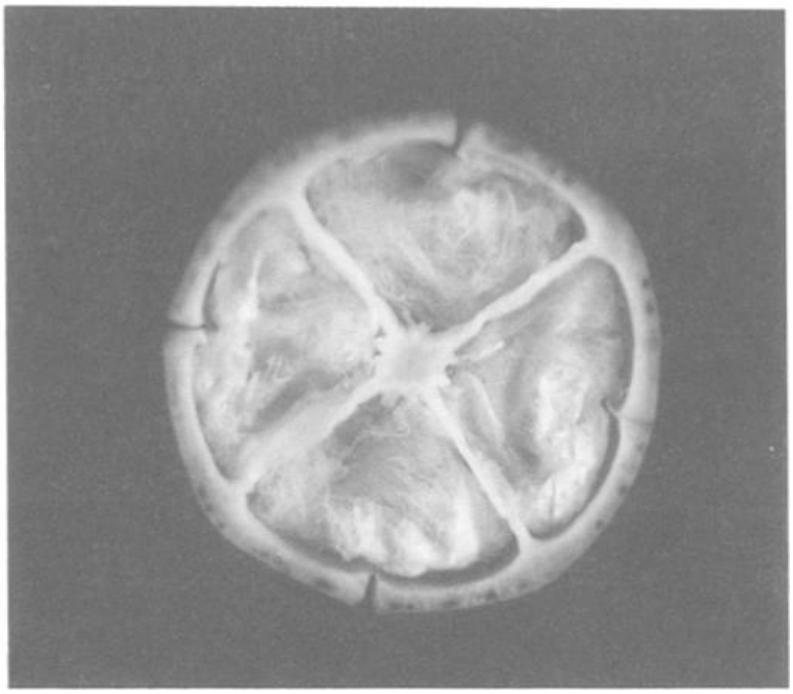

Figure 1. Image of cottonseed developing within a thick-walled boll divided into 4 locules. Reprinted from (Goynes & Lee, 1989).

The seed and fibers that grow within a given locule are called a lock, and the fungal infection in an open cotton boll is usually evidenced by the locks that go unfluffed and remain tight. The tight locks are caused by the weakening of the cotton fibers due to the exponential growth of *A. flavus* which causes problems throughout the milling process (Ashworth, Rice, McMeans, & M., 1971).

Cottonseed Processing

Typically, a ton of cottonseed produces around 1440 pounds of cottonseed meal and hulls, which ultimately is fed to livestock and poultry. Raw cottonseed is both food

and fiber and is made up of three parts: 1) linters, which are short fibers still connected to the seed; 2) hulls, the protective coating for the kernel; and 3) the protein and oil rich kernel. Products derived from a ton of cottonseed are as follows: meal (900lbs, 45%), hulls (27%), crude oil (16%), linters (8%), and waste (4%) (NCPA, 2020). The hulls for fiber, meal are sources of vegetable protein feed for animals, and linters are used as a chemical cellulose source in personal care products and high quality paper (Smith & Cothren, 1999).

Before cottonseed is processed into oil and cottonseed meal, cotton fibers are removed from the seed during the ginning process, producing fuzzy cottonseed. The cottonseed sent to an oil mill usually uncleaned and containing small amounts of other plant materials including soil and dust. The removal of foreign material is done by using a magnetic force to remove metal, pneumatic separation to remove sticks and pods, and screening equipment to remove weed seeds, sand, and soil (Pighinelli & Gambetta, 2012). Following cottonseed cleaning, the short linter fibers are removed to maximize the oil extracted from the seed due to the absorption ability of the cellulose fibers. The delinting step is unique to cottonseed because the linters tend to be bulky and occupy space during the extraction of the oil (O'Brien, Jones, King, Wakelyn, & Wan, 2005). Chemicals such as sulfuric acid and mechanical machines have been used to remove linters. Chemical delinting is usually for replanting of cottonseed or research otherwise mechanical removal is for oil extraction. The use of sulfuric acid completely removes fibers and is costly.

After removal of linters, the outer seed coat, known as the hull and rich in fiber and poor in oil and protein, needs to be removed. The removal of the hulls yields cottonseed meal with higher protein content (Kemper, 2005). The dehulling process

removes the lighter hull fraction by aspiration, agitation, and screening. A bar huller has a bar or knife-studded cylinder that rotates within another cylinder and the hulls are cut when they pass through the inner cylinder. The seed decorticator has two hardened steel rolls, both with longitudinal grooves. The seeds are fed into the decorticator and cut by the grooves and the difference in speed between the two rolls. The mechanical removal of seeds can lead to more broken and finer pieces which result in higher aflatoxin content when processing (Piedade et al., 2002).

After dehulling, the kernels are reduced in size or flaked to facilitate oil removal. In order to enhance flaking, the material is heated to 90-110°C to decrease the viscosity of the oil, resulting in good quality cake (Kemper, 2005). Cottonseed cake is the solid remaining after oil extraction. The flaking operation distorts the cottonseed cellular structure and facilitates separation of the material remaining (Pighinelli & Gambetta, 2012). Cottonseed is flaked by passing between two crushing rolls, the cylindrical rollers rotate opposite of each other creating thinner flakes. The flakes are cooked which break down the cell wall, reduce oil viscosity, control moisture content, coagulate protein, inactivate enzymes, and detoxify bound gossypol is bound (Cherry, 1983). The flakes are poured into the top kettle, heated for a period of time (~120 minutes), and swept into the kettle below (O'Brien et al., 2005). As the flakes are placed into the bottom kettle, water is evaporated and removed by vents until the desired moisture content is reached for the oil pressing process (O'Brien et al., 2005).

There are four types of processing systems which are used to extract oil: (1) hydraulic press, (2) screw press, (3) prepress solvent extraction, and (4) direct solvent

extraction. Dehulled cotton seeds contain around 34% oil and are suitable for mechanical pressing because of the high amount of extractable oil (O'Brien et al., 2005). The chosen processing will result in various amounts of aflatoxin content remaining in the cottonseed cake. Hydraulic pressing was the earliest type of batch pressing for cottonseed oil extraction. The open presses are fed with seeds wrapped in cloths and the plates gradually squeeze the oil from the seeds. However, this process is labor intensive. The screw press is similar to the hydraulic press, but this system has both vertical and horizontal presses to maximize pressure (O'Brien et al., 2005). This system pressure is gradually applied to the flakes while a screw conveys them from the feed end to the discharge end of the expeller barrel. Direct solvent extractions uses hexane, and the residual meal is heated to evaporate the solvent, which is collected and reused (Ziegler, Kadan, Freeman, & Spadaro, 1981). Solvent extraction yields about 11.5% more oil than a screw press , with less oil remaining in the cottonseed meal (O'Brien et al., 2005). The primary downside to this method is the use of hexane because of its classification as a Hazardous Air Pollutant (HAP) by the U.S. Environmental Protection Agency. The post-harvest process begins with whole fuzzy cottonseeds and ending with meal retrieval following oil extraction (Figure 2).

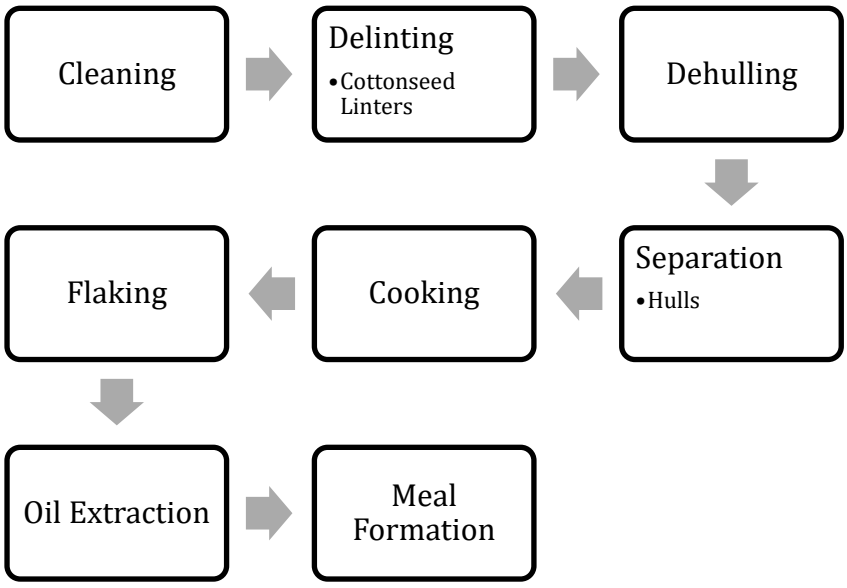

Figure 2. Cottonseed milling process from whole fuzzy seed to cottonseed meal.

Damage to Cottonseed during Post-Harvest Processing

Cottonseed contributes to almost 15% of farmers income from cotton (Jaime-Garcia & Cotty, 2003). Cottonseed value can be reduced by aflatoxin contamination if the gin cannot sell to the dairy or beef farmers (Wu et al., 2008). Mechanical damage to cottonseed is a major cause of cottonseed quality problems which is due to the mechanical fiber pickers or the mechanical gin (Delouche, 1981). In storage, when a cracked cottonseed is exposed to a seed that has been infected and the ideal fungus growing conditions are met the cracked seed has a high susceptibility to be contaminated (Cotty, Howell, Bock, & Tellez, 1997). A previous research study looked into the effect of cottonseed moisture levels and seed feed rate during ginning on cottonseed damage due to the high moisture content and the associated fungal growth (Shaw & Franks, 1962).

Another study showed that seed damage increased with moisture content, with the largest increase in damage at seed moisture levels above 12% (Columbus & Mangialard, 1996).

When contaminated seed is stored, *A. flavus* growth and consequent aflatoxin production primarily occur on the cottonseed kernel while some of the toxin is on the seed hulls (Ciegler, Kadis, & Ajl, 1973). Higher moisture content causes higher relative humidity of the air infected seed will be stored which encourages more growth of *A. flavus*. Fungal invasion and decreased physical qualities are proportional to increases moisture content and length of storage (Robertson, Chapman, & Wilson, 1984). Cellular respiration occurs at a higher rate when there is more fungal growth which also contributes to higher moisture content. The product of water is a key product which this equation is below:

$$C_6H_{12}O_6 + O_2 \rightarrow CO_2 + H_2O + energy$$

During cellular respiration, sugar is broken down to carbon dioxide, water and Adenosine triphosphate (ATP). The ATP can then be used for fungal nutrition, metabolism, growth, and reproduction (Walker & White, 2018). The initial water content is low and increases as the growth of fungal species increases (Richards, 1927). Free fatty acids are also produced and lower the oil quality (Eckel, Borra, Lichtenstein, & Yin-Piazza, 2007). Triglycerides are split by enzymes due to hydrolysis that result in the release of free fatty acids and the hydrolysis is dependent on temperature and moisture (Hammond, 2003). To limit the spread of aflatoxin, cottonseed must be stored at a moisture content of less than 10% (w.b) and dehulled seed should be stored at less than 9% (w.b) (O'Brien et al., 2005). If the moisture content rises, then there is a need for drying the stored cottonseed and an air-cooling system is essential for the successful storage of

cottonseed. Not only does moisture content assist in the increase of aflatoxin levels, but the temperature is dependent on the ambient temperature and degree of ventilation in the storage area. Temperature should be kept below 60°F because optimal growth of aflatoxin is above 77°F when being stored (O'Brien et al., 2005). Cottonseed seed damage during harvesting, transportation, cleaning, and the ginning process can also assist in the spread of aflatoxin (Searcy et al., 2010).

Atmospheric Cold Plasma

Atmospheric cold plasma (ACP) is used in various different industries including textiles for the enhancement of product quality, durability, and improving bonding characteristics (Choudhary, Dey, Bhattacharyya, & Ghosh, 2018). ACP has also been used on odor emissions which tend to have negative effects on the surrounding and communities (Andersen, Feilberg, & Beukes, 2012). Beneficial effects have been shown in cancer treatment by eradicating cancer cells *in-vitro* without damaging the normal cells and significantly reduced tumor size *in-vivo.* ACP is a novel treatment system that is multi-faceted and is showing promise in multiple fields (Keidar et al., 2013).

ACP has significantly impacted food production, agriculture, medicine, and environmental sectors (Bourke, Ziuzina, Boehm, Cullen, & Keener, 2018). ACP has been shown to have decontaminating effects on various microbial communities and minimal impact on food quality and the environment (Pankaj, Wan, & Keener, 2018). This novel non-thermal treatment technology has been shown to successfully inactivate a variety of microbes and inactivate mycotoxins on agricultural products such as sprout seeds, alfalfa seeds, carrots, cucumbers, pears, distillers wet grains, wheat grain, barley, and whole

peppercorns (Butscher, Loon, Waskow, Rohr, & Schuppler, 2016; Los et al., 2018; McClurkin-Moore, Ileleji, & Keener, 2017; Mošovská et al., 2018; Shi, Stroshine, & Ileleji, 2017; Wang et al., 2012). A research study's results showed that there was a 99.3% reduction in the reduction of *A. flavus* and the aflatoxin content was decreased by 90% in ground nuts (Devi, Thirumdas, Sarangapani, Deshmukh, & Annapure, 2017). ACP has also been shown to disinfect *A. flavus* on beef jerky, brown rice cereal , maize, and hazelnuts (Dasan, Boyaci, & Mutlu, 2016; Haelim et al., 2016; Shi, Ileleji, Stroshine, Keener, & Jensen, 2017; Siciliano et al., 2016; Suhem, Matan, Nisoa, & Matan, 2013).

In a recent study, higher resistance was reported in fungal spores compared to bacteria cells due to the difference in cytology, morphology, reproductive cycles, and growth (Dasan et al., 2016). The treatment is greatly affected by the physiochemical and physiological properties of cottonseed which include moisture content, protein concentration, nitrogen levels, germination, growth, and overall yield which creates reactive gas species (RGS). RGS react with microbes that may form on the infected commodity that results in the decontamination for a few hours post-treatment (Surowsky, Fischer, Schlueter, & Knorr, 2013). Detoxification of mycotoxin production has been shown to serve as a method to replace current inefficient mycotoxin removal strategies (Figure 3). Research shows that there are at least two different ways that ACP degrades aflatoxins (Shi, Cooper, Stroshine, Ileleji, & Keener, 2017). The first pathway is when a water molecule, hydrogen molecule, or aldehyde group is added to aflatoxin. This pathway relies on the hydrogen atom and hydroxyl radical which are made by the ACP system by the process of hydration and hydrogenation. The second pathway relies on the formation

of hydroxyl radical, hydrogen peroxide, and ozone which lead to epoxidation and oxidation.

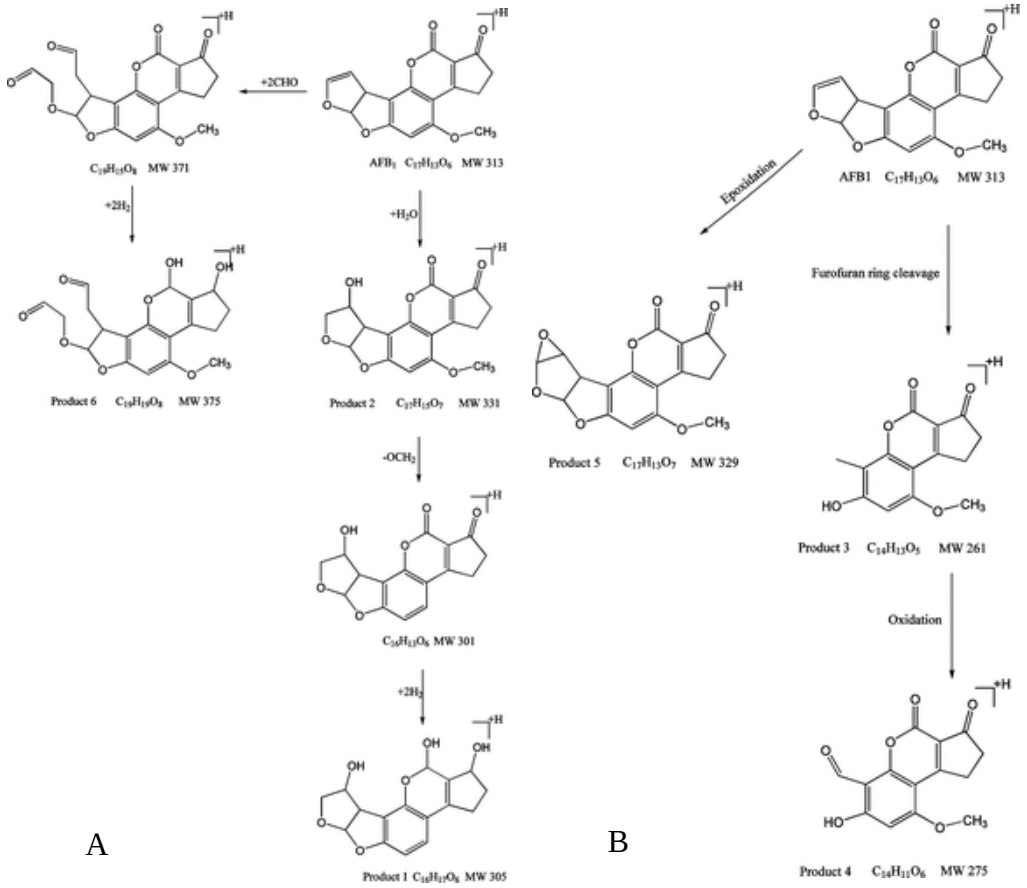

Figure 3. Two degradation pathways of aflatoxin B_1 using atmospheric cold plasma: a) addition of hydorgen molecule or aldehyde group and b) epoxidation and oxidation reactions. This figure was reprinted from (Shi, Cooper, et al., 2017).

There is potential that ACP can be used to treat *A. flavus* in cottonseed and decrease microbial load. There has been previous research that has used physical and chemical methods to decontaminate molds and mycotoxins in animal feeds. Heat, irradiation, and ultraviolet light have been shown to lower microbial growth and microbial loads (Doyle, Applebaum, Brackett, & Marth, 1982; Samarajeewa, Sen, Cohen, & Wei, 1990). Similarly, chemical treatments including chlorine, hydrogen peroxide, ozone, bisulfite,

ammonia, and other acids have been shown to have detoxifying effects (Dwarakanath, Rayner, Mann, & Dollear, 1967; Samarajeewa et al., 1990). Even though these treatments have been shown to decrease microbial load and mycotoxin levels, there is a considerable of deterioration in food quality (Holdsworth, 1997). The other nonthermal technologies, irradiation and high-pressure, are somewhat effective, but are time consuming and tedious during treatments (Devi et al., 2017). Using ACP can be an effective treatment in comparison to the aforementioned treatments, which can save time and cost. ACP can be an effective treatment for *Aspergillus flavus* in cottonseed.

The treatment is greatly affected by the physiochemical and physiological parameters which include moisture content, protein concentration, nitrogen levels, germination, growth, and overall yield which creates antimicrobial species that react with any microbes that may be formed on the infected commodity that results in the decontamination for a few hours post-treatment (Surowsky et al., 2013). ACP can be induced in ambient conditions with the input of energy causing the neutral gases to ionize, this process can be done inside or outside of a package. The dielectric barrier discharges ionize the gases by applying a high voltage in an interdieletric space (N. Misra et al., 2015). Reactive oxygen species (ROS) such as hydroxyl radicals, singlet oxygen molecules, superoxide anions, and ozone are responsible for the deactivation of microbes (Hiroshi et al., 2013). Barrier discharges are comprised between two electrodes at differential potentials, separated by dielectric materials. The prevention of an electric arc is due to the barrier limiting the electrodes current flow. The reaction mechanisms involve

the vibration, excitation, dissociation, attachment, and ionization of the molecular species by causing strong responses in the applied magnetic field (Fridman et al., 2008).

There has a been little to no research studying the effects of ACP on cottonseed nutritional composition and microbial load. *A. flavus* has been inactivated using ACP and demonstrated to cause cell leakage and loss of viability (Suhem et al., 2013). Using ACP can also lead to the destruction of DNA and changes in cell morphology in fungal spores (N. Misra et al., 2019). The effects of plasma on fungi are shown in Figure 4.

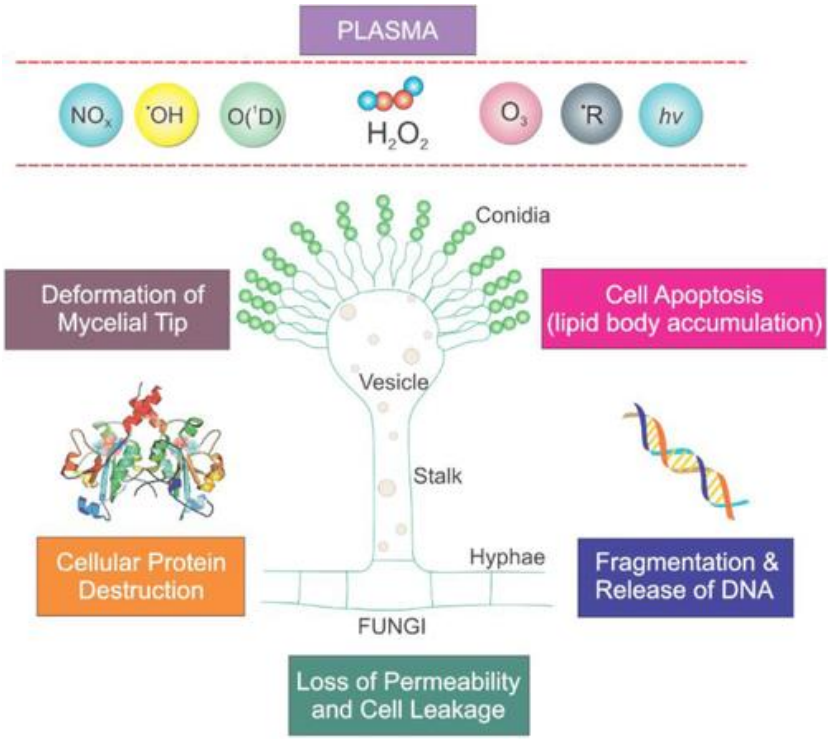

Figure 4. A summary of the effects of cold plasma species in fungal cells resulting in inactivation. This figure was reprinted from (N. Misra et al., 2019).

Cottonseed is not currently packaged, but an upscaled treatment system can be constructed to treat larger quantities of infected cottonseed after the inoculated cottonseed from the healthy samples. The novel treatment can be upscaled and use in a large

processing facility for cottonseed. This research focuses on the laboratory scale of treatment within a bag, but the promising results shows the possible impacts on treating cottonseed by using a larger treatment container. Upscaling the ACP treatment would involve creating larger dielectric barriers, increase size of electrodes, and add the effect of gyration to ensure the surface of the cottonseed are exposed to the RGS between the barriers. After the cottonseed is identified as being contaminated, these cottonseeds can then be treated quickly, lost cost, and at high volumes.

CHAPTER III

INCREASED MOISTURE CONTENT EFFECTS ON KEY PHYSICAL PROPERTIES OF FUZZY COTTONSEED; A MEASURED RESPONSE IN MICROBIAL ACTIVITY, COLOR, AND AFLATOXIN CONTENT

Introduction

Physical properties are important for proper design of machines and processes to harvest, handle, and store cottonseed (Udomkun et al., 2017). During the storage of cottonseed, abiotic factors, such as high humidity and ambient temperatures, are linked to fungal infection of cottonseed (Lillehoj, Wall, & Bowersm, 1987). When ideal conditions for *Aspergillus flavus* growth on cottonseed occur, discoloration, rotting, and development of off-odors and off-flavors. Changes in the physical properties of cottonseed can be used to monitor and detect this deterioration due to post-harvest fungal contamination and subsequent production of aflatoxin.

Physical properties have been used for sorting clean from mycotoxin-contaminated stored grains and other high-value products such as cereals, maize, cocoa beans, and coffee beans (Karlovsky et al., 2016). Such physical properties include: dimensions, projected area, sphericity, mass, volume, density, and surface area, these properties may also be important in cottonseed processing. Size and sphericity affect cleaning and storage of cottonseed in seed bins. after the removal of fibers at cotton gins (Shaw, 1962). The projected area of cottonseed affects drag force, which ultimately affects pneumatic conveying (Ashley, Thomas, Holt, & Valco, 2018). In the design of air ducts for cottonseed storage, cottonseed surface area must be considered to produce even airflow

throughout the aeration process (Groves & Bourland, 2010). Volume, mass, and density are important in the cleaning of undesirable products in cottonseed on oscillating chaffers (Azadzadeh, 2014). Likewise, color, moisture content and color analysis are often used to separate out spoiled grain which could lead to major quality issues in stored agricultural commodities including rice, wheat, and soybeans (Champathi Gunathilake, Bhat, Singh, & Tharanga Kahandawala, 2019; Nadvornikova, Banout, Herak, & Verner, 2018; Ponce-García, Ramírez-Wong, Escalante-Aburto, Torres-Chávez, & Serna-Saldivar, 2017).

Two studies showed correlations between multiple physical properties in fuzzy and delinted cottonseed. The first study showed a linear relationship between moisture content and cottonseed dimensions, sphericity, 1000 seed mass, projected area, and true density (Ozarslan, 2002). Manimehalai and Viswanathan (2006) demonstrated that the individual mass of a cottonseed affects similar physical properties in fuzzy cottonseed (Manimehalai & Viswanathan, 2006). The results mirrored the previous study, as mass positively correlated with increased dimensions, sphericity, 1000 seed mass, projected area, particle density, and volume. These studies showed the importance of physical properties in processing delinted and fuzzy cottonseed. Higher microbial load can change various physical properties such as mass, density, aflatoxin content, and cottonseed appearance in cottonseed (Koltun, Gardner, Dollear, & Rayner, 1973). The study showed that aflatoxin content increased with decreasing seed density. However, no explanation for this relationship was given.

Cateye fluorescence, yellow-greenish fluorescence observed in the linters, was used in another study to determine the relationship between density, aflatoxin

contamination, and physical appearance under a long-wave ultraviolet light. Lower density was associated with higher aflatoxin content, but the results were not discussed. From this study, sorting of ginned fuzzy cottonseed was ineffective due to seeds having cateye fluorescence, but no aflatoxin contamination (Lee, Cucullu, Pons, & Russell, 1977). The previous studies suggest that sorting cottonseeds by a few physical properties, such as physical appearance and mass, would not be successful. However, the use of multiple physical properties for sorting may be promising. There is no research correlating multiple physical properties and aflatoxin levels in cottonseed.

Previous research has shown that sorting corn by size and density can result in an 84% reduction of aflatoxin by removing the fine material (Shi, Stroshine, et al., 2017). In fact, cleaning and sorting of other agricultural commodities before storage can lead to better storage techniques by removing smaller kernels and fine materials (Ojiambo et al., 2018). In fact, best practices are to clean and sort agricultural commodities that are susceptible to contamination before storage by removing smaller kernels and fine materials because this improves aflatoxin management practices (Ojiambo et al., 2018).

A better understanding of cottonseed physical properties and their relationship to aflatoxin and moisture content is needed, which can lead to more efficient postharvest sorting operations. This information is especially important for designing equipment to handle fuzzy cottonseed during the postharvest processing of cottonseed (Figure 2). Currently, the only sorting involved in the processing of cottonseed is the removal of unwanted material such as sticks and dirt during cleaning. With the intention to combat the negative economic impact of aflatoxin contamination of cottonseed, this study

explored the usage of physical properties to sort cottonseed prior to processing. Specific objectives for this study were to: 1) determine the differences between dimensions, projected area, sphericity, mass, volume, particle density, and surface area distributions of healthy and contaminated cottonseed, 2) determine the effects of physical properties on aflatoxin levels, 3) understand the effect of moisture content of cottonseed on aflatoxin levels. The conclusions from this study can be applied to develop techniques to reduce aflatoxin contamination of cottonseed during post-harvest sorting operations.

Methods and Material

Clean fuzzy cottonseed was obtained from the Cotton Gin Lab on Texas A&M University's campus. Initially, the moisture content was determined by oven drying three 5 gram (g) samples of cottonseed at 105 ± 2 ℃ for 14.5 hours (Griffin Jr, 1980). The initial moisture content of the seeds sampled was determined to be 8.3% (d.b.). There was a total of three groups which included an experimental group and two control sample groups. Each group had 350 grams (g) of fuzzy cottonseed and were labeled as the following: 1) clean (control), 2) wetted (control), 3) inoculated (experimental). A concentration of 9.6×10^4 CFU/g of *Aspergillus flavus* (Carolina Biological Supply Company, Burlington, NC) was concentrated into Triton X-100 solution and Aflatoxin B_1 (150 ppb) dissolved in DMSO (Aflatoxin B1 5MG, Sigma Aldrich, Darmstadt, Germany) *A. Flavus* and aflatoxin B1 was added to the inoculated by spraying then mixing every 30 minutes for 3 hours.

The same amount of water was added to the wetted cottonseed sample. Both the inoculated and wetted sample group were thoroughly mixed every 30 minutes for 3 hours.

Cottonseed samples at the desired moisture content of 25% were prepared by using the following equation (Izli, 2015):

$$Q = \frac{W_i (M_f - M_i)}{100 - M_f}$$

where Q is the mass of the water added (g), W_i the initial mass of the sample (g), M_i the initial moisture content of the sample (% d.b.), and M_f is the final moisture content of the sample (% d.b.). The inoculated and wetted sample were placed in the freezer at 5℃ for 7 days to allow for uptake of water and limit the microbial growth without the influence of humidity and temperature. The inoculated and wetted cottonseed sample were then placed in a relative humidity and temperature chamber (Percival Scientific, Inc., Perry, IA) at 25℃ and 85% relative humidity (rh) for 21 days which ensured that any presence of toxigenic *A. flavus* had the opportunity to produce additional aflatoxins (Ellis, Smith, Simpson, Ramaswamy, & Doyon, 1994b). Fifty random seeds were taken from the control, wetted, and inoculated sample and examined for the experimental physical properties.

To measure aflatoxin levels the Envirologix Aflatoxin Flex kit (Envirologix Inc., Portland, ME)) was used for this experiment. The Envirologix Aflatoxin Flex kit is approved by the Association of Official Analytical Chemists (AOAC) and the Grain Elevator and Processing Society (GEAPS). Twenty-five grams of cottonseed was removed from the clean, wetted, and inoculated was ground and tested in triplicates. The ground cottonseed was combined with 100 milliliters (ml) of 50% ethanol and the elution buffer pouch of the Envirologix kit. The sample was vigorously shaken for two minutes and filtered with an approved filter. 100µl of the liquid was obtained and added to 100µl of

the Envirologix kit's DB5 buffer. A test strip was added for 5 minutes and the color change of the test strip was determined by the Envirologix system color scanner, which has a minimum detection threshold of 1ppb (QuickScan, Envirologix Inc., Portland, ME).

Plate counts were used to assess microbial growth. *A. flavus* was counted by identifying the fungus in color and growth stages. Immediately after removing the wetted and inoculated cottonseed sample from the relative humidity and temperature chamber, 5g from all three groups were removed and placed in a sterile 15ml tube with 8.3 ml of 0.05% Triton X 100 solution. The samples were then shaken for 2 minutes. For the control, uninoculated, and inoculated groups, 100 µL of the wash was plated onto 100 mm × 15 mm sterile petri dishes containing Potato Dextrose Agar (Sigma Aldrich, Merck KGaA, Darmstadt, Germany). Three replicates from each group were prepared and plated. After 7 days of incubation at 25°C, colony forming units (CFUs) per ml were counted to compare microbial growth of different treatments. Growth curves were developed by plotting the mean colony diameters (mm) of the diluted samples against 72 hours of incubation time to estimate logistic regression of the growth curves. The logistic growth model equation used:

$$\frac{dN}{dt} = r_{max}(\frac{K-N}{K})N$$

Where rmax is the growth rate, K is the carrying capacity, and N is the population size currently present. This model was chosen because of the limited growth capability and resources which result in which result in the lag phase, exponential phase, and the death phase.

To evaluate the color differences between the samples a HunterLab Labscan XE spectrocolorimeter (Hunter Associates Laboratory, Inc., Reston, VA) was used with a port size of 10.1 mm and a view area of 6.35 mm. The Hunter L, a, b is a uniform color scale that plots the differences in points in the color space which is organized in a cube form. The *L* axis represents the darkest black at *L*=0 and brightest white at *L*=100. The *a* axis represents green and red, with green correlating to the negative direction and red in the positive direction. The *b* axis represents blue and yellow, with blue correlating to the negative direction and yellow in the positive direction. The colorimeter was calibrated before each measurement, using black and white color tiles. Color of the cottonseed was assessed as a possible way to sort infected cottonseed. Three readings for each sample were tested with the colorimeter.

Physical Properties

Major, minor, and intermediate diameters were measured by a digital caliper to the nearest 0.1 mm. A total of 50 seeds were randomly selected and characterized individually from each of the cottonseed sample groups. The sphericity was calculated by using the equation:

$$S = \frac{(LWT)^{\frac{1}{3}}}{L}$$

where: S is the sphericity, the major, minor, and intermediate diameters are length (L), width (W), and thickness (T) respectively (Ozarslan, 2002).

Projected area

Using the relationship suggested by Manimehalai et al. (2006), the projected area of cottonseed was calculated by:

$$A = \frac{\pi L L_1}{4}$$

$$L_1 = \frac{L+W}{2}$$

where A is the projected area in mm^2, L is the length of the seed, and L_1 is the average of length and width in mm. The same 50 seed dimensions from the previous section were used to calculate the projected area.

The surface area was calculated by modeling the cotton seed as a cone with a hemispherical base with the diameters obtained previously (Hodson, 1920). The shape was used due to the nonuniform, conical shape, of cottonseed makes it difficult to measure the surface area. Since there is no exact equation for calculating the surface area of a cottonseed, the following equation was designed specifically for this work to model the shape (Hodson, 1920):

$$SA_S = 4\pi\left(\frac{W}{2}\right)^2$$

$$SA_C = \pi\left(\frac{W}{2}\right)l$$

$$SA_{S+C} = 4\pi\left(\frac{W}{2}\right)^2 + \pi\left(\frac{W}{2}\right)\sqrt{\left(L-\frac{W}{2}\right)^2 + \left(\frac{W}{2}\right)^2}$$

where: SA_S is the surface area of the sphere, SA_C is the surface area of the cone, SA_{S+C} is the surface area of the cone with a hemispherical base, and l is the length of the cone (Figure 5). Figure 5 is a sketch of the modeled shape, combining the cone and hemispherical base, next to the sketch of a cottonseed.

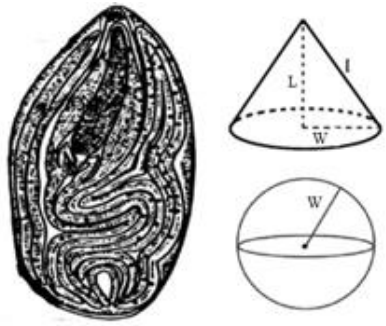

Figure 5. Sketch of surface area of the modeled shape for cottonseed as a cone with a hemispherical base. This figure was adapted (Ritchie, Bednarz, Jost, & Brown, 2007).

A pycnometer (Micromeritics, USA; Model AccuPyc 1330) determined the volume of individual cottonseed in order to calculate the density, helium was used as a fill for the empty spaces in the pycnometer around the cottonseed. The same individual seed mass was measured an electronic balance of .001g accuracy (American Weigh, USA; Mode GEMNI-20). These properties were measured in triplicates and averaged.

Statistical Analysis

Statistical analyses were performed using Origin software (OriginLab, Northhampton MA). The one-way ANOVA was used to determine if the treatment effect was significant. A Shapiro-Wilk test was performed to determine if the sample data was drawn from a normally distributed population. Tukey's test was used to compare means. A 5% level of significance was used for all statistical tests.

$$F = \frac{\Sigma n_j \left(\overline{X}_j - \overline{X}\right)^2 / (k-1)}{\Sigma\Sigma \left(\overline{X} - \overline{X}_j\right)^2 / (N-k)}$$

Where F is the degrees of freedom, n_j is the sample size, j^{th} group, $\overline{X}_j$ is the sample mean in the j^{th} group, and $\overline{X}$ is the overall mean.

$$W = \frac{\left(\sum_{i=1}^{n} a_i x_{(i)}\right)^2}{\left(\sum_{i=1}^{n} x_i - \bar{x}\right)^2}$$

Where x_i is the order random sample values and a_i are constants generated from the covariances, variance, and means of the sample from a normally distributed sample.

$$HSD = \frac{M_i - M_j}{\sqrt{\frac{MS_w}{n_h}}}$$

Where $M_i - M_j$ is the difference between the pair of the means to calculate this M_i should be larger than M_j. MS_w is the mean square within, the n is the number in the group.

Results and Discussion

Seed Appearance, Moisture Content, Microbial Analysis, and Aflatoxin Levels

The Hunter *L,a,b* analysis analyzed the color of the cottonseed sample groups before testing physical parameters (Table 1). After averaging values, the cottonseed appeared to be either a white, green, or black color indicating a difference in appearance between the three sample groups. The control cottonseed had higher *L* values, which correlated to a whiter sample. This control cottonseed should have a whiter color, as the linters from the cotton are white and microbial degradation was not expected with these samples. The wetted sample that was placed in the relative humidity temperature chamber had negative *a* value, indicating the sample had a green tint. The green tint indicates microbiological growth on the surface of the cottonseed. This would be expected since the wetted sample was stored at a temperature and humidity which would encourage microbial growth. The inoculated sample had *L* values that were lower than the control and wetted sample, indicating a greyish color as *L* spans the white to black spectrum. This darker

color can be attributed to the presence of microbes.

	Hunter *L*,*a*,*b* Measurements		
Cottonseed Sample	*L*	*a*	*b*
Control	46.9[a]	1.46 [a]	6.86
Wetted	40.6[a]	-0.3 [b]	6.74
Inoculated	23.7[b]	1.42 [a]	5.64

Table 1. The average Hunter Lab XE scanner values of Cottonseed Samples. [a,b] = significant at $\alpha = 0.05$.

Cottonseed Sample	Microbial Load (CFU/ml)	Moisture Content, (%d.b.)	Aflatoxin Level (ppb)
Control	$1.5\times 10^{4\,a}$	8.29 %	0.00
Wetted	$1.2\times 10^{8\,a}$	25.9%	0.00
Inoculated	$6.7\times 10^{14\,b}$	48.9%	>380

Table 2. The averages of the microbial load, moisture content, and aflatoxin levels in the control, and inoculated sample. [a,b] = significant at $\alpha = 0.05$

After the 21 days of storage, moisture content was 8.29%, 25.9%, and 48.9% (d.b.) for the control, wetted, and inoculated sample, respectively (Table 2). The wetted and inoculated sample were initially stored with the same moisture content, but the values changed over time. The inoculated sample had significantly higher microbial growth in comparison to the wetted and control sample. A 4-log and 10 log-difference of microbial growth compared to wetted and control sample, respectively (Table 2).

The moisture content was higher in the inoculated sample because of the larger microbial load actively growing on the sample during storage. The significant increase of growth is indicative to the available moisture in the sample, therefore decreasing moisture in storage can lead to a less hospitable environment for the microbial growth to occur. Similar studies with stored rice, maize, sorghum, chestnuts, and distillers wet grains show

that moisture content plays a large role in encouraging the growth of *Aspergillus flavus* and the subsequent mycotoxin production in stored grains (Aydin, Aksu, & Gunsen, 2011; McClurkin & Ileleji, 2015; Mpuchane, Taligoola, Gashe, & Matsheka, 1997; Prencipe et al., 2018).

Aflatoxin levels were only detected in the inoculated sample. The results showed aflatoxin levels of 0 ppb, 0 ppb, and above 380 ppb for the control, wetted, and inoculated sample, respectively (Table 2). The inoculated sample tested above the FDA action level of 20 ppb. The difference between the control and the inoculated sample results will be used in the determination of the effect of aflatoxin on the cottonseed sample and the resulting understanding of sorting of seed based on moisture content and the culmination of physical properties in this work.

Microbial Growth Analysis and Modeling

The logistics growth model showed that there was a significantly slower growth rate in the control cottonseed sample. The inoculated sample had a faster growth rate when compared to wetted sample was due to the higher population size present on the inoculated sample. The R-squared (R^2) values indicate the accuracy of the primary model developed with the R^2 values being 0.99, 0.96, and 0.94 for the control, wetted, and inoculated sample, respectively. The R-squared (R^2) values indicate the accuracy of the primary model developed with the R^2 values being 0.99, 0.96, and 0.94 for the control, wetted and inoculated sample, respectively.

The higher values represent smaller differences between the observed data and predicted values. As can be seen in Figure 6, the larger initial population size caused the

rate to be faster the carrying capacity to be at a higher microbial load. There were limited resources such as PDA and space available on the plate the death of the fungal cells plateaued The *A. flavus* concentration in the inoculated sample was significantly higher than the other two sample groups which resulted in an increased amount of growth occurring. This means that there is less intraspecific competition occurring which allow for more population sustainability. A study verified that there is an inverse relationship between final community size and inoculum dilution (Franklin, Garland, Bolster, & Mills, 2001).

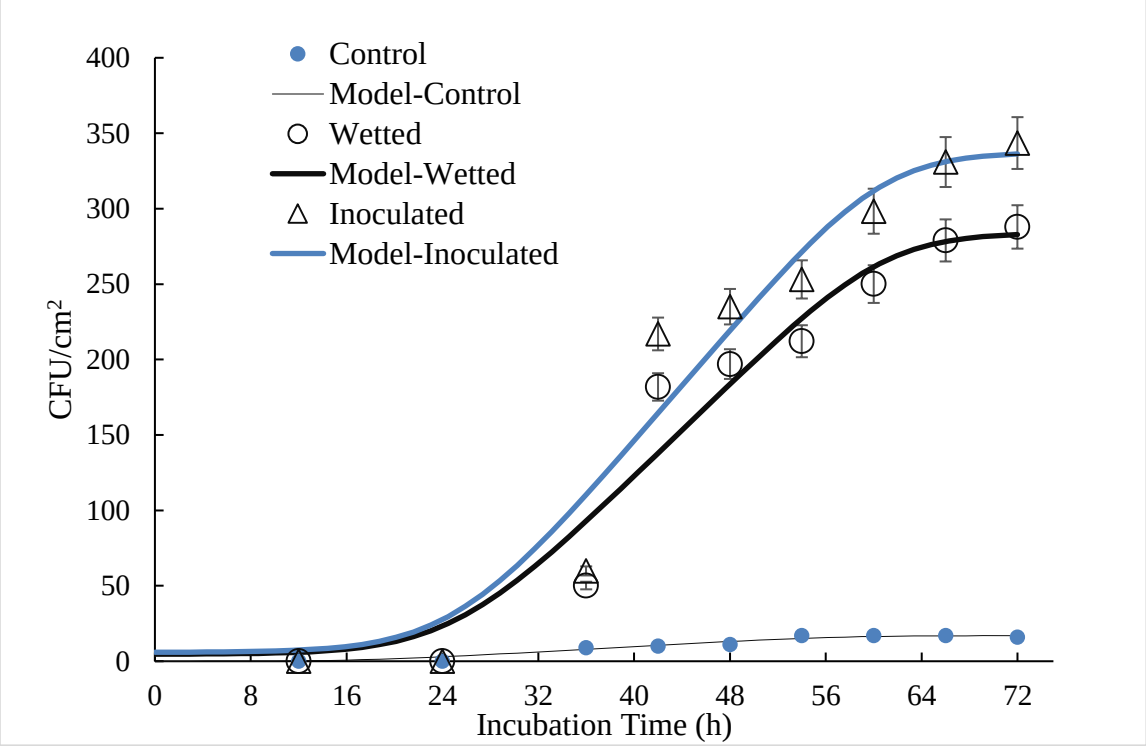

Figure 6. The modeling of predicted values in the microbial growth over 72 hours.

Seed Dimensions and Sphericity

The average major, minor, and intermediate diameter was larger as microbial growth increased. The increase of growth contributed to higher moisture content resulting in the swelling of the seed. The intake of water in to the intercellular spaces of the cottonseed caused the dimensions to get larger. Similar to previous studies, this study shows that there is a positive correlation between moisture content and size dimensions which was reported by Ozarslan (2002) for delinted cottonseed and for rice (Kibar & Ozturk, 2008; Ozarslan, 2002). The sphericity of the seed was not significantly different between the sample groups (Table 3, Figure 7d). This result suggests that the roundness of the cottonseed would not be useful in identifying infected seeds for sorting methods during processing.

The Shapiro-Wilk test indicated that cottonseed major, intermediate, and minor diameters were normally distributed. The growth of *A. flavus* did not have an impact on the sphericity and the shape of cottonseed was not significantly different between the three cottonseed groups. These results show that the dimensions alone cannot indicate that the seed is infected or not. If the cotton ginner can identify the variety of cottonseed and adjust machinery for the desired seeds because cottonseed varies in size, then an implementation of sorting can be successful by adjusting for the typical size for the specific variety. Sieves can help with separating by size of seed and this results from this research suggest larger sized seeds can indicate a high moisture content.

High moisture is associated with higher microbial growth, which may lead to subsequent mycotoxin production. The combination of seed dimensions, moisture content,

and color can indicate the susceptibility of cottonseed being infected. Similar to Nahiemien et al. (2006), we found that values of the sphericity in fuzzy cottonseed were not significantly different with respect to sample groups tested. However, Shi et al. (2017) reported sphericity being statistically significant between moldy and healthy corn kernels. *A. flavus* did not have a significant impact on cottonseed because of the consumption of the fungus is mostly occurring inside the hull which allowed for the seed shape to stay the same. Unlike the cottonseed, *A. flavus* consumption on a corn is on the whole exposed kernel which significantly changes the shape.

In Figure 7, the mean and the distribution of seed size in the control, wetted and the inoculated sample groups are shown. For this specific cottonseed variety, the seed with a major diameter exceeding 10.1 mm could be considered infected and removed from the process stream (Figure 7a). If the contaminated seed lot is poorly sampled and gets to the oil milling facility, then there is a need to be able to separate some and larger seed with high moisture content may be infected. Adding in a sorting step based on dimensions can help eliminate potential infected cottonseed. The point at which the distribution of the control and wetted sample overlap in size indicates an ideal threshold for determining if the cottonseed is contaminated. The major diameter distributions for the two samples intersects around 9.2 mm. Anything above 9.2 mm should be removed from the sample due to the possibility of additional cottonseed being contaminated. Similar results were found with the minor diameter, with infected seeds being larger than 4.9 mm. The intermediate diameter threshold was 5.2 mm (Figure 7b and 7c). Figure 7d shows overlap

of the control and inoculated cottonseed samples, also indicating there was no significant difference in sphericity between the two groups.

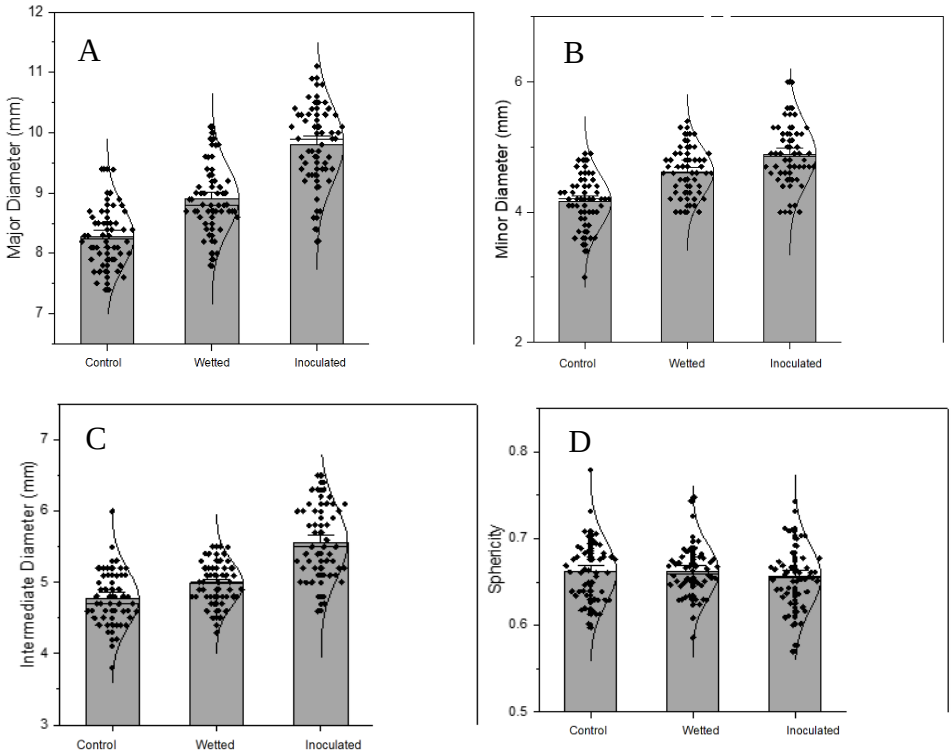

Figure 7. Distribution results showing differences in control, wetted, inoculated sample on major diameter (a), minor diameter (b), intermediate diameter (c), and sphericity (d).

Physical Properties	Treatment				
	Control	Wetted	Inoculated	F Value	Prob>F
Major Diameter (mm)	8.3 [a]	8.9 [b]	9.8 [c]	94.23	4.595E-27
Intermediate Diameter (mm)	4.7 [a]	5.0 [b]	5.6 [c]	50.31	2.266E-17
Minor Diameter (mm)	4.2 [a]	4.6 [b]	4.9 [c]	38.03	4.445E-14
Sphericity	0.66	0.66	0.65	0.510	0.6013
Projected Area (mm^2)	40.53 [a]	47.09 [b]	56.72 [c]	107.6	1.641E-29
Surface Area (mm^2)	234.4 [a]	302.8 [b]	368.4 [c]	66.57	2.598E-21
Mass (g)	0.094 [a]	0.106 [b]	0.106 [b]	4.175	0.017
Volume (cm^3)	0.066 [a]	0.067 [a]	0.080 [b]	9.308	1.562E-4
Density (g/cm^3)	1.433 [a]	1.592 [b]	1.321 [c]	106.7	2.382E-29

Table 3. The average measurements of the dimensions, sphericity, mass, volume, density, surface areas, and projected area for the control, wetted, and inoculated sample groups. [a,b,c] = significant at $\alpha = 0.05$

Projected Area

The average projected area increased with the increase in microbial load. The projected area was calculated from the measured diameters. The increase in the cottonseed's dimensions due to the swelling of the seed which increased the projected area. At the 0.05 level, the population means were different, indicating the possibility of sorting seeds during pneumatic conveying of the seed (Table 3). Cottonseed projected area was normally distributed. Similar results were shown for bare and fuzzy cottonseed, legume seeds, and spinach seeds (Altuntas & Demirtola, 2007; Kilickanm A., Ucer, & Yalcin, 2010; Ozarslan, 2002). Determining design parameters for cleaning and separating agricultural products is essential for processing cottonseed (Ramesh et al., 2015). The inoculated sample has a large distribution for projected area (41mm to 72mm). This value could be used to determine if the sample is contaminated with potentially toxigenic fungal

species. The distribution of the projected area of the seeds showed that for this cottonseed, a projected area greater than 47 mm^2 could be considered infected (Figure 8a).

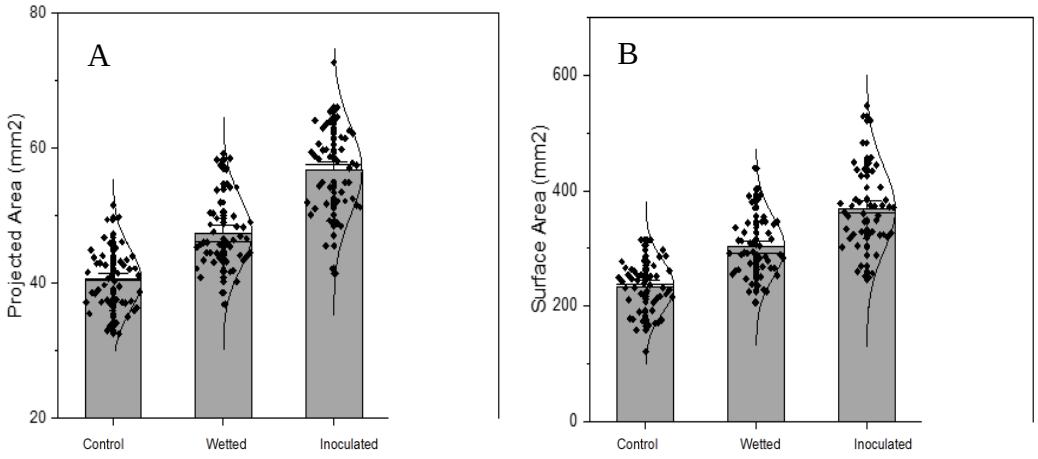

Figure 8. Distribution results showing significant differences in control and inoculated sample on projected area (a) and surface area (b).

Surface Area

Similar to projected area, surface area is dependent on the cottonseed dimensions. As the dimensions increased due to microbial growth and increased seed swelling the surface area also increased in size. Surface area is not used in sorting, but it has potential to be used with estimating with machine vision. Few studies have calculated the surface area of cottonseed. However, we noted the surface area increased with the increase of *A. flavus* growth. Surface area was normally distributed, and treatment means were significantly different. Any cottonseed larger than 300 mm^2 should be considered infected (Figure 8b).

Individual Seed Mass, Volume, and Density

The average individual seed mass was different, but differences were small (Table 3). Individual seed mass did not increase between wetted and inoculated samples even though there was a difference in moisture content and microbial load (Table 3). The Shapiro-Wilk test showed that the data from the control and wetted sample groups for the volume and mass were drawn from a normally distributed population. However, the inoculated sample for the mass and volume was not from a normally distributed population. At the 0.05 level, the control's mass was significantly different from the other two cottonseed sample groups. This result indicates that there is not a possibility of sorting cottonseed based on mass alone similar to results from the study by Cucullu (1977). Table 3 indicates that there were differences between the distribution of wetted and inoculated samples, but not as significant as other physical properties. Similar results were reported for bare cottonseed and flax seed (Coşkuner & Karababa, 2007; Ozarslan, 2002). The inoculated samples have a wide distribution of seed mass (Figure 9a). The majority of the sample mass for the inoculated samples were within the same range as the control samples, below 0.115 g. When a sample mass is greater than 0.115 g it should be rejected as it is likely that it is contaminated with *A. flavus* and aflatoxin that will proliferate in storage, leading to major quality issues.

Similar results were seen for seed volume, since this property is dependent on seed dimensions. With regards to density, the control, wetted, and inoculated sample groups were all from a normally distributed population. The density decreased with the increase in microbial load. In the inoculated sample, *A. flavus* breaks down the protein-rich kernel

inside the hull (Koltun 1973). The significant difference between the inoculated and wetted sample suggest that with the addition of *A. flavus* the density of the cottonseed changed. Even though there were similarities in mass there was consumption of the kernel that decreased the kernel density significantly. The fungal consumption of the kernel led to the increased production of aflatoxin in the inoculated samples. The distribution of the cottonseed's density showed that any cottonseed below 1.4 g/cm^3 would have to be removed by sorting to decrease the chance of future contamination (Figure 9c).

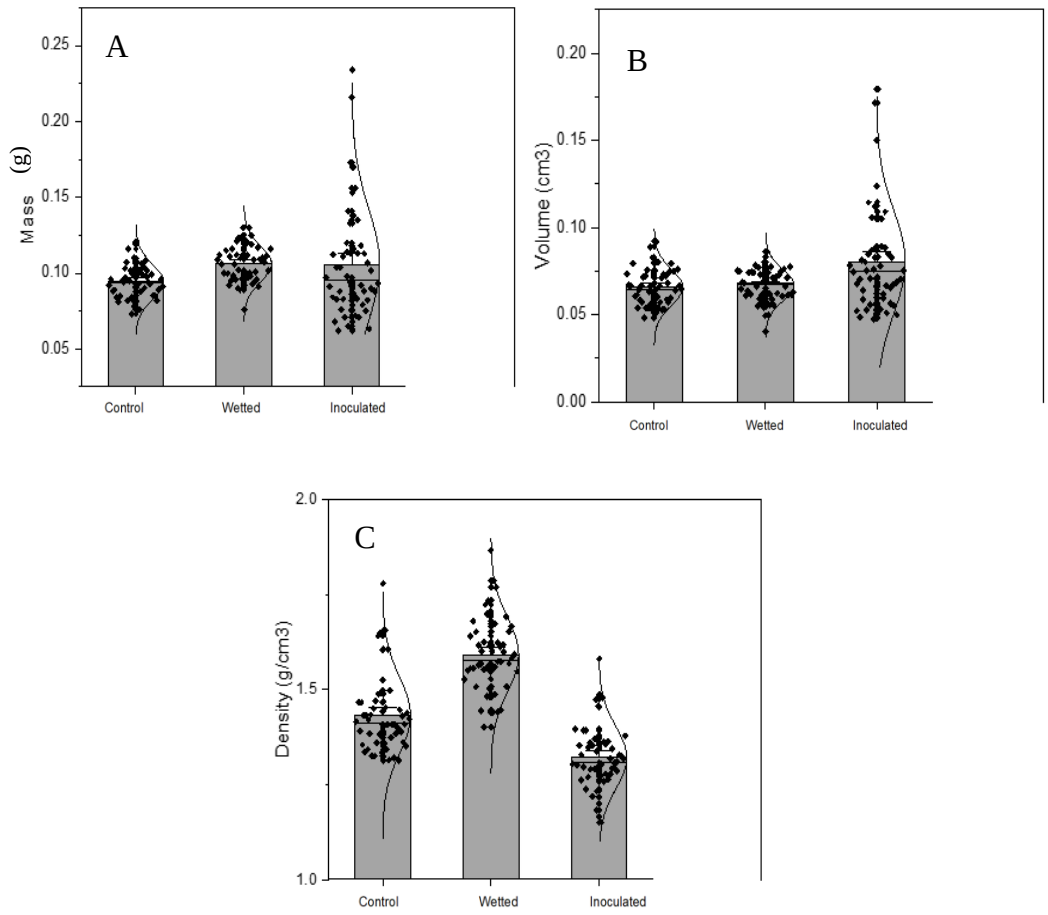

Figure 9. Distribution results showing significant differences in control and inoculated sample on mass (a), volume (b), and density (c).

Conclusions

This study shows the potential of separating according to physical properties cottonseed that can be indicative to microbial load and aflatoxin contamination. Infected cottonseeds could be identified based on physical appearance, dimensions, projected area, surface area, or density mass, volume, and sphericity of the seed would likely not be useful because there were no significant differences between the samples when aflatoxin was introduced. Sorting based on the physical properties could lead to cleaner and healthier feed and allow for the infected seeds to be treated for future use in animal feed production or harvesting. By identifying the differences in physical properties of cottonseed samples with high microbial levels and aflatoxin above the FDA action levels, can also provide for better storage conditions by sorting out infected cottonseed, reducing the instances in storage. The addition of moisture and aflatoxin increased the presence of microbial communities after 21 days of storage at 25℃ with 85% rH in the inoculated sample. Future work from this group will focus on treatment methods to reduce microbial contamination and aflatoxin levels in cottonseed. A recommendation for future studies is to determine what effect *A. flavus* has on cottonseed quality, for example, nutritional composition or tensile strength of the seed. Future studies can lead to understanding the impact of the *A. flavus* of cottonseed physical properties.to make Cottonseed sorting can be more feasible in addition to the other physical properties that were examined in this research study.

CHAPTER IV

INCREASE IN AFLATOXIN LEVELS DURING COTTONSEED PROCESSING

Introduction

Typically, a ton of cottonseed produces around 1440 pounds of cottonseed meal and hulls which ultimately is fed to dairy and beef cattle. Aflatoxin-infected cottonseed can have negative changes in the odor, taste, appearance, and nutritional value (Goldblatt, 1968). Cottonseed provides a good supply of protein, fat, and fiber that is ideal for livestock rations (Osborne & Mendel, 1917). The largest market for cottonseed meal, the dairy industry, is negatively affected by aflatoxin contaminated animal meal (Wu et al., 2008). Aflatoxin levels are tested to ensure the animal feed is within the action levels recommended by the U.S. Food and Drug Administration (*FDA Compliance Guide*, 2019).

For the dry-grind process for dried distillers' grains and solubles (DDGS), studies have shown that mycotoxin accumulation can increase up to three times in the co-products (Bennett & Richard, 1996; Bothast, Bennett, Vancauwenberge, & Richard, 1992; Murthy et al., 2005; Wu & Munkvold, 2008a). Throughout the postharvest processing of cottonseed, aflatoxin levels mostly likely increase when the whole cottonseed is milled into cottonseed meal and the toxin is now highly concentrated. Aflatoxin contamination can be a problem in crushing cottonseed due to infected cottonseeds' susceptibility to breakage, further contamination of end products, and toxicity to the consumer.

The cottonseed crushing process consists of cleaning, delinting, hull removal, kernel flaking, oil extraction, and meal formation. Cleaning consists of removing materials

such as leaves, stems, and dirt. Delinting either uses acid for complete removal of linters, or is mechanically done, leaving the remaining percent by weight is 1-2% (Holt et al., 2017). If the cottonseed is being used only for oil and meal, seed is mechanically delinted, which is less dangerous for workers because there are no hazardous chemicals used (Delouche, 1986). After the seed is delinted, the hulls and kernels are separated by friction and sieves. If the cottonseed is being used only for oil and meal, seed is mechanically delinted, which is less dangerous for workers because there are no hazardous chemicals used (Delouche, 1986). After the seed is delinted, the hulls and kernels are separated by friction and sieves.

The hulls may be blended with cottonseed meal which offer advantages in transportation, ease of handling, and protein content (Blasi & Drouillard, 2002). In industry, the hulls and kernels are sold separately as well. Following dehulling, the kernels are flaked (flattened) and then the flakes are placed in a solvent for the extraction of the oil. The use of extrusion, applying high heat and pressure following oil extraction can decrease aflatoxin levels by 50%, with the increase in passes through the extruder (Buser & Abbas, 2001). After four passes through the extruder, aflatoxin levels decreased by 23% each pass. Even though there was a decrease in aflatoxins, valuable time was wasted by increasing the number of passes, high aflatoxin levels were still present. The are many reports on using extrusion to reduce aflatoxin levels in cottonseed meal, but many of the processes are conflicting in the optimal pressure and heat needed during extrusion (Buser, 1999). There is a need to accurately see how much of an increase in aflatoxin levels can occur when processing cottonseed. This information leads to accurately determining how

much aflatoxin will be present after processing. The meal can then be treated using to reduce aflatoxin levels based on the starting amount without the need for multiple passes through an extruder.

Other than meal, cottonseed can be processed for the use of planting for the next harvest season. There is a great importance in having seed that can easily flow through planting machinery because fuzzy seeds can result in clumping throughout the planting process which results in the use of sulfuric acid to delint the seeds (*The Encyclopedia of Seeds: Science, Technology and Uses,* 2006). The remaining cottonseed not being used for replanting will be used for meal. Previous research has shown the impact of mechanical processing on aflatoxin levels, no research has been shown on how acid delinting will affect aflatoxin levels.

Typically, cottonseed meal is tested for crude protein, fat acidity, and aflatoxin levels. Cottonseed meal has similar protein degradability to soybean meal and is important for muscle growth in cattle. Fat acidity levels are measured because it serves as an indication of quality loss due to cottonseed deterioration (White, 2000). Proper storage and reduction of moisture content usually contribute to lower fat acidity levels. Previous studies have shown a positive correlation between aflatoxin levels and free fatty acid content (Bulaong & Dharmaputra, 2002). Fat acidity levels increase because of the breakdown of fats by *A. flavus.*

Evaluating the concentrated amount in cottonseed meal can help in predicting if cottonseed meal will be below FDA action levels. Understanding the effect of acid delinting on cottonseed aflatoxin levels can show impact of different processing methods.

There have been no studies that measure the increase of aflatoxin levels when concentrated to cottonseed meal. This study quantifies the increase in aflatoxin concentration throughout crushing based on properties of the whole cottonseed. The objectives of the research was to 1) quantify the increase in aflatoxin levels throughout the post-harvest processing by analyzing the whole seed and the ending cottonseed meal, 2) record the differences in aflatoxin levels and microbial load in different cottonseed processing, and 3) observe the changes in fat acidity levels and crude protein content in aflatoxin infected cottonseed.

Materials and Methods

Clean fuzzy cottonseed was obtained from the Cotton Gin Lab on Texas A&M University campus. For this study, 3 kg of cottonseed was stored at ambient temperature. Initially, the moisture content was determined by oven drying three 5g samples of cottonseed at 105 ± 2 ℃ for 14.5 hours (Griffin Jr, 1980). The initial moisture content of the seeds sampled was determined to be 7.9% (d.b.). The cottonseed was separated into two 250 grams of fuzzy cottonseed that would be mechanically and acid delinted which each included control, uninoculated (16.3%), and the aflatoxin inoculated (21.9%) sample group. A concentration of 9.6×10^4 CFU/g of *Aspergillus flavus* (Carolina Biological Supply Company, Burlington, NC) was concentrated into Triton X-100 solution and Aflatoxin B_1 (150 ppb) dissolved in DMSO (Aflatoxin B1 5MG, Sigma Aldrich, Darmstadt, Germany) *A. Flavus* and aflatoxin B1 was added to the inoculated sample by spraying then mixing every 30 minutes for 3 hours.

The uninoculated and inoculated sample were then placed in the relative humidity and temperature chamber (Percival Scientific, Inc., Perry, IA) at 25℃ with 85% relative humidity (rh) for 21 days which ensured that any presence of toxigenic *Aspergillus flavus* had the opportunity to metabolize into aflatoxin (Ellis, Smith, Simpson, Ramaswamy, & Doyon, 1994a).

Acid and Mechanical Delinting Process

In a 100ml beaker, 75ml of dilute 10% sulfuric acid and a Triton X-100[3] (surfactant), at .15% by volume was added to the acid to ensure complete wetting of the fuzzy cottonseed for acid delinting. One hundred grams of fuzzy cottonseed of each sample group was placed in sterile stainless-steel bowls with the acid solution and stirred for three minutes with a wooden spoon. The acid was then neutralized by 100 ml 1:1 sodium bicarbonate/water solution and washed 4 times with deionized water. Following delinting, seeds were dried in an oven for 4-6 hours at 105 ± 2 ℃.

One hundred grams of fuzzy cottonseed of each sample group was dehulled in a rice dehuller to get mechanically delinted and dehulled cottonseed. The processing rate was 20g/min and the products of the dehulling were separated by using a size 30 sieve (0.600 mm openings). The acid delinted cottonseed was dehulled by hand.

After the hulls, kernels, and fuzz were separated for each process, the kernels were flaked in order to maximize oil extraction. A modified barley mill was used to flake the seed (Figure 10). After the flaking process, the flakes were cooked at 190 °F (87.8 °C) for 120 minutes (O'Brien et al., 2005).

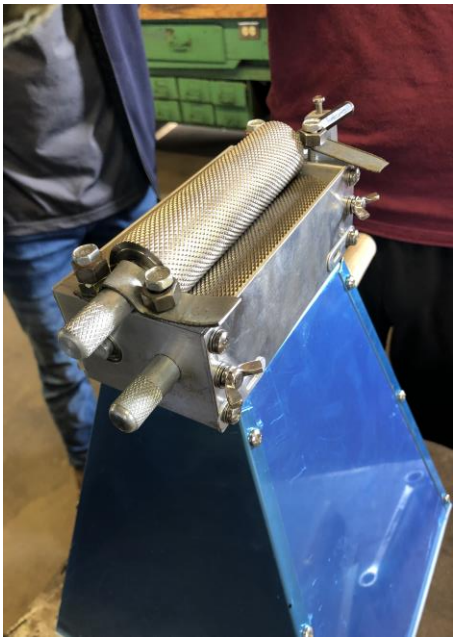

Figure 10. The modified barley mill was used to flake the whole kernels.

Meal Retrieval from Oil Extraction

For oil extraction, 375 ml of 95% ethanol was added to 25.0g of flaked cottonseed meats, corresponding to a solvent to solid ratio of 15:1 (Saxena, Sharma, & Sambi, 2011). The flaked kernels and extraction solvent were stored at 4 C for 24 hours. The oil solvents were filtered out of the solids and the meal was placed in the oven at 105 ± 2 ℃ for 2 hours.

Microbial Analysis

The number of colonies was counted for each sample group to measure the colony forming units (CFUs). Immediately after removing the wetted and inoculated sample from relative humidity and temperature chamber, 5g from each sample group was removed and placed in a sterile 15ml tube with 8.3 ml of 0.05% Triton X-100 solution. The samples were then shaken for 2 minutes. For each sample group, 100 µL of the wash was plated

onto 100 mm × 15 mm sterile Petri-dishes containing Potato Dextrose Agar (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany). Three replicates for each sample group were prepared and plated.

Aflatoxin Levels

To measure aflatoxin levels the Envirologix Aflatoxin Flex kit (Envirologix Inc., Portland, ME)) was purchased and used for this experiment. The Envirologix Aflatoxin Flex kit is approved by the Association of Official Analytical Chemists (AOAC) and the Grain Elevator and Processing Society (GEAPS). Twenty-five grams of cottonseed was removed from each sample group, ground and tested in triplicate. The ground cottonseed was combined with 100ml of 50% ethanol and the elution buffer pouch. After the sample was vigorously shaken for 2 minutes and filtered with an approved coffee filter, 100µl of the liquid was obtained and added to 100µl of the Envirologix kit's DB5 buffer. A test strip was added for 5 minutes and the color change of the test strip was determined by the Envirologix system color scanner with the minimum detection of 1ppb (QuickScan, Envirologix Inc., Portland, ME). The whole fuzzy cottonseed and the meal were analyzed for control, uninoculated, and inoculated sample groups.

Protein and Fat Acidity Analysis

Protein content was determined by combustion. Each cottonseed sample group was analyzed three times. The whole fuzzy cottonseed seed and meal was weighed (~2 mg) and analyzed for nitrogen on an Elementar vario MICRO analyzer (St. Joseph, MI). Oxygen was used for quantitative combustion via injection. The combustion tube temperature was 1,100℃, reduction tube temperature was 800℃, and the temperature of

the Temperature Programmed Desorption (TPD) trap column was 59℃. A conversion factor of 5.3 was used to convert nitrogen into protein, derived from reported cottonseed meal amino acid profiles (Jones, 1931).

Fat Acidity values were measured by titration according to AACC standards 02-02 (AACC, 1995) and expressed as the milligrams of potassium hydroxide (KOH) required to neutralize the free fatty acids from 100 g of cottonseed (mgKOH/100g). The method involved extracting free fatty acids from milled cottonseed using purified toluene and titrating with a CO_2 free standard solution of 0.0178N KOH. The reported titration values are an average of the three replications for each sample group.

Statistical Analysis

Statistical analyses were performed using Origin software (OriginLab, Northhampton MA). One-way ANOVA, Shapiro-Wilks, and Tukey Test was used to statistically analyze sample groups to determine the differences in means and the normality of the data between the control, wetted, and inoculated sample groups. The one-way ANOVA statistically analyzed if the groups had significantly different means when the p-value is smaller than 0.05.

$$F = \frac{\Sigma n_j \left(\overline{X}_j - \overline{X}\right)^2 / (k-1)}{\Sigma\Sigma \left(\overline{X} - \overline{X}_j\right)^2 / (N-k)}$$

Where F is the degrees of freedom, n_j is the sample size, j^{th} group, $\overline{X}_J$ is the sample mean in the j^{th} group, and $\overline{X}$ is the overall mean.

The Shapiro-Wilk determines if the sample data has been drawn from a normally distributed population.

$$W = \frac{\left(\sum_{i=1}^{n} a_i x_{(i)}\right)^2}{\left(\sum_{i=1}^{n} x_i - \bar{x}\right)^2}$$

Where x_i is the order random sample values and a_i are constants generated from the covariances, variance, and means of the sample from a normally distributed sample. The Tukey Test compares the means and signifies if the sample groups are significantly different from each other.

$$HSD = \frac{M_i - M_j}{\sqrt{\frac{MS_w}{n_h}}}$$

Where $M_i - M_j$ is the difference between the pair of the means to calculate this M_i should be larger than M_j. MS_w is the mean square within, the n is the number in the group.

Results and Discussion

Microbial Load

The mean values of CFU's of the acid-delinted whole seed were 70, 1.3×10^3, and 7.0×10^5 CFU/ml for the control, uninoculated, and inoculated sample groups, respectively. The mean values of the mechanically-delinted whole seed which were 66, 1.7×10^3, and 7.3×10^5 CFU/ml for the control, uninoculated, and inoculated sample groups, respectively. The CFU initial population size was similar in each sample group for the two different delinting processes, so differences in microbial counts is due to the treatments. The inoculated whole cottonseed samples were significantly different from the other two whole seed sample groups for both processes. The acid-delinted inoculated

whole seed was also significantly different than the mechanical-delinted whole cottonseed sample.

The mean of colony forming units for acid-delinted cottonseed meal had the averages of 2.6×10^{3} CFU/ml, 3.5×10^{5} CFU/ml, and 5.16×10^{9}CFU/ml for the control, uninoculated, and inoculated cottonseed meal sample. There was a 2-log increase in the control and uninoculated whole cottonseed to the cottonseed meal. There was a 4-log increase in the inoculated sample group when the whole seed was milled into cottonseed meal (Figure 11). Since the colony forming units were higher in the inoculated sample initially it was to be expected that the meal would be higher than the control and uninoculated sample groups.

Similarly, the colony forming units for the mechanically-delinted cottonseed meal had the averages of 2.9×10^{3} CFU/ml, 4.5×10^{7} CFU/ml, and 1.48×10^{10} CFU/ml. Similar to the acid-delinted control sample, there was a 2-log increase from the control whole cottonseed to the cottonseed meal. From these results we can conclude, that at minimum there will be a 2-log increase in microbial growth when cottonseed is being processed. There was a 4-log increase in the mechanical-delinted wetted sample. This indicates that the acid affected the microbial growth, but did not completely stop the microbial growth of *A. flavus.* There was a 5-log increase in the inoculated whole cottonseed to the cottonseed meal. The increase between the uninoculated and the inoculated sample was different due to the higher initial concentration of *A. flavus* being added to the inoculated sample.

In figure 11, there was a 10-log difference from the mechanical delinted control whole seed sample to the inoculated mechanical delinted cottonseed meal. The Shapiro-Wilk test showed that for the acid delinted whole seed, mechanical delinted whole seed, acid delinted cottonseed meal, and the mechanical delinted cottonseed meal all showed that the data was significantly drawn from a normally distributed population for the control, wetted, and inoculated sample groups. At a 5% level of significance, the inoculated cottonseed meal was significantly different for each sample group for both delinting processes. The delinted whole seed for both processes were significantly different than the meal in for the control, uninoculated, and inoculated sample group. The conclusions from the difference in microbial load in cottonseed sample groups indicates at minimal the cottonseed farmer should expect a minimum of a 2-fold increase in microbial growth in samples.

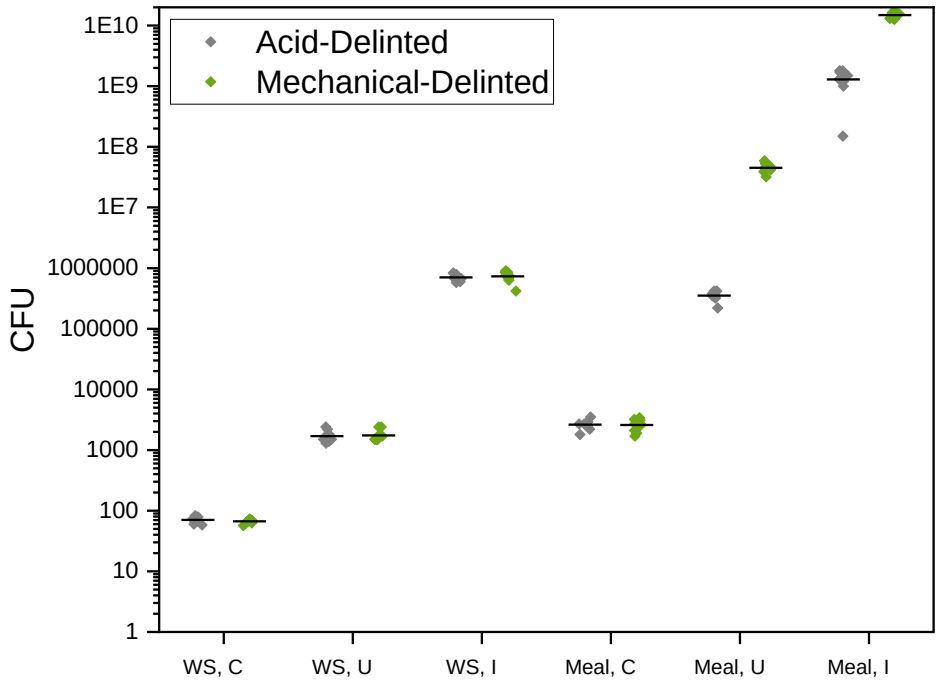

Figure 11. The average colony forming units for the acid-delinted and mechanical delinted whole seed (WS) and meal for the control (C), uninoculated (U), and inoculated (I) sample groups.

These results are similar to a previous research study studying the effects of the milling process on the microbial load of products milled from various wheat varieties (Sabillón Galeas, 2014). There was an increase in mold by 0.74 log CFU/g, indicating the impact of processing and the concentrated microbial growth. Another study showed an increase of microbial load yam flour in Nigeria. This research did not only show that there was increase in microbial load throughout the milling process, but there was a significantly higher microbial count in the commercial milled product versus the laboratory milling process (Somorin, Bankole, Omemu, & Atanda, 2011). Even though this research experiment focused on laboratory-scale milling processes, commercial milling will likely affect the microbial count on cottonseed.

Aflatoxin Levels

No aflatoxin was found in the control or uninoculated sample, which was expected since they were not intentionally inoculated. There was aflatoxin detected in the inoculated whole seed and meal. There was a 3-fold increase in aflatoxin content in the acid-delinted seed compared to the 4-fold increase that occurred in the mechanical-delinted meal (Figure 12). Consistent with microbial count data, there was higher aflatoxin level in cottonseed meal that was mechanical-delinted. The sulfuric acid eliminated some of the aflatoxin content and microbial load, but not entirely. The mechanical-delinted samples had fuzz in the meal, which could have aflatoxins attached to the fibers, leading to an increase in concentration.

With the inoculated sample, for both delinting methods, the hulls had a lower concentration of aflatoxin, compared to the kernels. Similar results were found in peanut kernels, aflatoxin contamination was present in the hulls when the peanuts were machine processed (Blankenship, Cole, Sanders, & Hill, 1984). Aflatoxin levels were shown previously to be higher in the kernels than the hulls, with meats containing 10,200 ppb, compared to 390-ppb in the cottonseed hulls (Whitten, 1970). These similar results show that the removal of hulls does not completely eliminate aflatoxin contamination and the remaining meal concentration will still be more concentrated. In this laboratory experiments, the hull material has been shown to be less of a consumable substrate for *Aspergillus flavus*, but there are still aflatoxins present on the hulls. The inoculated cottonseed kernels had higher values than the hulls. Aflatoxins are higher in the meal when

compared to whole kernels because the aflatoxins wont dissolve until the kernel is crushed. These results can have impact on detoxification practices by showing the best time to treat infected cottonseeds after the crushing process has taken place. Similar to distiller's grains, the processing of cottonseed can lead to an increase aflatoxin concentration. A processing diagram was developed and adapted from previous research showing the modeling of mycotoxins in ethanol co-products (Figure 12) (Wu & Munkvold, 2008b). This model can be further researched by an economist to analyze the loss of feed and animals due to aflatoxin infected cottonseed meal.

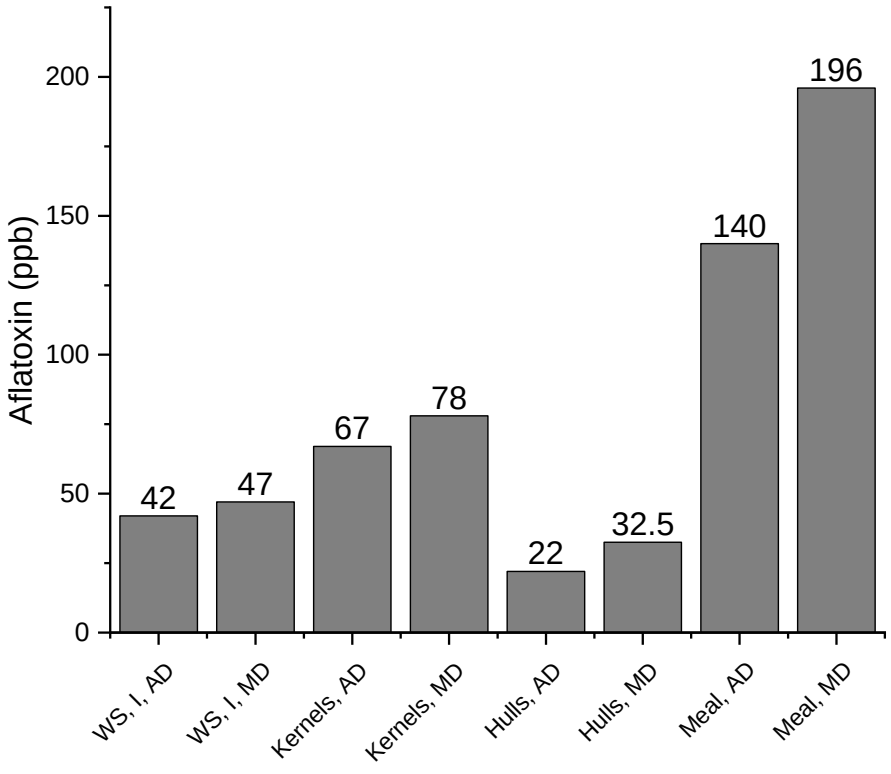

Figure 12. The aflatoxin content in the acid delinted and mechanical delinted whole cottonseed and cottonseed meal. The inoculated sample's hulls and kernels were also tested.

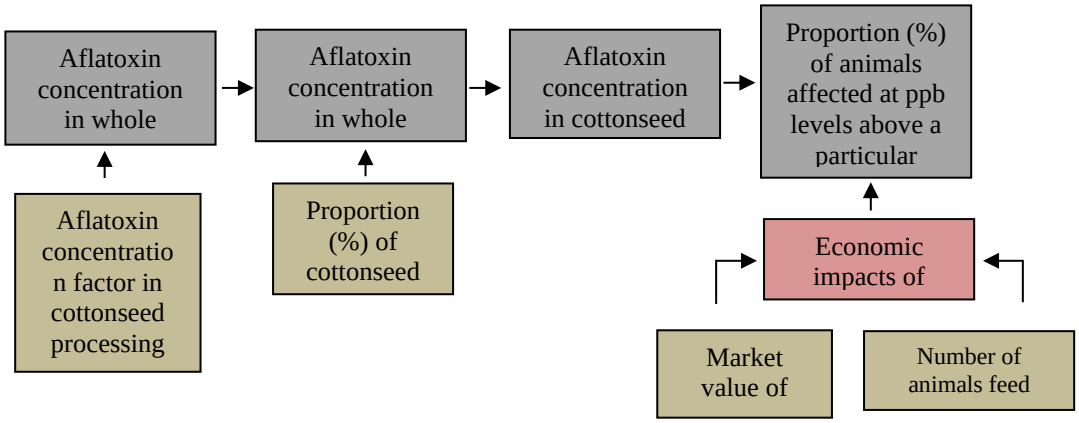

Figure 13. Factors influencing economic impacts on livestock industry of aflatoxins in cottonseed. This figure was adapted from distiller's grains article (Wu & Munkvold, 2008b)

Protein Levels

As expected, the protein content became more concentrated during the milling process. The acid-delinted whole seed average protein content levels were 20 %, 19%, and 13% for the control, uninoculated, and the inoculated samples, respectively. Similarly, the average protein content was 22%, 19%, 13% in the mechanical delinted whole cottonseed for the control, uninoculated, and the inoculated samples, respectively. The protein content decreased due to the presence of microbes on the uninoculated sample and *A. flavus* on the inoculated sample. The consumption of the kernels occurred which resulted in differences in the protein content among the three sample groups. The protein content loss was greater in the inoculated sample because there was a higher concentration of microbes, compared to the uninoculated sample. protein concentration can increase the microbial load in oilseeds (Mellon & Cotty, 1998; Stuart, 1940). When the ANOVA test was run at level 0.05 there was a significant difference in population means (Prob>F, 3.060E-33).

The protein content increased in the acid delinted meal which was 38%, 27%, and 18% for the control, uninoculated, and inoculated sample groups, respectively. Similarly, the mechanical delinted meal increased in protein content resulting in 35%, 28%, and 26% from the control, uninoculated and inoculated sample groups. There was higher concentration in the mechanical delinted inoculated group which was unexpected. In Figure 14, the distribution of the mechanical delinted meal protein content was and this result suggests that an increase in aflatoxin content and microbial load may cause more variability in protein content within a sample. This result indicates the importance of sampling variation which has been shown to be the largest area for determining mycotoxins with food commodities (Coker et al., 1995). Studies have shown skewed results in large amounts of feed when sampling due to the distribution of aflatoxins among the sample (Mallmann et al., 2014). There was a decrease in protein content in the meal when the microbial load was increased (Figure 14). There was little effect observed from the different delinting processes and the change in protein content was due to the presence of microbial growth. Cottonseed meal serves as an ideal substrate for Aspergillus flavus digestion.

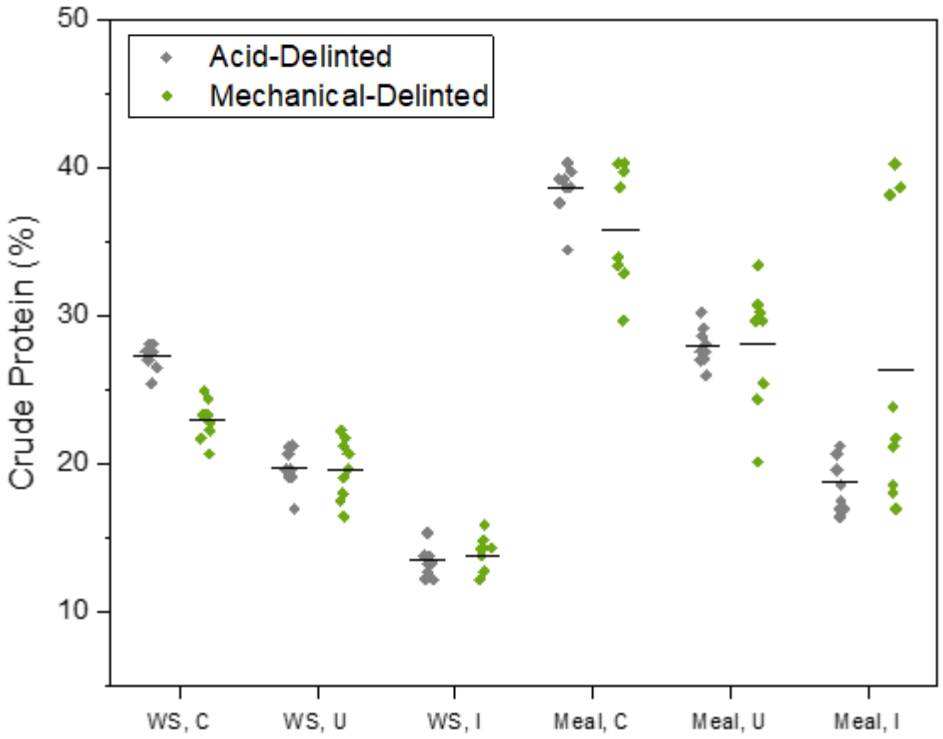

Figure 14. The average protein content for the acid delinted and mechanical delinted whole seed and meal for the control, uninoculated, and inoculated sample groups.

Fat Acidity Levels

The fat acidity levels decreased during the milling process due to the oil being extracted. The acid delinted whole cottonseed average free fatty acid content levels were 1.16%, 2.4%, and 9.1% for the control, uninoculated, and the inoculated samples, respectively (Figure 15). Similarly, in the mechanical delinted whole cottonseed the average free fatty acid content was 1.23%, 3.13%, 11.63% for the control, uninoculated, and the inoculated samples, respectively. The fat acidity levels were higher in the sample groups that had higher microbial growth. The fat acidity levels decreased when whole cottonseed was milled. The acid-delinted meal had fat acidity levels 0.83%, 1.36%, and

2.50% for the control, uninoculated, and inoculated sample groups, respectively. Similarly, the mechanically-delinted meal increased in fat acidity levels resulting in 0.80%, 1.63%, and 2.86% from the control, uninoculated and inoculated sample groups. In Figure 15, the differences in fat acidity levels were due to the presence of microbial growth. *A. flavus* has been shown to be a lipase-producing species which can contribute to the deterioration of agricultural products with high lipid concentrations (Sarıyar & Heperkan, 2003).

This increase in fat acidity levels in the meal with higher microbial load has also been observed in hazelnuts, corn, wheat, peanuts, and soybeans (Liu et al., 2016; Sarıyar & Heperkan, 2003). There was little effect observed from the different delinting process and the change in fat acidity levels was due to the presence of microbial growth. When the ANOVA test was run at level 0.05 there was a significate difference in population means (Prob>F, 2.262E-5). The Shapiro-Wilk test showed that the data from was drawn from a normally distributed population. Previous research has shown that higher microbial load have been shown to have a positive correlation protein levels and fat acidity levels in rapeseed (Stepien, Wojtkowiak, & Pietrzak-Fiecko, 2017).

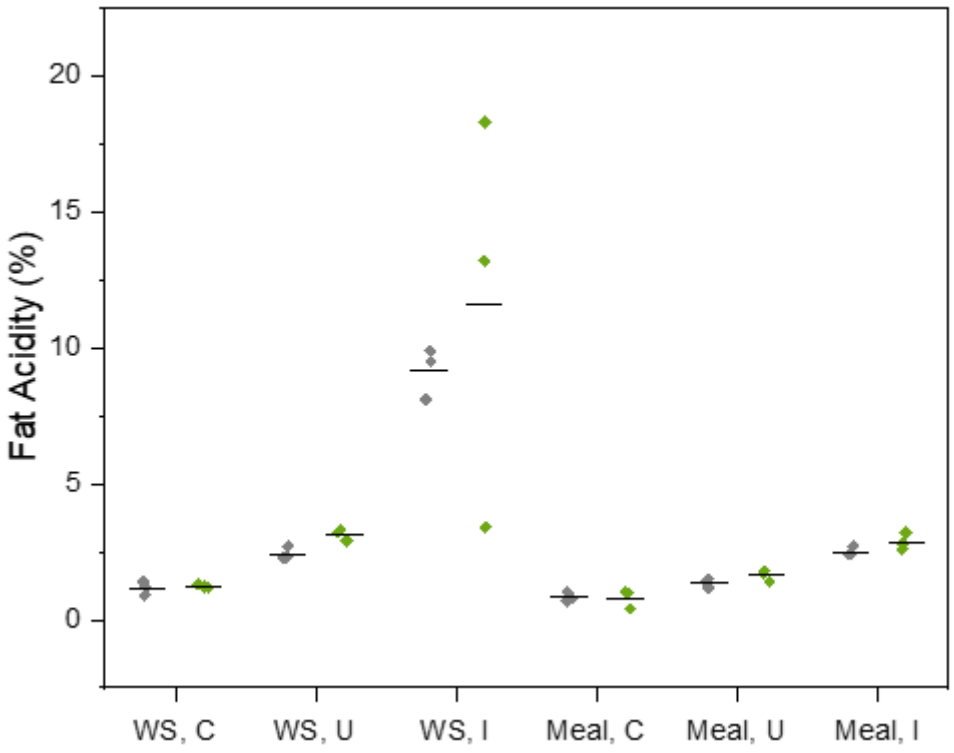

Figure 15. The average fat acidity levels for the acid-delinted and mechanical-delinted whole seed and meal for the control, uninoculated, and inoculated sample groups.

Conclusions

This research shows the effects of microbial growth and aflatoxin content on nutritional components, free fatty acids and protein levels, of cottonseed. Similar to other studies on grains, the protein content and fat acidity levels were affected due to the presence of microbes, including *A. flavus*. When aflatoxin levels and microbial concentration increased, there was a decrease in protein content in the cottonseed meal and an increase in fat acidity levels. There were also significant differences in the effects of acid-delinting and mechanical-delinting and its effect on aflatoxin content and

microbial growth. Acid-delinted cottonseed meal had lower microbial load than the mechanical-delinted cottonseed meal. Higher microbial load contributed to lower protein concentration and higher fat acidity levels. These nutritional components were changed because the microbial growth played a role in consuming the cottonseed meal. Future studies can look at the effect of commercial processing of cottonseed meal to determine if there is more growth associated with large production-scale equipment. This study represents a starting point for determining changes in microbial load and aflatoxin levels throughout processing.

CHAPTER V

HIGH VOLTAGE ATMOSPHERIC COLD PLASMA TREATMENT EFFECTS ON MICROBIAL LOAD ON COTTONSEED MEAL

Introduction

Contamination of cottonseed with aflatoxins can lead to economic losses because availability for oil production and utilization as a feed is reduced. For cotton production, growing seasons with high rain totals often come with high aflatoxin levels. Aflatoxins are mycotoxins produced by the common fungi, *Aspergillus flavus*, under specific temperature and moisture conditions (Filazi & Tansel, 2013). Improper storage of cottonseed can lead to increased microbial and insect activity, thereby reducing quality of the product (Hams & Ayres, 1977; Kumar & Kalita, 2017; Pitt & Hocking, 2009). Aflatoxin B1 is categorized as a group I carcinogen by the World Health Organization (Marchese et al., 2018). The amount of aflatoxin content in cottonseed used as a feed has a direct impact on livestock through acute toxicity, reduced growth rates and weight, and immunosuppression at low doses (Park, 2002). Better management and treatment practices will allow us to protect our cottonseed supply from post-harvest losses due to aflatoxin contamination. One strategy is to identify treatment methods that can reduce mycotoxins and microbiological species.

There is a need to set up control and treatment methods for post-harvest protection to reduce the risk of spreading aflatoxin content within the cottonseed feed. There have been previous treatments which include thermal, chemical, and biological treatments for

A. flavus and the subsequent production of aflatoxin. Thermal inactivation of microbial and mycotoxin production are done by boiling the infected seeds in aqueous solutions (Park, 2002). Even though aflatoxins are typically destroyed in the process, there are studies that show that the *A. flavus* spores restart developing because of the acidification of the cottonseed product (Price & Jorgensen, 1985). Chemical treatments including ammoniation have been shown to alter the effects of *A. flavus*, but there is an increase in meal toxicity in dairy cattle (Smalley & Bicknell, 1982). Biological treatments, including atoxigenic strains of *A. flavus* were created to block to infection of the toxigenic strain of *A. flavus*. A study showed that an atoxigenic strain, NRRL 35739, had slower growth which did not allow for it to out compete the toxigenic strain (Pennerman, Yin, Bennett, & Hua, 2019). There are other atoxigenic strains that work well, but in the long term there is still mold development occurring which can have a negative impact during storage. Other non-thermal treatments such as ozone have been shown to be effective, but it can have treatment effects on chemical components (Pandiselvam et al., 2019). Ozone is considered as a toxic air pollutant and can cause problems in the human respiratory tract (Mudway & Kelley, 2000). In comparison, Atmospheric Cold Plasma (ACP) is not expected to have a negative effect on nutritional quality of food, although one challenge in the food industry is upscaling the system (Mandal, Singh, & Pratap Singh, 2018). There are few commercialized cold plasma treatment systems, although there are many patented designs (Weltmann et al., 2018).

The non-thermal, chemical free treatment has shown great potential on a laboratory scale and combines UV radiation, ozone, free radicals, charged particles, oxygen radicals,

and other reactive gas species (Lacombe et al., 2015). ACP is created by a dielectric barrier discharge (DBD) at high voltage and low amperage between two electrodes separated by insulating dielectric barriers (Pankaj et al., 2014). In-package treatments have been used with the DBD to inactivate spoilage microorganisms (N. N. Misra et al., 2016). Polypropylene is a polymer food packaging product that is low density, low cost, and has a high melting point(Pankaj & Keener, 2017). Food packaging materials are used in order to protect commodities from deterioration, damage or outside contamination (Pankaj et al., 2014). The combination of commodity, packaging and fill, which includes air, O_2, CO_2, N_2, or He, can help decrease microbial load (Connolly et al., 2013).

Antimicrobial effects occur through direct attack on microbial structures, such as cellular envelopes, DNA, and proteins (Colagar, Sohbatzadeh, Mirzanejhad, & Omran, 2010; Dasan et al., 2016; Selcuk, Oksuz, & Basaran, 2008; Song et al., 2009). Gases within the plasma field are ionized, producing reactive gas species capable of sterilization (Figure 16). ACP has proven to be effective in treating Aspergillus *spp.* in hazelnuts, peanuts, pistachios, tomato seeds, wheat, barley, oat, rye, maize and lentils (McClurkin-Moore et al., 2017; Surowsky et al., 2013). The use of ACP provides a novel way to continuously treat cottonseed meal to protect the commodity from *Aspergillus flavus* which lead to post-harvest loss. ACP is not only a surface decontaminate; it has been shown to penetrate into tomato seeds (Zhou, Huang, Yang, & Chen, 2011). The potential of ACP is promising to decontaminate infected cottonseed.

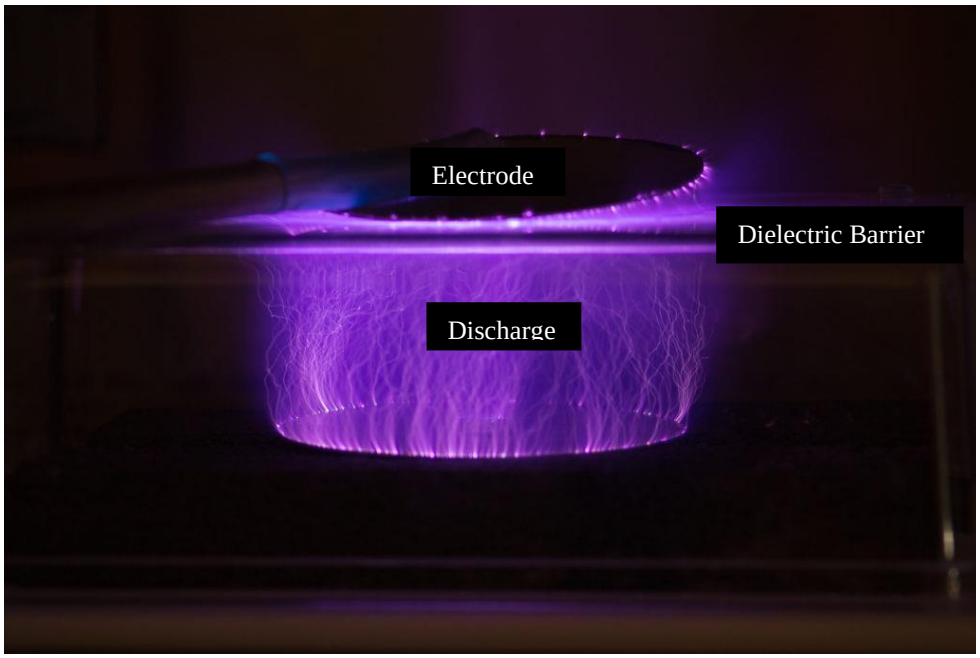

Figure 16. Dielectric barrier ACP system (Photo by Jessica Rae Spence).

There has been scarce economic analysis, but a few have shown that the technology can be affordable when atmospheric air is used instead of expensive Nobel gases. The inclusion of equipment design and recurrent cost (power and inducer gas) will help estimate the operational cost (Muhammad et al., 2018).The rise in wattage consumed from the laboratory scale to industrial scale will be in regards to the size and capacity of the plasma equipment being used. The plasma system has been estimated to have the cost of power consumption in kWh as $0.05. Implying that for every 1000 hours of operation, approximately $4500 for the electricity cost (Niemira, 2012). Sorting infected seeds prior to treatment can help decrease the amount of cottonseed that need to be treated so the cost for cottonseed post-harvest treatment is feasible for decreasing microbial load.

The objectives of this research were to 1) determine treatment parameters for using ACP to treat in-packaged cottonseed meal samples and 2) observe the effects of ACP on physical changes of cottonseed meal including fat acidity levels, crude protein levels, microbial load, and water activity of 28 days. This research can be implemented on a larger scale with minimal operation cost for cottonseed processors. The treatment should implement gyration and larger scale dielectric barriers to increase the discharge area. The movement will allow for the cottonseed meal surfaces can be treated.

Materials and Methods

Raw Material

Clean, mechanically-delinted cottonseed was obtained from the United Ag-El Campo site. For this study, 30 kg of cottonseed was stored at ambient temperature. Initially, the moisture content was determined by oven drying three 5g of cottonseed at 105 ± 2 ℃ for 14.5 hours (Griffin Jr, 1980). The initial moisture content of the seeds was 8.3% d.b. The sample was milled by using a commercial hammer mill with a processing rate of 15kg/h (Great Wall Instruments, N.A.)

Plasma Operating Parameters

The dielectric barrier system used an AC Dielectric Test set, model number 6CP120/60.75 (Phenix Technologies, Accident, MD). The output voltage could be varied from 0-70 kilovolts with 125 milliamperes current output.

Packaging Description and Treatment Parameters

A polypropylene bag with the dimensions of 9.5 in. by 10 in. was filled with 150g of cottonseed meal. The bag was filled with modified atmospheric packaging (MAP) gas

(5% C_2, 65% O_2, 30% N_2) until the bag reached a height of 30 mm. The bag was then sealed and placed between the dielectric barriers, which had a gap of 50.0 mm.

In order to test the efficacy of the ACP system, the use of methylene blue discoloration. Methylene Blue decolorization was used to quantify ionizing species produced during the treatments using ACP. Methylene Blue is an indicator of the oxidation effect occurring within the packaged sample (Ehrampoush, Moussavi, Ghaneian, Rahimi, & Ahmadian, 2011). The importance of this test shows the greatest discoloration percentage which correlates to the most effect voltage and time to treat the commodity. In addition to the methylene blue test, the initial testing parameters including voltage and time were determined by treating 5 g of *Aspergillus flavus* inoculated cottonseed meal with 50 kV for 3 minutes, 50 kV for 5 minutes, 70 kV for 1 minute, 70 kV for 3 minutes, and 70 kV for 5 minutes. The testing was done in triplicates, a total of 15 treatments and the samples were stored for 24 hours in 4℃. The treatments were then washed and observed for microbial growth.

Product Characteristics

Three sample groups were prepared which included the control, inoculated, and ACP-treated meal. A concentration of 1.18×10^5 CFU/g of *Aspergillus flavus* (Carolina Biological Supply Company, Burlington, NC) was concentrated into Triton X-100 solution and Aflatoxin B_1 (250 ppb) dissolved in DMSO (Aflatoxin B1 5MG, Sigma Aldrich, Darmstadt, Germany) *A. Flavus* and aflatoxin B1 was added to the inoculated and ACP-treated 5 kg sample group by spraying then mixing every 30 minutes for 3 hours. The inoculated and ACP treated sample groups were placed in the relative humidity and

temperature chamber (Percival Scientific, Inc., Perry, IA) at 25℃ with 85% relative humidity (rH) for 21 days which ensured that any presence of toxigenic *Aspergillus flavus* had the opportunity to produce aflatoxin (Ellis, et. al., 1994). Triplicates were done for each sample group tested. The samples were treated and stored for 28 days in the freezer, outside, and in a control environment.

Microbial analysis

Colony forming units (CFUs) were measured on day 1, day 7, day 14, day 21, and day 28 at 37℃ with 85% relative humidity (rH), outside at varying temperatures and relative humidity, and in the freezer at -23 ℃ with 47% relative humidity (Appendix A). at the same time each testing day, 5g from each sample group was removed and placed in a sterile 15ml tube with 8.3 ml of 0.05% Triton X-100 solution. The samples were then shaken for 2 minutes. For each sample group, 100 µL of the wash was plated onto 100 mm × 15 mm sterile Petri-dishes containing Potato Dextrose Agar (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany). Three samples for each treatment combination? were prepared and plated.

Fat Acidity, Water Activity, and Protein Levels

Water activity was calculated by using a water activity meter (CNYST-160, Lu'an, China). The range of readings of 0~1.0 aw and ± 0.02 aw accuracy. This measurement reflects the amount of free water in food, stability of food, and the likelihood of microbial growth that could negatively affect food quality.

Protein content was determined by combustion. A sample of meal from each experimental run was weighed (~150 mg) into a tin foil and was analyzed for nitrogen on

an Elementar vario MICRO analyzer (St. Joseph, MI). A conversion factor of 5.3 was used to convert nitrogen into protein, as this value was found to agree with values derived from reported cottonseed meal amino acid profiles.

Fat acidity values were measured by titration according to AACC standards 02-01 and 02-03 (AACC, 1995) and expressed as the milligrams of potassium hydroxide (KOH) required to neutralize the free fatty acids from 100 g of cottonseed (mgKOH/100g). The method involved extracting free fatty acids from milled DWGS using purified toluene and titrating with a CO_2 free standard solution of 0.0178N KOH. The reported titration values are an average of the three replicates.

Statistical Analysis

Statistical analyses were performed using Origin software (OriginLab, Northhampton MA). Two-way ANOVA was used to statistically analyze sample groups to determine the differences in means between aflatoxin treatments and storage conditions. A significance level of 0.05 was used. Where the sum of squares of the main effect A, effect B, and interaction effect.

The Tukey Test compares the means and signifies if the sample groups are significantly different from each other.

$$HSD = \frac{M_i - M_j}{\sqrt{\frac{MS_w}{n_h}}}$$

Where $M_i - M_j$ is the difference between the pair of the means to calculate this M_i should be larger than M_j. MS_w is the mean square within, the n is the number in the sample.

Results and Discussion

Testing Parameters

The methylene blue sample results had the highest percentage of discoloration in the 1 minute at 70 kV, 3 minutes at 70 kV, and 5 minutes at 70 kV (Figure 17).

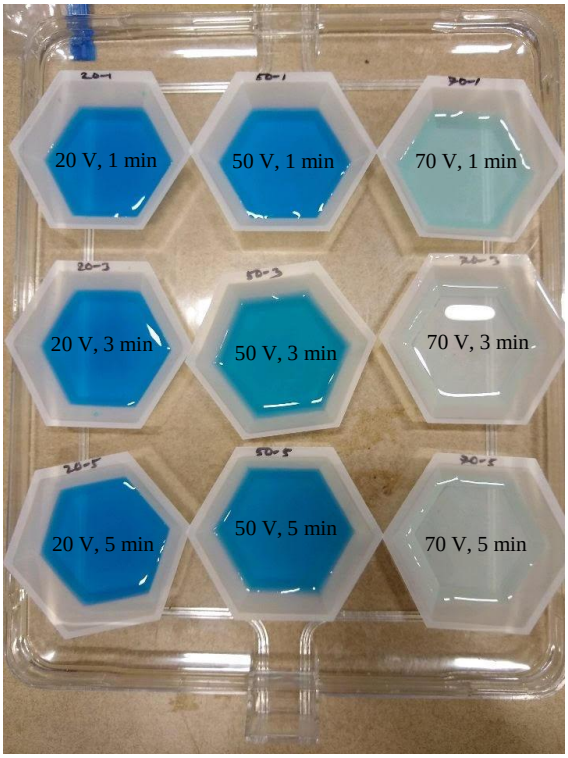

Figure 17. Methylene Blue decolorization after ACP treatment system at 20 kV for various times: 1, 3, 5 minutes; 50 kV for various times: 1, 3, 5 minutes; 70 kV for various times: 1, 3, 5 minutes.

Table 4 shows the reduction in microbial count and percent discoloration of Methylene Blue. The treatment parameter was chosen to treat the remainder of the inoculated cottonseed sample group. The best treatment parameters were 70 kV for 3 minutes due to the highest microbial load reduction and percent discoloration of Methylene Blue.

Sample	Microbial Count (Log_{10}CFU/g)	Log Reduction	% discoloration of Methylene Blue
Control	4.18(0.07)	0	N/A
50 kV, 3 min.	1.79(0.06)	2.39	46.68
50 kV, 5 min.	1.56(0.09)	2.62	72.07
70 kV, 1 min.	1.41(0.04)	2.77	95.73
70 kV, 3 min	1.35(0.06)	2.83	99.68
70 kV, 5 min.	1.51(0.06)	2.67	98.47

Table 4. Analysis of CFU values for the tested treatment parameters with MAP gas.
n = 3, three replicates per treatment
The value in parenthesis is the standard deviation.

Microbial Load Reduction

The control group had lower microbial growth over time compared to the inoculated and the ACP treated sample groups (table 5). The microbial growth increased overtime and there was a higher microbial load in the inoculated sample. More growth occurred in the relative humidity chamber and outside than the freezer. The relative humidity chamber had higher values of microbial load due to the optimal environmental parameters that encouraged growth of *Aspergillus flavus*. The outside temperature varied from 2.61℃ to 25℃, and relative humidity ranged from 77% to 99% (Appendix A). Freezer conditions were not favorable for growth. In the relative humidity chamber, the inoculated sample had a 0.20 log increase and the ACP-treated sample had a 0.11 decrease from day 0 to day 28. Over 28 days, the inoculated sample had a 1.88 log increase and the ACP-treated samples had a log reduction of 1.40 in comparison to the inoculated sample.

Over 28 days, the control sample outside had a microbial load increase of 0.32. In comparison the inoculated sample microbial load increased by 1.27 over 28 days and the ACP treated sample had a reduction of 1.02. The increase in growth over 28 days for all

of the samples was due to the high relative humidity and optimal temperatures that encouraged microbial growth. In comparison to the constant temperature and relative humidity in the chamber encouraged more growth in the inoculated sample and the ACP treated sample group over 28 days. The freezer did not have an increase in growth over 28 days due to the cold temperatures and low relative humidity which did not encourage growth of microbes.

Results for *A. flavus* reduction in hazelnuts showed a 4.4 log reduction when the cold plasma technology was treated for 30 minutes (Sen, Onal-Ulusoy, & Mutlu, 2019). Other studies showed a 2.5 log reduction of *Aspergillus flavus* on red peppers when treated for 30 minutes and a 1.45 log reduction in groundnuts when treated at 24 minutes (Devi et al., 2017; Kim, Lee, & Min, 2014). The results from this current study showed the effectiveness of using ACP for reducing *Aspergillus flavus* concentration in cottonseed. There was a statistically significant main effect interaction of storage conditions on the protein values $F(2, 402) = 9.727$, $p = 7.51\times 10^{-5}$. There was a statistically significant main effect interaction of sample group on the protein values $F(2, 402) = 20.15$, $p = 4.63\times 10^{-9}$. Appendix B shows ANOVA test results on the effect of storage conditions and sample groups on protein content. Appendix B shows ANOVA test results on the microbial load.

		Log_{10}(CFU/ml)				
Sample	Temperature	Day 1	Day 7	Day 14	Day 21	Day 28
Control	RH/T	2.91±2.03	2.67±2.41	2.88±2.47	2.93±2.03	3.49±3.24
Control	Outside	2.69±1.79	2.98±2.39	2.77±2.29	2.76±2.18	3.01±2.67
Control	Freezer	2.36±1.57	2.45±1.98	2.45±1.75	2.42±2.12	2.43±2.00
Inoculated	RH/T	3.11±2.20	3.30±2.63	3.38±2.79	3.17±2.60	4.79±4.32
Inoculated	Outside	3.42±2.94	3.40±3.19	3.40±2.98	3.91±3.21	4.28±4.00
Inoculated	Freezer	2.66±2.15	2.75±2.03	2.77±2.02	2.67±1.75	2.68±2.02
ACP	RH/T	2.80±2.11	2.66±2.33	2.67±2.10	3.17±3.08	3.39±2.84
ACP	Outside	2.85±1.90	2.66±2.37	2.70±1.95	2.91±1.99	3.25±3.12
ACP	Freezer	1.89±1.11	2.22±2.19	2.61±2.16	2.33±1.77	2.10±2.07

Table 5. Average values of CFU/ml over 28 days in the control, inoculated, and ACP treated sample groups.
n = 3, three replicates per treatment
The value after ± is the standard deviation.

Protein Content

Protein levels decreased over time in all of the different storage conditions (Table 6). Over 28 days, the control sample had a 26.2% decrease in the relative humidity chamber, 19.5% decrease outside, and a negligible decrease in the freezer. microbial growth played a large role in the degradation of the protein content available in the meal. A similarly decrease occurred in the inoculated and the ACP treated sample groups. The inoculated sample group had the lowest starting protein content amount and decreased by 43.9%, 20.28% and a negligible amount in the relative humidity chamber, outside, and in the freezer, respectively. The ACP-treated sample decreased by 6.8%, 9.8%, and a negligible amount in the relative humidity chamber, outside, and in the freezer, respectively. The treatment had an effect on the inoculated and ACP treated sample

groups. The ACP treatment showed to preserve the protein content over 28 days even though there was a slight decrease in the content it was still higher than the sample that was inoculated. ACP treatment can maintain protein content, that is valued by the livestock producers feeding cottonseed products. Similar effects of ACP on protein content have been shown in mango flour, radish sprouts, and wheat (Abidin, Rukunudin, Zaaba, & Wan Omar, 2018; Kim et al., 2014; Saberi, Modarres-Sanavy, Zare, & Ghomi, 2018). There was a statistically significant main effect interaction of storage conditions on the protein values F (2, 44) = 3.868, p = .0300. There was a statistically significant main effect interaction of sample group on the protein values F (2, 44) = 7.844, p = 0.001. Appendix B shows ANOVA test results on the effect of storage conditions and sample groups on protein content.

		Protein Content				
Sample	Temperature	Day 1	Day 7	Day 14	Day 21	Day 28
Control	RH/T	34.2±0.82	30.5±0.79	29.2±0.81	26.3±0.80	25.2±0.79
Control	Outside	30.2±0.42	27.6±0.45	28.1±0.44	30.6±0.54	24.3±0.40
Control	Freezer	38.7±0.22	31.2±0.21	30.7±0.24	32.7±0.22	30.5±0.21
Inoculated	RH/T	28.9±1.49	28.3±1.54	24.6±1.44	21.4±1.40	16.2±1.34
Inoculated	Outside	28.1±1.08	30.0±1.22	30.2±1.18	24.4±1.04	22.4±1.11
Inoculated	Freezer	31.1±0.36	29.9±0.43	27.7±0.32	26.3±0.41	27.4±0.39
ACP	RH/T	30.9±0.44	31.9±0.43	31.9±0.42	28.4±0.36	28.8±0.37
ACP	Outside	30.7±0.40	31.9±0.38	33.4±0.39	28.1±0.44	27.7±0.48
ACP	Freezer	30.6±0.27	32.6±0.28	31.1±0.32	32.4±0.30	29.0±0.29

Table 6. Average values of protein levels over 28 days in the control, inoculated, and ACP treated sample groups.
n = 3, three replicates per treatment
The value after ± is the standard deviation.

Fat Acidity

Fat acidity levels increased over 28 days due to microbial growth in all the sample groups (Table 7). The increase in fat acidity values are due to improper storage, mold

growth, and high respiratory activity (Becker, 2008). The highest fat acidity values were associated with the inoculated samples which showed an increase 18.0%, 15.1%, and a negligible increase for the relative humidity chamber, outside, and freezer conditions. There were lower levels associated with the ACP treated samples which had 11.9% increase in the relative humidity chamber, a decrease in 4.4% in the outside condition, and a negligible change in the freezer. There is limited data on the effect of *Aspergillus flavus* on the fatty acid composition in cottonseed. In distiller's wet grain solubles there will be about three times higher fungal concentration than that of corn due to the presences of microbial growth and deterioration (McClurkin-Moore, 2015).

ACP treatment can prevent further degradation from occurring in the cottonseed sample when *Aspergillus flavus* is present and does not cause an increase in fat acidity values. Similar results have been seen in white grape juice, mandarins, and melons (Pankaj, Wan, Colonna, & Keener, 2017; Tappi et al., 2016; Won, Lee, & Min, 2017). controlling the fat acidity levels can limit the amount of deterioration in cottonseed meal stored in undesirable conditions, promoting a longer safe storage period for the cottonseed. There was a statistically significant main effect interaction of storage conditions on the protein values $F(2, 44) = 8.506$, $p = 9.43\times 10^{-4}$. There was a statistically significant main effect interaction of sample group on the protein values $F(2, 44) = 1051$, $p = 1.17\times 10^{-32}$. Appendix B shows ANOVA test results on the effect of storage conditions and sample groups on fat acidity levels.

		Fat Acidity				
Sample	Temperature	Day 1	Day 7	Day 14	Day 21	Day 28
Control	RH/T	4.3	4	4.2	4.4	4.7
Control	Outside	4	4.3	3.9	3.9	4.4
Control	Freezer	3.5	3.6	3.7	3.7	3.9
Inoculated	RH/T	13.3	13.4	13.7	15.3	15.7
Inoculated	Outside	13.2	13.5	13.5	13.8	15.2
Inoculated	Freezer	13.2	13.1	13.2	13.3	13.2
ACP	RH/T	8.4	7.2	7.5	8.9	9.4
ACP	Outside	8.3	7.3	7.5	7.9	8.1
ACP	Freezer	7.3	7.4	7.5	7.4	7.2

Table 7. Average values of fat acidity levels over 28 days in the control, inoculated, and ACP treated sample groups.

Water Activity

Water activity increased in all of the sample groups throughout the 28-day storage time (Table 8). Over 28 days, the control sample there was an increase of 28.6%, 20.37%, and 9.1% increase in the relative humidity chamber, outside, and freezer storage temperatures. In comparison there was a 14.46%, 17.5%, and negligible increase in the relative humidity temperature chamber, outside, and freezer storage temperatures. The ACP treated group had an increase of 6.06%, decrease in 7%, and a negligible change in the relative humidity temperature chamber, outside, and freezer conditions.

The ACP-treated samples had a smaller increase in water activity, corresponding to a decrease in *A. flavus* growth within the samples. The control and inoculated samples had higher increase of growth due to the higher microbial load. The ACP treatment has limited the increase in water activity which preserves the cottonseed more when it is being stored because microbial growth was limited. This shows the importance of drying samples even after treatment to ensure the limit of *A. flavus* growth. Water activity correlates with mold growth in storage (Peleg M., Corradini M G., & D., 2015). There was

a statistically significant main effect interaction of storage conditions on the protein values F (2, 44) = 229.8, p = 3.16×10^{-21}. There was a statistically significant main effect interaction of sample group on the protein values F (2, 44) = 61.83, p = 2.26×10^{-12}. Appendix B shows ANOVA test results on the effect of storage conditions and sample groups on water activity levels.

		Water Activity				
Sample	Temperature	Day 1	Day 7	Day 14	Day 21	Day 28
Control	RH/T	0.63±0.03	0.68±0.04	0.72±0.03	0.76±0.02	0.81±0.03
Control	Outside	0.54±0.04	0.54±0.03	0.56±0.05	0.58±0.03	0.65±0.04
Control	Freezer	0.44±0.01	0.46±0.01	0.47±0.02	0.44±0.01	0.48±0.01
Untreated	RH/T	0.83±0.08	0.85±0.07	0.86±0.09	0.93±0.10	0.95±0.90
Untreated	Outside	0.8±0.06	0.85±0.06	0.86±0.04	0.92±0.05	0.94±0.05
Untreated	Freezer	0.46±0.02	0.47±0.01	0.44±0.01	0.45±0.02	0.46±0.02
ACP	RH/T	0.66±0.04	0.67±0.05	0.66±0.08	0.73±0.09	0.7±0.06
ACP	Outside	0.7±0.04	0.68±0.05	0.64±0.03	0.67±0.04	0.65±0.06
ACP	Freezer	0.48±0.01	0.48±0.01	0.48±0.01	0.46±0.02	0.45±0.02

Table 8. Average values of water activity levels over 28 days in the control, untreated, and ACP treated sample groups.
n = 3, three replicates per treatment
The value after ± is the standard deviation.

Conclusions

The research shows the impact of ACP on decreasing the microbial load on *Aspergillus flavus* on infected cottonseed meal. The research showed that ACP had a significant microbial load reduction in the optimal growing temperatures. This non-thermal treatment also allowed for the preservation of the protein content, fa acidity levels, and water activity compared to the untreated sample. This novel treatment can serve as a possible treatment for whole cottonseed and cottonseed meal. This treatment uses reactive oxygen species to decrease the microbial load on a variety of commodities.

CHAPTER VI

CONCLUSIONS AND RECOMMENDATIONS

This research can help the cottonseed industry with decontaminating and eliminating *A. flavus* and aflatoxins from cottonseed. The first objective results can be used for sorting cottonseed based on physical parameters such as density, dimensions, surface area, and projected area. Instead of using just a singular physical property, using a culmination of properties can be more effective in separating infected seed from healthy cottonseed. There are currently no sorting efforts that happen in cottonseed processing, but implementing a sorting practice can help decrease *A. flavus* and aflatoxin content. A recommendation from this objective would be to incorporate aeration in order to separate cottonseed based on density or use a density gravity table. Lower density and high moisture content can be associated with high levels of aflatoxin above the FDA action levels.

Objective two results conclude that during processing of cottonseed, contaminated meal will at least increase by twice the amount in the starting whole cottonseed. There is potential to use this process to understand when the best time to treat cottonseed during after separating based on physical properties. The recommendation would design a system that can penetrate whole fuzzy cottonseed and doesn't slow down processing time. Objective three results conclude that ACP has the potential to be used to decontaminate infected cottonseed meal. Additional research can be done to optimize treatment times, gas composition, voltage levels, and packaging depth to successfully alleviate *A. flavus* growth and aflatoxin. Longer exposure time to ACP can lead to greater decontamination.

Even though there aren't a lot of commercialized systems to treat cottonseed, bit a design can be developed and patented. Using larger dielectric barriers can help treat larger sample sizes and gyration can help with RGS to treat the surface and penetrate the cottonseed hull. Economic analysis would help with understanding how much cottonseed can be processed with system because typically a cottonseed ginner would process almost 60 bales an hour. A specialized design can lead to decontaminating infected cottonseed.

Overall, cottonseed is an agricultural commodity that has potential to be used as cooking oil, animal feed, animal roughage, and biodiesel. Cottonseed has an economic impact and is negatively affected by *A. flavus* contamination which optimal environmental conditions are met. Implementing sorting and a treatment system can lead to decreasing cottonseed contamination.

REFERENCES

Abidin, N. S. A., Rukunudin, I. H., Zaaba, S. K., & Wan Omar, W. A. (2018). The effect of atmospheric cold plasma (ACP) treatment on colour, water activity, antioxidant activity and total phenolic content of mango flour noodles during storage. *International Food Research Journal, 25*(4), 1444-1449.

Al-Shikli, R. A., A.Abdulrasool, A., & Al-Hiti, M. M. (2010). Effect of Some Storage Conditions upon the Survival of Some Fungal Spores. *Iraqi J Pharm Sci, 19*(2), 1-10.

Altuntas, E., & Demirtola, H. (2007). Effect of moisture content on physical properties of some grain legume seeds. *New Zealand Journal of Crop and Horticultural Science, 35*(4), 423-433. doi:10.1080/01140670709510210

Andersen, K. B., Feilberg, A., & Beukes, J. A. (2012). Use of non-thermal plasma and UV-light for removal of odour from sludge treatment. *Water Sci Technol, 66*(8), 1656-1662. doi:10.2166/wst.2012.367

Ashley, H., Thomas, J., Holt, G., & Valco, T. (2018). Cottonseed Air-Handling and Storage Requirements. *The Journal of Cotton Science, 22*, 47-59.

Ashworth, L. J., Rice, R. E., McMeans, J. L., & M., B. C. (1971). The Relationship of Insects to Infection of Cotton Bolls by Aspergillus flavus. *Phytopathology, 61*(5), 488-&.

Aydin, A., Aksu, H., & Gunsen, U. (2011). Mycotoxin levels and incidence of mould in Turkish rice. *Environ Monit Assess, 178*(1-4), 271-280. doi:10.1007/s10661-010-1688-9

Azadzadeh, A. H. (2014). *Some Physical Properties of Cotton Seed ('Varamin' Cultivar) at Different Moisture Contents*. Paper presented at the ISHS Acta Horticulturae 1054.

Becker, R. (2008). *Fatty acids in food cereal grains and grain products*. New York: Food Science and Technology.

Bennett, G. A., & Richard, J. L. (1996). Influence of processing on Fusarium mycotoxins in contaminated grains. *Food Technology, 50*, 235-238.

Blankenship, P. D., Cole, R. T., Sanders, T. H., & Hill, R. A. (1984). Effect of geocarposphere temperature on pre-harvest colonization of drought-stressed peanuts by Aspergillus flavus and subsequent aflatoxin contamination. *Mycopathologia, 85*, 69-74.

Blasi, D. A., & Drouillard, J. (2002). Cottonseed Feed Products for Beef Cattle. *Kansas State University Agricultural Experiment Station and Cooperative Extension Service*.

Bothast, R. J., Bennett, G. A., Vancauwenberge, J. E., & Richard, J. L. (1992). Fate of Fumonisin B1 in Naturally Contaminated Corn during Ethanol Fermentation. *Applied and Environmemtal Microbiology, 58*(1).

Bourke, P., Ziuzina, D., Boehm, D., Cullen, P. J., & Keener, K. (2018). The Potential of Cold Plasma for Safe and Sustainable Food Production. *Trends Biotechnol, 36*(6), 615-626. doi:10.1016/j.tibtech.2017.11.001

Bulaong, S. S. P., & Dharmaputra, O. S. (2002). Fungal Population, Aflatoxin and Fatty Acid Contents of Peanuts Packed in Different Bag Types. *Biotropia, 19*, 1-25.

Buser, M. D. (1999). *Update on the Extrusion of Cottonseed and Cotton Gin Waste Mixtures*. Paper presented at the Beltwide Cotton Conference, Memphis, TN.

Buser, M. D., & Abbas, H. K. (2001). Mechanically Processinf Cottonseed to Reduce Gossypol and Aflatoxin Levels. *Journal of Toxicology - Toxin Reviews, 20*, 161-278.

Butscher, D., Loon, H., Waskow, A., Rohr, P., & Schuppler, M. (2016). Plasma Inactivation of Microorganisms of Sprout Seeds in Dielectric Barrier Discharge. *International Journal of Food Microbiology, 238*, 222-232.

Carvajal-Campos, A., Manizan, A. L., Tadrist, S., Akaki, D. K., Koffi-Nevry, R., Moore, G. G., . . . Puel, O. (2017). Aspergillus korhogoensis, a Novel Aflatoxin Producing Species from the Cote d'Ivoire. *Toxins (Basel), 9*(11). doi:10.3390/toxins9110353

Champathi Gunathilake, D. M. C., Bhat, J., Singh, I. R., & Tharanga Kahandawala, K. A. (2019). Dynamics of the physical properties of soybean during storage under tropical condition. *Legume Research - an International Journal*(of). doi:10.18805/lr-447

Cherry, J. P. (1983). Cottonseed oil. *Journal of the American Oil Chemists' Society, 60*(2). doi:https://doi.org/10.1007/BF02543519

Choudhary, U., Dey, E., Bhattacharyya, R., & Ghosh, S. K. (2018). A Brief Review on Plasma Treatment of Textile Materials. *Advvance Research in Textile Engineering, 3*(1), 388-397.

Ciegler, A., Kadis, S., & Ajl, S. J. (1973). Microbial toxins. A comprehensive treatise. *Food and Cosmetics Toxicology, 11*, 664-665. doi:10.1016/S0015-6264(73)80346-3

Coker, R. D., Nagler, M. J., Blunden, G., Sharkey, A. J., Defize, P. R., Derksen, G. B., & Whitaker, T. B. (1995). Design of sampling plans for mycotoxins in foods and feeds. *Nat Toxins, 3*(4), 257-262; discussion 280. doi:10.1002/nt.2620030417

Colagar, A. H., Sohbatzadeh, F., Mirzanejhad, S., & Omran, A. V. (2010). Sterilization of Streptococcus pyogenes by afterglow dielectric barrier discharge using O2 and CO2 working gases. *Biochemical Engineering Journal, 51*(3), 189-193. doi:10.1016/j.bej.2010.06.017

Columbus, E. P., & Mangialard, G. J. (1996). Cottonseed Moisture and Seed Damage at Gins. *Transactions of the ASAE, 39*(5), 1671-1621.

Connolly, J., Valdramidis, V. P., Byrne, E., Karatzas, K. A., Cullen, P. J., Keener, K. M., & Mosnier, J. P. (2013). Characterization and antimicrobial efficacy againstE. coliof a helium/air plasma at atmospheric pressure created in a plastic package. *Journal of Physics D: Applied Physics, 46*(3). doi:10.1088/0022-3727/46/3/035401

Coşkuner, Y., & Karababa, E. (2007). Some physical properties of flaxseed (Linum usitatissimum L.). *Journal of Food Engineering, 78*(3), 1067-1073. doi:10.1016/j.jfoodeng.2005.12.017

Cotty, P. J., Howell, D. R., Bock, C., & Tellez, A. (1997). *Aflatoxin Contamination of Bt Cottonseed*. Retrieved from

Dasan, B. G., Boyaci, I. H., & Mutlu, M. (2016). Inactivation of aflatoxigenic fungi (Aspergillus spp.) on granular food model, maize, in an atmospheric pressure fluidized bed plasma system. *FSTA - Food Science and Technology Abstracts, 70*, 1-8.

Delouche, J. C. (1981). *Harvest and Post-Harvest Factors Affecting the Quality of Cotton Planting Seed and Seed Quality Evaluation.* Paper presented at the Beltwide Cotton Production Research Conference Proceeding, Mississipi Agriculturasl and Forestry Experiment Station.

Delouche, J. C. (1986). Harvest and Post-Harvest Factors Affecting the Quality of Cotton Planting Seed and Seed Quality Evaluation. In J. R. Mauney & J. M. Stewart (Eds.), *Cotton Physiology*.

Devi, Y., Thirumdas, R., Sarangapani, C., Deshmukh, R. R., & Annapure, U. S. (2017). Influence of cold plasma on fungal growth and aflatoxins production on groundnuts. *Food Control, 77*, 187-191. doi:10.1016/j.foodcont.2017.02.019

Doyle, M. P., Applebaum, R. S., Brackett, R. E., & Marth, E. H. (1982). Physical, Chemical and Biological Degradation of Mycotoxins in Foods and Agricultural Commodities. *J Food Prot, 45*(10), 964-971. doi:10.4315/0362-028X-45.10.964

Dwarakanath, C. T., Rayner, E. T., Mann, G. E., & Dollear, F. G. (1967). Reduction of Aflatoxin Levels in Cottonseed and Peanut Meals by Ozonization *The Journal of the American Oil Chemists' Society, 45*.

Eckel, R. H., Borra, S., Lichtenstein, A. H., & Yin-Piazza, S. Y. (2007). Understanding the complexity of trans fatty acid reduction in the American diet: American Heart Association Trans Fat Conference 2006: report of the Trans Fat Conference Planning Group. *Circulation, 115*(16), 2231-2246. doi:10.1161/CIRCULATIONAHA.106.181947

Ehrampoush, M. H., Moussavi, G. H. R., Ghaneian, M. T., Rahimi, S., & Ahmadian, M. (2011). Removal of Methylene Blue Dye from Textile Simulated Sample Using Tubular Reactor and TiO2/UV-C Photocatalytic Process. *Journal of Environmental Health Science and Engineering ·, 8*(1), 35-40.

Ellis, W. O., Smith, J. P., Simpson, B. K., Ramaswamy, H., & Doyon, G. (1994a). Growth of and aflatoxin production by Aspergillus fiat:us in peanuts stored under modified atmosphere packaging (MAP) conditions. *International Journal of Food Microbiology, 22*, 173-187.

Ellis, W. O., Smith, J. P., Simpson, B. K., Ramaswamy, H., & Doyon, G. (1994b). Growth of and aflatoxin production by Aspergillus flavus in peanuts stored under modified atmosphere packaging (MAP) conditions. *International Journal of Food Microbiology, 22*, 173-187.

The Encyclopedia of Seeds: Science, Technology and Uses. (2006). (M. Black, J. D. Bewley, & P. Halmer Eds.). Cambridge, MA: CABI Publishing.

FDA Compliance Guide. (2019).
Filazi, A., & Tansel, U. (2013). Occurrence of Aflatoxins in Food. In *Aflatoxins - Recent Advances and Future Prospects*.
Filtenborg, O., Frisvad, J. C., & Thrane, U. (1996). Moulds in food spoilage. *International Journal of Food Microbiology, 33*(1), 85-102. doi:https://doi.org/10.1016/0168-1605(96)01153-1
Fisher, M. C., Henk, D. A., Briggs, C. J., Brownstein, J. S., Madoff, L. C., McCraw, S. L., & Gurr, S. J. (2012). Emerging fungal threats to animal, plant and ecosystem health. *Nature, 484*(7393), 186-194. doi:10.1038/nature10947
Franklin, R. B., Garland, J. L., Bolster, C. H., & Mills, A. L. (2001). Impact of dilution on microbial community structure and functional potential: comparison of numerical simulations and batch culture experiments. *Appl Environ Microbiol, 67*(2), 702-712. doi:10.1128/AEM.67.2.702-712.2001
Fridman, G., Friedman, G., Gutsol, A., Shekhter, A. B., Vasilets, V. N., & Fridman, A. (2008). Applied Plasma Medicine. *Plasma Processes and Polymers, 5*(6), 503-533. doi:10.1002/ppap.200700154
Frisvad, J. C., Hubka, V., Ezekiel, C. N., Hong, S. B., Novakova, A., Chen, A. J., . . . Houbraken, J. (2019). Taxonomy of Aspergillus section Flavi and their production of aflatoxins, ochratoxins and other mycotoxins. *Stud Mycol, 93*, 1-63. doi:10.1016/j.simyco.2018.06.001
Gamba, R. R., Colo, C. N., Correa, M., Astoreca, A., Alconada, T., Antoni, G., & Peláez, A. L. (2015). Antifungal Activity against Aspergillus parasiticus of Supernatants from Whey Permeates Fermented with Kefir Grains. *Advances in Microbiology, 05*(06), 479-492. doi:10.4236/aim.2015.56049
Goldblatt, L. A. (1968). Aflatoxin and its Control. *Economic Botany, 22*, 51-62. doi:https://doi.org/10.1007/BF02897744
Goynes, W. R., & Lee, S. L. (1989). Aspergillus flavus Infection of Developing Cottonseed: Microscopical Determination of Mycelial Progression and Associated Aflatoxin Formation. *Arch. Environ. Contain. Toxicol, 18*, 421-429.
Griffin Jr, A. C. (1980). Cotton Moisture Measurements—Effects of Laboratory Procedures. *Transactions of the ASAE, 23*(1), 177-0179. doi:https://doi.org/10.13031/2013.34548
Groves, F. E., & Bourland, F. M. (2010). Estimating Seed Surface Area of Cottonseed. *The Journal of Cotton Science, 14*, 74-81.
Haelim, L., Hae, Y. I., Hyun, J. L., Joo, Y. P., Wonho, C., & Cheorun, J. (2016). *Flexible Thin-Layer Plasma in Package: Inactivation Effect of Bacteria and Mold and Physiochemical Properties in Beef Jerky*. Paper presented at the 62nd International Congress of Meat Science and Technology, Bangkok, Thailand.
Hammond, E. W. (2003). *Encyclopedia of Food Sciences and Nutrition*.
Hams, T. A. P., & Ayres, J. C. (1977). Factors Affecting Aflatoxin Contamination of Cottonseed I. Contamination of Cottonseed with Aspergillus flavus at Harvest and during Storage. *Journal of the American Oil Chemists Society, 54*, 219-224.
Hiroshi, H., Takayuki, O., Jia, F., Keigo, T., Kenji, I., Masaru, H., & Masafumi, I. (2013). Inactivation effects of neutral reactive-oxygen species on Penicillium

digitatum spores using non-equilibrium atmospheric-pressure oxygen radical source. *Appl Phys Lett, 103*. doi:10.1063/1.4824892
Hodson, E. A. (1920). Lint frequency in cotton with a method for determination.
Holdsworth, S. D. (1997). *Thermal Processing of Packaged Foods* (2 ed.). New York.
Holt, G., Wedegaertner, T., Wanjura, J., Pelletier, M., Delhom, C., & Duke, S. (2017). Development and Evaluation of a Novel Bench-Top Mechanical Cotton Seed Delinter for Cotton Breeders. *The Journal of Cotton Science, 21*, 18-28.
Horn, B. W., Moore, G. G., & Carbone, I. (2009). Sexual Reproduction in Aspergillus flavus. *Mycologia, 101*(3), 423-429. doi:10.3852/09-011
Izli, N. (2015). Effect of moisture on the physical properties of three varieties of kenaf seeds. *J Food Sci Technol, 52*(6), 3254-3263. doi:10.1007/s13197-014-1369-8
Jaime-Garcia, R., & Cotty, P. J. (2003). Aflatoxin Contamination of Commercial Cottonseed in South Texas. *Phytopathology, 93*(9), 1190–1200.
Jones, D. B. (1931). Factors for Converting Percentages of Nitrogen in Foods and Feeds into Percentages of Proteins. 1-21.
Karlovsky, P., Suman, M., Berthiller, F., De Meester, J., Eisenbrand, G., Perrin, I., . . . Dussort, P. (2016). Impact of food processing and detoxification treatments on mycotoxin contamination. *Mycotoxin Res, 32*(4), 179-205. doi:10.1007/s12550-016-0257-7
Keidar, M., Shashurin, A., Volotskova, O., Ann Stepp, M., Srinivasan, P., Sandler, A., & Trink, B. (2013). Cold atmospheric plasma in cancer therapy. *Physics of Plasmas, 20*(5). doi:10.1063/1.4801516
Kemper, T. G. (2005). Oil Extraction. In *Bailey's Industrial Oil and Fat Products*.
Kibar, H., & Ozturk, T. (2008). Physical and mechanical properties of soybean. *Int. Agrophys, 22*(3), 239-244.
Kilickanm A., Ucer, N., & Yalcin, I. (2010). Some physical properties of spinach (Spinacia oleracea L.) seed. *African Journal of Biotechnology, 9*(5), 648-655.
Kim, J. E., Lee, D. U., & Min, S. C. (2014). Microbial decontamination of red pepper powder by cold plasma. *Food Microbiol, 38*, 128-136. doi:10.1016/j.fm.2013.08.019
Klich, M. (2007). Aspergillus flavus: the major producer of aflatoxin. *Mol Plant Pathol, 8*(6), 713-722. doi:10.1111/j.1364-3703.2007.00436.x
Klich, M., & Chimielewski, M. A. (1985). Nectaries as Entry Sites for Aspergillus flavus in Developing Cotton Bolls. *Applied and Environmental Microbiology, 50*(3), 602-604.
Koltun, S. P., Gardner, J. R., Dollear, F. G., & Rayner, E. T. (1973). Physical Properties and Aflatoxin Content of Individual
Cateye Fluorescent Cottonseeds *Journal of the American Oil Chemists' Society, 51*, 178-180.
Kotinagu, K., Mohanamba, T., & Kumari, L. R. (2015). Assessment of aflatoxin B1 in livestock feed and feed ingredients by high-performance thin layer chromatography. *Vet World, 8*(12), 1396-1399. doi:10.14202/vetworld.2015.1396-1399

Kumar, D., & Kalita, P. (2017). Reducing Postharvest Losses during Storage of Grain Crops to Strengthen Food Security in Developing Countries. *Foods, 6*(1). doi:10.3390/foods6010008

Lacombe, A., Niemira, B. A., Gurtler, J. B., Fan, X., Sites, J., Boyd, G., & Chen, H. (2015). Atmospheric cold plasma inactivation of aerobic microorganisms on blueberries and effects on quality attributes. *Food Microbiol, 46*, 479-484. doi:10.1016/j.fm.2014.09.010

Lee, L. S., Cucullu, A. F., Pons, W. A., & Russell, T. E. (1977). Separation of aflatoxin-contaminated cottonseed based on physical characteristics of seed cotton and ginned seed. *Journal of the American Oil Chemists' Society, 54*(3), 238A-241A.

Lillehoj, E. B., Wall, J. H., & Bowersm, E. J. (1987). Preharvest Aflatoxin Contamination: Effect of Moisture and Substrate Variation in Developing Cottonseed and Corn Kernels. *Applied and Environmental Microbiology, 53*(3), 584-586.

Liu, J., Sun, L., Zhang, N., Zhang, J., Guo, J., Li, C., . . . Qi, D. (2016). Effects of Nutrients in Substrates of Different Grains on Aflatoxin B1 Production by Aspergillus flavus. *Biomed Res Int, 2016*, 7232858. doi:10.1155/2016/7232858

Los, A., Ziuzina, D., Akkermans, S., Boehm, D., Cullen, P. J., Van Impe, J., & Bourke, P. (2018). Improving microbiological safety and quality characteristics of wheat and barley by high voltage atmospheric cold plasma closed processing. *Food Res Int, 106*, 509-521. doi:10.1016/j.foodres.2018.01.009

Mallmann, A. O., Marchioro, A., Oliveira, M. S., Rauber, R. H., Dilkin, P., & Mallmann, C. A. (2014). Comparison of the efficiency between two sampling plans for aflatoxins analysis in maize. *Brazilian Journal of Microbiology, 45*(1), 35-42.

Mandal, R., Singh, A., & Pratap Singh, A. (2018). Recent developments in cold plasma decontamination technology in the food industry. *Trends in Food Science & Technology, 80*, 93-103. doi:10.1016/j.tifs.2018.07.014

Manimehalai, N., & Viswanathan, R. (2006). Physical Properties of Fuzzy Cottonseeds. *Biosystems Engineering, 95*(2), 207-217. doi:10.1016/j.biosystemseng.2006.06.008

Marchese, S., Polo, A., Ariano, A., Velotto, S., Costantini, S., & Severino, L. (2018). Aflatoxin B1 and M1: Biological Properties and Their Involvement in Cancer Development. *Toxins (Basel), 10*(6). doi:10.3390/toxins10060214

McClurkin-Moore, J. D. (2015). *Shelf-Life Improvement of Distillers Wet Grains with Solubles.* (Doctor of Philosophy (PhD). Purdue University,

McClurkin-Moore, J. D., Ileleji, K. E., & Keener, K. M. (2017). The Effect of High-Voltage Atmospheric Cold Plasma Treatment on the Shelf-Life of Distillers Wet Grains. *Food and Bioprocess Technology, 10*(8), 1431-1440. doi:10.1007/s11947-017-1903-6

McClurkin, J. D., & Ileleji, K. E. (2015). The effect of storage temperature and percentage of condensed distillers solubles on the shelf-life of distillers wet grains stored aerobically. *Journal of Stored Products Research, 62*, 58-64. doi:10.1016/j.jspr.2015.03.002

Mellon, J. E., & Cotty, P. J. (1998). Effects of Oilseed Storage Proteins on Aflatoxin Production by Aspergillus flavus. *Journal of the American Oil Chemists' Society, 75*(9), 1085-1089.

Mellon, J. E., Cotty, P. J., & Dowd, M. K. (2007). Aspergillus flavus hydrolases: their roles in pathogenesis and substrate utilization. *Appl Microbiol Biotechnol, 77*(3), 497-504. doi:10.1007/s00253-007-1201-8

Misra, N., Kaur, S., Tiwari, B. K., Kaur, A., Singh, N., & Cullen, P. J. (2015). Atmospheric pressure cold plasma (ACP) treatment of wheat flour. *Food Hydrocolloids, 44*, 115-121. doi:10.1016/j.foodhyd.2014.08.019

Misra, N., Yadav, B., Roopesh, S., & Jo, C. (2019). Cold Plasma for Effective Fungal and Mycotoxin Control in Foods: Mechanisms, Inactivation Effects, and Applications. *Comprehensive Reviews in Food Science and Food Safety, 18*(1), 106-120. doi:10.1111/1541-4337.12398

Misra, N. N., Pankaj, S. K., Segat, A., & Ishikawa, K. (2016). Cold plasma interactions with enzymes in foods and model systems. *Trends in Food Science & Technology, 55*, 39-47. doi:10.1016/j.tifs.2016.07.001

Misra, N. N., Yepez, X., Xu, L., & Keener, K. (2019). In-package cold plasma technologies. *Journal of Food Engineering, 244*, 21-31. doi:10.1016/j.jfoodeng.2018.09.019

Mitchell, N. J., Bowers, E., Hurburgh, C., & Wu, F. (2016). Potential economic losses to the US corn industry from aflatoxin contamination. *Food Addit Contam Part A Chem Anal Control Expo Risk Assess, 33*(3), 540-550. doi:10.1080/19440049.2016.1138545

Mošovská, S., Medvecká, V., Halászová, N., Ďurina, P., Valík, L., Mikulajová, A., & Zahoranová, A. (2018). Cold Atmospheric Pressure Ambient Air Plasma Inhibition of Pathogenic Bacteria on the Surface of Black Pepper. *Food Research International, 106*, 862-869. doi:10.1016/j.foodres.2018.01.066

Mpuchane, S. F., Taligoola, H. K., Gashe, I. Z., & Matsheka, M. (1997). A Mycological Study of Stored Maize and Sorghum Grains. *Botswana Society, 29*, 81-91.

Mudway, I. S., & Kelley, F. J. (2000). Ozone and the Lung a Sensitive Issue. *Molecular Aspects of Medicine, 21*, 1-48.

Muhammad, A. I., Liao, X., Cullen, P. J., Liu, D., Xiang, Q., Wang, J., . . . Ding, T. (2018). Effects of Nonthermal Plasma Technology on Functional Food Components. *Comprehensive Reviews in Food Science and Food Safety*. doi:Doi:10.1111/1541-4337.12379

Murthy, G. S., Townsend, D. E., Meerdink, G. L., Beargen, G. L., Tumbleson, M. E., & Singh, V. (2005). Effect of Aflatoxin B1 on Dry-Grind Ethanol Process. *Cereal Chemistry, 82*(3), 302-304.

Nadvornikova, M., Banout, J., Herak, D., & Verner, V. (2018). Evaluation of physical properties of rice used in traditional Kyrgyz Cuisine. *Food Sci Nutr, 6*(6), 1778-1787. doi:10.1002/fsn3.746

NCPA. (2020). Products.

Niemira, B. A. (2012). Cold plasma decontamination of foods. *Annual Review of Food Science and Technology, 3*(1), 125-142. doi:doi.org/10.1146/annurev-food-022811-101132
O'Brien, R. D., Jones, L. A., King, C. C., Wakelyn, P. J., & Wan, P. J. (2005). Cottonseed Oil. In *Edible Oil and Fat Products: Edible Oils*.
Ohta, T. (2016). Plasma in Agriculture. In N. N. Misra, O. Schluter, & P. J. Cullen (Eds.), *Cold Plasma in Food and Agriculture: Fundamentals and Applications* (pp. 382): Elsevier Science & Technology.
Ojiambo, P. S., Battilani, P., Cary, J. W., Blum, B. H., & Carbone, I. (2018). Cultural and Genetic Approaches to Manage Aflatoxin Contamination: Recent Insights Provide Opportunities for Improved Control. *Phytopathology, 108*(9), 1024-1037. doi:10.1094/PHYTO-04-18-0134-RVW
Osborne, T. B., & Mendel, L. B. (1917). The use of Cotton seed as Food. *The Journal of Bioloigcal Chemistry, 29*(2), 289-317.
Ozarslan, C. (2002). Physical Properties of Cotton Seed. *Biosystems Engineering, 83*(2), 169-174. doi:10.1016/S1537-5110(02)00151-4
Pandiselvam, R., Mayookha, V. P., Kothakota, A., Sharmila, L., Ramesh, S. V., Bharathi, C. P., . . . Srikanth, V. (2019). Impact of Ozone Treatment on Seed Germination-A Systematic Review. *Ozone: Science & Engineering*, 1-16. doi:10.1080/01919512.2019.1673697
Pankaj, S. K., Bueno-Ferrer, C., Misra, N. N., Milosavljević, V., O'Donnell, C. P., Bourke, P., . . . Cullen, P. J. (2014). Applications of cold plasma technology in food packaging. *Trends in Food Science & Technology, 35*(1), 5-17. doi:10.1016/j.tifs.2013.10.009
Pankaj, S. K., & Keener, K. M. (2017). Cold plasma: background, applications and current trends. *Current Opinion in Food Science, 16*, 49-52. doi:10.1016/j.cofs.2017.07.008
Pankaj, S. K., Wan, Z., Colonna, W., & Keener, K. M. (2017). Effect of high voltage atmospheric cold plasma on white grape juice quality. *J Sci Food Agric, 97*(12), 4016-4021. doi:10.1002/jsfa.8268
Pankaj, S. K., Wan, Z., & Keener, K. M. (2018). Effects of Cold Plasma on Food Quality: A Review. *Foods, 7*(1). doi:10.3390/foods7010004
Park, D. L. (2002). Effect of Processing on Aflatoxin. *Mycotoxins and Food Safety*, 173-179.
Park, D. L., & Stoloff, L. (1989). Aflatoxin Control-How a Regulatory Agency Managed Risk from an Unavoidable Natural Toxicant in Food and Feed *Regulatory Toxicology And Pharmacology, 9*, 109-130.
Peleg M., Corradini M G., & D., N. M. (2015). *On Modeling the Effect of Water Activity on Microbial Growth and Mortality Kinetics*. New York, NY.
Pennerman, K. K., Yin, G., Bennett, J. W., & Hua, S. T. (2019). Aspergillus flavus NRRL 35739, a Poor Biocontrol Agent, May Have Increased Relative Expression of Stress Response Genes. *J Fungi (Basel), 5*(2). doi:10.3390/jof5020053

Piedade, F. S., Fonseca, F., Glória, E. M., Calori-Domingues, M. A., Piedade, S. M. S., & Barbin, D. (2002). Distribution of aflatoxins in contaminated corn fractions segregated by size. *Brazilian Journal of Microbiology, 33*(1), 12-16. doi:10.1590/S1517-83822002000100002

Pighinelli, A. L. M. T., & Gambetta, R. (2012). *Oilseeds*: IntechOpen.

Pitt, J. I., & Hocking, A. D. (2009). *Fungi and Food Spoilage* (3 ed.). New York: Springer-Verlag.

Ponce-García, N., Ramírez-Wong, B., Escalante-Aburto, A., Torres-Chávez, P. I., & Serna-Saldivar, S. O. (2017). Grading Factors of Wheat Kernels Based on Their Physical Properties. In *Wheat Improvement, Management and Utilization*.

Poor, M., Balint, M., Hetenyi, C., Goder, B., Kunsagi-Mate, S., Koszegi, T., & Lemli, B. (2017). Investigation of Non-Covalent Interactions of Aflatoxins (B1, B2, G1, G2, and M1) with Serum Albumin. *Toxins (Basel), 9*(11). doi:10.3390/toxins9110339

Prencipe, S., Siciliano, I., Gatti, C., Garibaldi, A., Gullino, M. L., Botta, R., & Spadaro, D. (2018). Several species of Penicillium isolated from chestnut flour processing are pathogenic on fresh chestnuts and produce mycotoxins. *Food Microbiol, 76*, 396-404. doi:10.1016/j.fm.2018.07.003

Price, R. L., & Jorgensen, K. V. (1985). Effects of Processing on Aflatoxin Levels and on Mutagenic Potential of Tortillas made from Naturally Contaminated Corn.

Ramesh, B., Reddy, B. S., Veerangoud, M., Anantachar, M., Sharanagouda, H., & Shanwad, U. K. (2015). Properties of Cotton Seed in Relation to Design of a Pneumatic Seed Metering Device. *Indian Journal of Dryland Agricultural Research and Development, 30*(1). doi:10.5958/2231-6701.2015.00011.1

Rapisarda, V., Loreto, C., Malaguarnera, M., Ardiri, A., Proiti, M., Rigano, G., . . . Bertino, G. (2016). Hepatocellular carcinoma and the risk of occupational exposure. *World J Hepatol, 8*(13), 573-590. doi:10.4254/wjh.v8.i13.573

Richards, F. J. (1927). The Relation Between Respiration and Water Content in Higher Fungi: With a Note on the Effect of Light on Respiration. *The New Phytologist, 26*(3), 187-201.

Ritchie, G. L., Bednarz, C. W., Jost, P. H., & Brown, S. M. (2007). Cotton Growth and Development. *The University of Georgia Cooperatiive Extension*.

Robertson, J. A., Chapman, G. W., & Wilson, R. L. (1984). Effect of moisture content of oil type sunflower seed on fungal growth and seed quality during storage. *Journal of the American Oil Chemists' Society, 61*, 768-771.

Saberi, M., Modarres-Sanavy, S. A. M., Zare, R., & Ghomi, H. (2018). Amelioration of Photosynthesis and Quality of Wheat under Non-thermal Radio Frequency Plasma Treatment. *Sci Rep, 8*(1), 11655. doi:10.1038/s41598-018-30200-7

Sabillón Galeas, L. E. (2014). *Understanding the Factors Affecting Microbiological Quality of Wheat Milled Products: From Wheat Fields to Milling Operations.* (Masters Thesis).

Samarajeewa, U., Sen, A. C., Cohen, M. D., & Wei, C. I. (1990). Detoxification of Aflatoxins in Foods and Feeds by Physical and Chemical Methods (1). *J Food Prot, 53*(6), 489-501. doi:10.4315/0362-028X-53.6.489

Sanchis, V., & Cranfield, N. M. (2004). Environmental conditions affecting mycotoxins. In N. Magan & M. Olsen (Eds.), *Mycotoxins in food: Detection and control* (pp. 174-186). Abington, Cambridge: Woodhead Publishing Limited.

Sarıyar, L., & Heperkan, D. (2003). The role of Aspergillus flavus and Aspergillus niger in the hydrolysis of hazelnut fat. *International Journal of Food Science & Technology, 38*(4), 487-492. doi:10.1046/j.1365-2621.2003.00702.x

Saxena, D. K., Sharma, S. K., & Sambi, S. S. (2011). Comparative Extraction of Cottonseed Oil by n-Hexane and Ethanol. *Asian Research Publishing Network (ARPN) Journal of Engineering and Applied Sciences, 6*(1), 84-89.

Searcy, S. W., Willcut, M. H., Buschermohle, M. J., Wankura, J. D., Brashears, A. D., & Barnes, E. M. (2010). Seed Cotton Handling & storage. *Cotton Inc.*

Selcuk, M., Oksuz, L., & Basaran, P. (2008). Decontamination of grains and legumes infected with Aspergillus spp. and Penicillum spp. by cold plasma treatment. *Bioresour Technol, 99*(11), 5104-5109. doi:10.1016/j.biortech.2007.09.076

Sen, Y., Onal-Ulusoy, B., & Mutlu, M. (2019). Aspergillus decontamination in hazelnuts: Evaluation of atmospheric and low-pressure plasma technology. *Innovative Food Science & Emerging Technologies, 54*, 235-242. doi:10.1016/j.ifset.2019.04.014

Shaw, C. S. (1962). *Cottonseed Drying and Storage at Cotton Gins*: United States Department of Agriculture.

Shaw, C. S., & Franks, G. N. (1962). *Cottonseed Drying and Storage at Cotton Gins*: United States Department of Agriculture.

Shi, H., Cooper, B., Stroshine, R. L., Ileleji, K. E., & Keener, K. M. (2017). Structures of Degradation Products and Degradation Pathways of Aflatoxin B1 by High-Voltage Atmospheric Cold Plasma (HVACP) Treatment. *J Agric Food Chem, 65*(30), 6222-6230. doi:10.1021/acs.jafc.7b01604

Shi, H., Ileleji, K., Stroshine, R. L., Keener, K., & Jensen, J. L. (2017). Reduction of Aflatoxin in Corn by High Voltage Atmospheric Cold Plasma. *Food and Bioprocess Technology, 10*(6), 1042-1052. doi:10.1007/s11947-017-1873-8

Shi, H., Stroshine, R. L., & Ileleji, K. (2017). Differences in Kernel Shape, Size, and Density between Healthy Kernels and Mold Discolored Kernels and their Relationship to Reduction in Aflatoxin Levels in a Sample of Shelled Corn. *Applied Engineering in Agriculture, 33*(3), 421-431. doi:https://doi.org/10.13031/aea.12037

Siciliano, I., Spadaro, D., Prelle, A., Vallauri, D., Cavallero, M. C., Garibaldi, A., & Gullino, M. L. (2016). Use of Cold Atmospheric Plasma to Detoxify Hazelnuts from Aflatoxins. *Toxins (Basel), 8*(5). doi:10.3390/toxins8050125

Smalley, S. A., & Bicknell, E. J. (1982). Gossypol Toxicity in Dairy Cattle. *Compendium on Continuing Education for the Practising Veterinarian, 4*(9), S378-S381.

Smith, C. W., & Cothren, J. T. (1999). *Cotton : origin, history, technology, and production*. New York: J. Wiley.

Somorin, Y. M., Bankole, M. O., Omemu, A. M., & Atanda, O. O. (2011). Milling on the Microbiological Quality of Yam Flour in Southwestern Nigeria. *Research Journal of Microbiology, 6*, 480-487. doi:10.3923/jm.2011.480.487

Song, H. P., Kim, B., Choe, J. H., Jung, S., Moon, S. Y., Choe, W., & Jo, C. (2009). Evaluation of atmospheric pressure plasma to improve the safety of sliced cheese and ham inoculated by 3-strain cocktail Listeria monocytogenes. *Food Microbiol, 26*(4), 432-436. doi:10.1016/j.fm.2009.02.010

Stepien, A., Wojtkowiak, K., & Pietrzak-Fiecko, R. (2017). Nutrient content, fat yield and fatty acid profile of winter rapeseed (Brassica napus L.) grown under different agricultural production systems. *Chilean journal of agricultural research, 77*(3), 266-272. doi:10.4067/s0718-58392017000300266

Strosnider, H., Azziz-Baumgartner, E., Banziger, M., Bhat, R. V., Breiman, R., Brune, M. N., . . . Wilson, D. (2006). Workgroup report: public health strategies for reducing aflatoxin exposure in developing countries. *Environ Health Perspect, 114*(12), 1898-1903. doi:10.1289/ehp.9302

Stuart, L. S. (1940). Effect of Protein Concentration and Cysteine on Growth of Halophilic Basteria. *Journal of Agricultural Research, 61*, 267-275.

Suhem, K., Matan, N., Nisoa, M., & Matan, N. (2013). In Vitro and In Vivo Antifungal Activities of Various Gas Species Under Plasma Jet Treatment Against Brown Rice Cereal Spoilage Molds. *International Food Research Journal, 20*(2), 947-951.

Surowsky, B., Fischer, A., Schlueter, O., & Knorr, D. (2013). Cold plasma effects on enzyme activity in a model food system. *Innovative Food Science & Emerging Technologies, 19*, 146-152. doi:10.1016/j.ifset.2013.04.002

Tappi, S., Gozzi, G., Vannini, L., Berardinelli, A., Romani, S., Ragni, L., & Rocculi, P. (2016). Cold plasma treatment for fresh-cut melon stabilization. *Innovative Food Science & Emerging Technologies, 33*, 225-233. doi:10.1016/j.ifset.2015.12.022

Udomkun, P., Wiredu, A. N., Nagle, M., Muller, J., Vanlauwe, B., & Bandyopadhyay, R. (2017). Innovative technologies to manage aflatoxins in foods and feeds and the profitability of application - A review. *Food Control, 76*, 127-138. doi:10.1016/j.foodcont.2017.01.008

Walker, G. M., & White, N. A. (2018). Introduction to Fungal Physiology. In K. Kavanagh (Ed.), *Fungi: Biology and Applications* (3 ed.): John Wiley & Sons, Inc.

Wang, R. X., Nian, W. F., Wu, H. Y., Feng, H. Q., Zhang, K., Zhang, J., . . . Fang, J. (2012). Atmospheric-pressure cold plasma treatment of contaminated fresh fruit and vegetable slices: inactivation and physiochemical properties evaluation. *The European Physical Journal D, 66*(10). doi:10.1140/epjd/e2012-30053-1

Weltmann, K. D., Kolb, J. F., Holub, M., Uhrlandt, D., Šimek, M., Ostrikov, K., . . . Becker, K. (2018). The Future for Plasma Science and Technology. *Plasma Processes and Polymers, 16*(1). doi:10.1002/ppap.201800118

White, P. J. (2000). Fatty Acids in Oilseeds. In C. K. Chow (Ed.), *Fatty acids in foods and their health implications* (2 ed., pp. 1045). New York.

Whitten, M. E. (1970). Aflatoxins in Cottonseed Hulls. *Journal of American Oil Chemists' Society, 45*, 575-576.

Won, M. Y., Lee, S. J., & Min, S. C. (2017). Mandarin preservation by microwave-powered cold plasma treatment. *Innovative Food Science & Emerging Technologies, 39*, 25-32. doi:10.1016/j.ifset.2016.10.021

Wu, F., Liu, Y., & Bhatnagar, D. (2008). Cost-Effectiveness of Aflatoxin Control Methods: Economic Incentives. *Toxin Reviews, 27*(3-4), 203-225. doi:10.1080/15569540802393690

Wu, F., & Munkvold, G. P. (2008a). Mycotoxins in Ethanol Co-products: Modeling Economic Impacts on the Livestock Industry and Management Strategies. *Journal of Agricultural and Food Chemistry, 56*, 3900-3911.

Wu, F., & Munkvold, G. P. (2008b). Mycotoxins in Ethanol Co-products: Modeling Economic Impacts on the Livestock Industry and Management Strategies. *Journal of Agricultural and Food Chemistry, 56*(11), 3900-3911. doi:https://doi.org/10.1021/jf072697e

Zhou, Z., Huang, Y., Yang, S., & Chen, W. (2011). Introduction of a new atmospheric pressure plasma device and application on tomato seeds. *Agricultural Sciences, 02*(01), 23-27. doi:10.4236/as.2011.21004

Ziegler, G. M., Kadan, R. S., Freeman, D. W., & Spadaro, J. J. (1981). A direct Hexane Extraction Process for Glanded Cottonseed. *Journal of Food Science, 47*, 315-316.

APPENDIX A

TEMPERATURE AND RERLATIVE HUMIDITY SENSOR READINGS

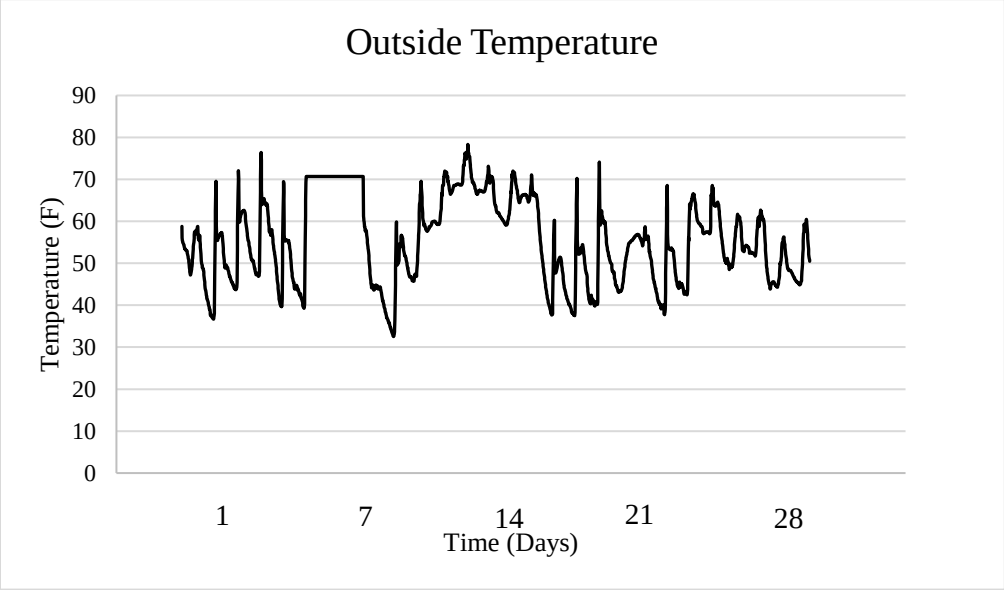

Figure 18. The outside sesor readings for the outside temperature readings.

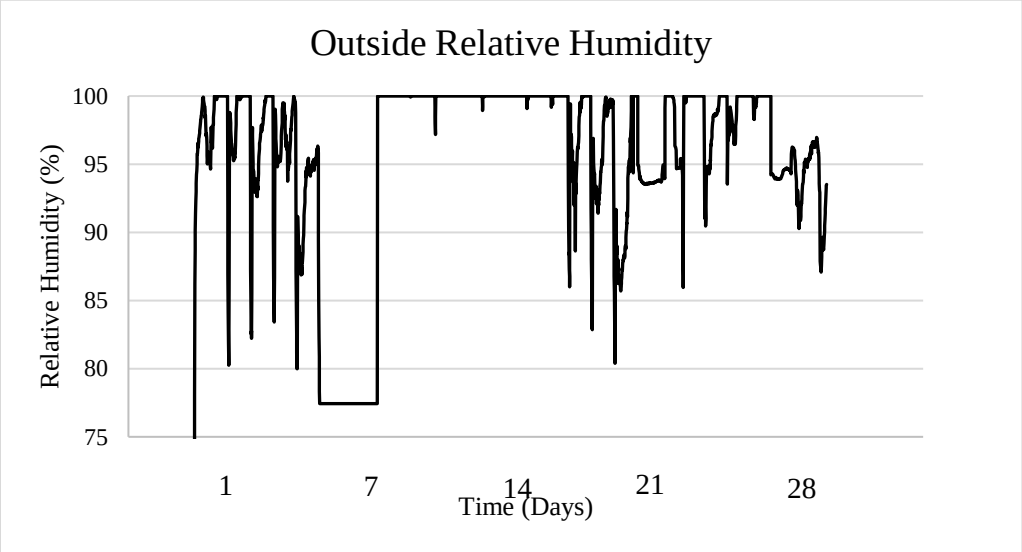

Figure 19. The outside sesor readings for the outside relative humidity readings.

APPENDIX B

ANOVA RESULTS

	DF	Sum of Squares	Mean Square	F-Value	P-Value
Temperature	2	1.727×10^{9}	8.635×10^{8}	10.31	4.308×10^{-5}
Sample	2	3.573×10^{9}	1.789×10^{9}	21.34	1.588×10^{-9}
Interaction	4	2.344×10^{9}	5.860×10^{8}	6.998	1.888×10^{-5}
Model	8	7.648×10^{9}	9.560×10^{8}	11.42	11.35×10^{-14}
Error	394	3.299×10^{10}	8.373×10^{7}	--	--
Corrected Total	402	4.064×10^{10}	--	--	--

Table 9. ANOVA test results for microbial growth.

	DF	Sum of Squares	Mean Square	F-Value	P-Value
Temperature	2	74.00	37.00	3.868	0.0301
Sample	2	150.1	75.05	7.844	0.0015
Interaction	4	42.54	10.63	1.112	0.3661
Model	8	266.6	33.33	3.484	0.0045
Error	36	344.4	9.567	--	--
Corrected Total	44	611.0	--	--	--

Table 10. ANOVA test results for protein levels.

	DF	Sum of Squares	Mean Square	F-Value	P-Value
Temperature	2	5.852	2.926	8.506	9.431×10^{-4}
Sample	2	723.2	361.6	1051	1.179×10^{-32}
Interaction	4	0.270	0.0676	0.196	0.9387
Model	8	729.4	91.17	265.0	1.288×10^{-29}
Error	36	12.38	0.344	--	--
Corrected Total	44	741.75	--	--	--

Table 11. ANOVA test results for fat acidity levels.

	DF	Sum of Squares	Mean Square	F-Value	P-Value
Temperature	2	0.768	0.384	229.8	3.165×10^{-21}
Sample	2	0.207	0.103	61.84	2.266×10^{-12}
Interaction	4	0.014	0.035	21.39	4.187×10^{-9}
Model	8	1.118	0.140	83.61	6.403×10^{-21}
Error	36	0.060	0.002	--	--
Corrected Total	44	1.178	--	--	--

Table 12. ANOVA test results for water activity levels.