APPLICATION OF ELECTRON BEAM TECHNOLOGY ON FREEZE-DRIED BERRIES FOR FUNGAL DECONTAMINATION

A Thesis

by

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ABSTRACT

Safety, quality, and sensory perception of foods are of paramount importance for food preservation. This study explores the combination of freeze-drying technology with electron beam processing (eBeam) processing to create a low microbial bioburden of food without compromising food quality. The hurdle approach in food processing and preservation operates by combining two or more techniques to extend food shelf life. Freeze-drying and eBeam processing are non-thermal processes that have complementary effects for food preservation. The purpose of this study was to understand the antimicrobial effects of eBeam processing on a freeze-dried berry medley. Freeze-dried berry medley consisting of strawberries, blackberries and raspberries was exposed to specific doses of eBeam radiation (3, 5, and 10 kGy) to isolate fungi that were resistant to these eBeam doses. The isolates that survived the eBeam processing were identified (*Aspergillus* sp., *Penicillium* sp., *Cladosporium* sp.) using Internal Transcribed Spacer (ITS) sequencing and their D_{10} values were determined. A dose validation study was then performed on the freeze-dried berries to show that 15 kGy was sufficient to eliminate all fungal spores/mycelia in the freeze-dried berry product. Quality attributes of the berries were analyzed for changes due to eBeam processing using multidimensional gas-chromatography – olfactometry – mass spectrometry (MDGC-O-MS). Four (4) volatile compounds showed significant increases ($P < 0.05$) by the eBeam treatment; 2-butenal, 3-methyl butenal, ethyl acetate, 2-furancarboxaldehyde. One (1) volatile compound showed significant decrease ($P < 0.05$) by the eBeam process; alpha pinene. Color attributes were tested for any changes due to eBeam processing using a colorimeter; no significant color changes were observed ($P < 0.05$) for L*, a*, or b* values of each individual berry except for the a* value of strawberries. Minimal changes to freeze-dried berry medley were observed with eBeam processing between 0 to 15 kGy.

DEDICATION

I dedicate this project to my wife, Stefanie Low Kalkstein O'Neil, and to my family & friends. This work would not be possible without their support.

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NOMENCLATURE

AFT	Advanced Food Technology
ARS	Acute Radiation Syndrome
ASL	Accelerated Shelf Life
BAM	Bacterial Analytical Manual
CCP	Critical Control Points
CDC	Center for Disease Control and Prevention
CFR	Code of Federal Regulation
D_{10}	Radiation dose required to inactivate 90% of a microbial population
DNA	Deoxyribonucleic Acid
DRBC	Dichloran Rose Bengal Chloramphenicol
DUR	Dose Uniformity Ratio
eBeam	Electron Beam
EOS	End Of Shelf-life
FD	Freeze-Dry
FDA	Food and Drug Administration
HACCP	Hazard Analysis Critical Control Points
HPLC	High Performance Liquid Chromatography
IRB	Institutional Review Board
ISS	International Space Station
ITS	Internal Transcribed Spacer
kGy	kilo Gray
mAyNC	Metabolically Active Yet Not Culturable
MAP	Modified Atmospheric Packaging
MeV	Megaelectronvolt
MRE	Meal Ready-to-Eat
MDGC-O-MS	Multidimensional Gas-Chromatography – Olfactometry – Mass Spectrometry
NASA	National Aeronautics & Space Administration
NCEBR	National Center for Electron Beam Research

PBS	Phosphate Buffered Saline
SDA	Sabouraud Dextrose Agar
SFRF	Space Food Research Facility
SFSL	Space Food Systems Laboratory
SPME	Solid Phase Micro Extraction

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CHAPTER I

INTRODUCTION

New food processing technologies are emerging out into the market place and the road to commercialization of a novel processes can be treacherous. Each new processing technique requires research to ensure consumer safety. The Food and Drug Administration (FDA) regulates the uses of these processes but ultimately the acceptance into the consumer market are the driving forces toward commercialization (Junqueira-Gonçalves et al., 2011).

Electron beam technology currently has commercial applications in the medical industry for medical device sterilization (Gotzmann et al., 2018). The polymer industry is using extremely high doses (above 50 kGy) for the crosslinking of polymers to make plastics stronger (Drobny, 2013). The food industry uses the technology for pathogen prevention and phytosanitary processing (Pillai et al., 2014). There are also uses in the aseptic packaging in and processing industry for package surface sterilization (Pillai & Shayanfar, 2015).

The commercial freeze-drying industry was valued at $47 billion in 2016 and projected to grow to $66.5 billion by 2021. The industry is primarily focused on the preservation of foods for camping, military, breakfast cereals and long duration space flight (McHugh, 2018). Freeze-drying is commonly accompanied with a thermal kill step (cooking) to reduce the microbial load prior to the freeze-drying process. There are freeze-dried food products that do not have a kill step associated with the process (by design), as thermal processing will change the structure of the food by degrading the nutrient quality of fruits & vegetables (Lund, 1988). The application of eBeam processing can reduce the microbial load in these commercial products to improve the quality and safety of the food.

The hospital food industry is a potential market for the combination of freeze-drying with eBeam processing for microbial bioburden reduction of hospital foods. Immunosuppressed hospital patients require a diet free from microbial contamination (Smith et al., 2014). Neutropenia is a very low count of neutrophils in the blood which can be onset by chemotherapy (DeMille et al, 2006). The neutropenic diet is being utilized in various countries such as Brazil (Vicenski et al, 2012). These low white blood cell counts make the immune systems of these patients drastically suppressed that even the smallest amount of an opportunistic organism can have major consequences on patient health and outcome (Farkas, 2016). Fruits and vegetables have natural fungal contamination that remains even after minimal processing (Ribes, 2018). The exploration of new food processing techniques and combination of food preservation methods is a clear potential solution for the decontamination of fruits and vegetables for the immunocompromised patient. Majority of commercially sterile food options are thermostabilized (canned food), which limits the type of fresh food products available for the immunocompromised. The combination of freeze-drying with eBeam processing can produce a product that is commercially sterile, mitigating risk of infection, and can be performed while in the final package, preventing any post process contamination. Both processing techniques are non-thermal and have complementary principles to retain quality. The freeze-drying aspect of the process removes the water from the food product. This controls the water available for microbial metabolic activity (Nester et al., 2007). The combination with eBeam processing in the package inactivates the microbes in the food sample without adding heat to the product and prevents post process contamination (Pillai & Shayanfar, 2015).

The research on neutropenic diets is currently unclear on the efficacy of the diet to mitigate infection during chemotherapy due to inconclusive studies based on small sample sizes and poorly

designed metrics (Jubelirer, 2011), However, hospitals still provide foods that have low microbial counts for the immunosuppressed (Trifilio et al., 2012; Moody et al., 2002). The cooked food diet follows the same principles of the neutropenic diet where anyone with a compromised immune system is fed a strict diet of cooked foods to lower the risk of infection from all microorganisms. There are no standard practices or menu items for the neutropenic diet but there are menu restrictions for the cooked food diet (Mank & Davies, 2008). These cooked food diets degrade the quality of the foods leaving the menu with no fresh items.

A very small niche market is the application of combining freeze-drying with eBeam in the space industry for serving as food for astronauts on the space station, space tourist and astronauts on long duration missions to other planets and heavenly bodies. The same value can be proposed with long durations space flight. Commercial companies are starting to privatize an independent space station (Axiom, Boeing, SpaceX) and these companies can benefit from this research when designing a food system (Crawley, 2018). Personal experience in the Space Food System Laboratory and Space Food Research Facility, freeze-drying berries without any microbial inactivation intervention has a high potential to fail the microbiological standards for yeast and mold that have been set by the National Aeronautics and Space Administration for space flight. These freeze-dried berries have been removed from the menu due to inconsistent microbial acceptance, leaving a gap in the diet of astronauts. Fresh fruits and vegetables are sent to the International Space Station (ISS) but with no refrigeration and the transport time, these fresh items do not last for more than a day or two on station. eBeam processing of fruits and vegetables is a solution to increase the menu options for astronauts. Fresh foods are not even an option for long duration space flight beyond lower earth orbit (i.e., Mars). Space is a stressful environment

(physically and mentally), where long durations space flights will create similar situations of microbial susceptibility and food fatigue.

The world population is rapidly growing, and a safe and robust food system is necessary to meet the consumer demand (Shayanfar & Pillai, 2018). The use of eBeam processing has proven to be successful in reducing the bioburden of microbial populations in a food stuffs without degrading the quality (flavor, color, aroma, texture etc.) of the product (Shayanfar et al., 2016). It has been reported that a 1 to 2 log reduction in overall microbial bioburden can be achieved using less than 1 kGy dose (Shayanfar et al., 2014). eBeam processing has been performed on dry nuts and showed to induce lipid oxidation (rancidity) at doses between 0 to 10 kGy but there were minimal changes to organic acids. Berries are low in fats and high in acids, making berries a great candidate for eBeam processing (Sánchez-Bel et al., 2008). Utilizing a hurdle approach, the combination of eBeam technology with freeze-drying can have synergistic effects to extend the shelf life of foods and preserve quality while minimizing post processing contamination.

The hypothesis is that the fungal bioburden of freeze-dried berries is susceptible to eBeam processing. The specific objectives of this study were:

1. To isolate and identify radiation resistant fungi from a berry medley. The hypothesis is that radiation resistant fungi can be isolated from a berry medley prepared from commercially available berries.
2. To quantify the radiation resistance of eBeam resistant fungi. The hypothesis is that fungi on berries are susceptible to eBeam radiation.
3. To evaluate the effect of eBeam processing on sensory (color, flavor and aroma) attributes of the freeze-dried-eBeam treated berry medley. The hypothesis is that there will be minimal adverse effects of eBeam processing on freeze-dried berry medley.

CHAPTER II

LITERATURE REVIEW

2.1 USE OF BERRIES MEDLEYS

Fruits are a part of everyday life for consumers. Their-health benefits arising from their phytochemicals (carotenoids and polyphenols) make them essential for physical wellness (Bowen-Forbes et al., 2010; Jimenez-Garcia et al., 2013). Clinical studies have shown that diets high in berries reduce the risk of cardiovascular disease (CVD; Yang & Kortesniemi, 2015; Basu et al., 2010). Pomegranate and berry juices in the diet have shown to reduce the risk of prostate cancer (Malik & Mukhtar, 2006). The health benefits of a high fruit diet extend beyond prevention of CVD and cancer formation; fresh berries are also high in dietary fiber, both soluble and insoluble fiber that promote healthy digestion (Nile and Park, 2014). They have a high concentration of natural antioxidants such as thiols and ascorbic acid which reduce the formation of free radicals in foods (Gülçin, 2011). There are anti-inflammatory benefits with a berry diet. Freeze-dried strawberries were used to measure human inflammation and the results concluded that berry supplemented diets significantly reduce inflammatory responses (Joseph et al., 2014). The natural sugars and dietary fiber in berries have been shown to be beneficial in regulating blood glucose levels mitigating risks of type II diabetes (Shi et al., 2017).

Berries have numerous health benefits that promote a healthy diet which leads to the prevention of obesity and diabetes from anthocyanins acting on adipose tissue (Tsuda, 2016). Type II diabetes affects roughly 16.6 million human beings and causes a strain on the U.S. healthcare system of roughly 159 billion dollars as reported in 2007 (Dall et al., 2010). Obesity in the United States has continually trended upward and projected to continue (Thorpe et al., 2004). The cost of

obesity related healthcare was calculated over a three-year period (2008-2010) to be $1.1 billion dollars (Tremmel et al., 2017). The number one cause of death in the United States is cardiovascular disease (CVD) with a reported 900,000 deaths in 2016 (Roth & Johnson et al., 2018). A decrease in the number of diabetic patients and/or CVD healthcare expenses would have a significant cost savings effect that can be reallocated to fund cancer research.

2.2 MICROBIOLOGICAL HAZARDS ASSOCIATED WITH FRUITS AND VEGETABLES FOR FOOD SPOILAGE

Food spoilage and pathogenic fungi are present in fruits and vegetables and have the ability to survive and grow on any type of food product (FDA, 2001). Contamination of fruits can be accomplished through the soil, aerosols, or farm runoff where the fruits and vegetables are grown (Oluwadara et al., 2018). Spoilage organisms have the potential cause harm if ingested by humans, but they more commonly have detrimental sensory effects on the foods they grow on (Pitt, 2014). Few publications have been released on the financial impact of fungal spoilage for fruits because spoilage can go unreported. The estimated impact is approximately 2 - 10 million dollars annually since 2005 (Snyder & Worobo, 2018). Pathogenic fungi are concerning for the ability to produce mycotoxins that can cause adverse health consequences (Fernández-Cruz et al., 2010). Even a comprehensive food safety program can have weaknesses in the process that allow for pathogenic outbreaks in the berry industry. Electron beam processing has been proven to inactivate pathogenic organisms (Pillai et al., 2014). Non-thermal processes, such as eBeam, are viable options when combined with a comprehensive Hazard Analysis Critical Control Points (HACCP) program to prevent produce recalls (Smith & Pillai, 2004). Outbreaks occur in the produce industry every year and cause consumer illness. Very few outbreaks have occurred in the berry industry in the past 30

years. An outbreak of *E. coli* O157:H7 in Oregon was reported from a small market grower that killed one person and injured 16 others in 2011 (Palumbo et al., 2016). The use of this technology in the fresh produce industry could prevent these types of outbreaks (Espinosa et al., 2011; Palekar et al., 2015).

Establishing a Process for Fungi Elimination Criterion in Freeze-Dried Berries

New product development in thermal processing must comply with FDA standards for establishing a commercially sterile food (Title 21 U.S. Code of Federal Regulations, part 113.83). The processor is to first identify biological hazards that are of concern to human health. This would include organisms that are naturally present in high numbers or highly resistant to the process in question. *Clostridium botulinum* (a spore forming bacterium) is the organism of concern in the retort processing industry given its thermal resistance and ability to produce a neurotoxin that can be extremely harmful to humans if ingested (Nester et al., 2007). The goal is to prevent the organism's ability to metabolize substrates by controlling the food matrix (ie pH for preventing optimal growing conditions) or by total elimination of the organism with confidence. Challenge studies are performed in the canning industry with *C. botulinum* or other surrogates to ensure food safety. Once the biological hazards are identified, Critical Control Points (CCP's) are then assigned and recorded to the process to ensure process uniformity from batch to batch (Title 21 U.S. Code of Federal Regulations, part 113.89; Title 21 U.S. Code of Federal Regulations, part 113.100). The industry standard for the commercial sterility of a food product in a hermetically sealed container is a 12D process or a 12-log reduction. The 12D concept is based on the inactivation rate of worse case scenarios of microbial contamination. The D-value is the time required at a constant temperature to achieve a 90% reduction of the microorganisms. The same approach is taken with

other microbial inactivation processes. The rate of inactivation is set against a constant condition to determine microbe survival rates. The D_{10} value is the dose required to achieve a 90% reduction of the microorganism studied.

There are fungi that are known to be resistant to eBeam processing such as *Cladosporium* sp. (Jay et al., 2005). This organism is also commonly found in the soil which is known to contaminate fruits and vegetables during harvest. This can be considered an organism of concern with respect to fungal contamination on fruits and vegetables and can be used as an indicator organism. If *Cladosporium* spp. is present on a food prior to freeze-drying, then it will still be present after the process. The industry practice is to set the process based on this organism's resistance and natural bioburden to simulate a worst-case scenario. *Cladosporium* sp. is an organism that is resistant to radiation processing and can be used as a challenge organism for process validation. By setting the eBeam process dose to eliminate *Cladosporium* sp., all other fungi should be (theoretically) eliminated with the process. Once the minimum eBeam dose is established, the next step will be to study the effects of the process on the food matrix. Both thermal processing and eBeam processing are known to change the food matrix in a way that can be undesirable (Kim et al., 2009; Fan, 2014). Therefore, the effects of eBeam processing on the sensory and other attributes of berry medley need to be studied.

Microbes that have been isolated from food samples provide real-world contamination scenarios specific to berries. This is a method for establishing a target organism that is used to set an eBeam dose. Once extracted, the organism must be identified using morphological or molecular methods. There are key differences when working with lab cultures compared to environmental cultures. Environmental cultures are exposed to harsh environments which create resistance and resilience when re-exposure occurs to the same harsh environments. Using microbes that have

been cultured from food samples reflects a greater real-world contamination and inactivation scenario.

Identification of Microorganisms

Microorganisms that are extracted from food samples can be identified using morphological features but for improved accuracy, the organisms can be identified using molecular methods. With big data merging with metagenomics, it is becoming more and more common to identify organisms based on their genetic makeup (Gilbert, 2015; Donovan et al., 2018). A variety of DNA extraction kits are available commercially (Griffin et al., 2002). There are multiple methods for identification using the extracted DNA. A common protocol for fungi is PCR amplification of the Internal Transcribed Spacer (ITS) sequence using universal fungal primers (Buehler et al., 2017). This is a highly conserved region of ribosomal 18S and 5.8S rRNA genes (approximately 800 bp; Schoch et al., 2012). PCR amplified fragments of this sequence can then be uploaded and match to a known and validated database of organisms.

2.3 FREEZE-DRYING TECHNOLOGY

Freeze-drying (lyophilization) is a thermal process, designed to remove the free water from a food to control microbial growth and enzymatic activity, however, the temperatures achieved during processing are not high enough to generate microbial lethality but low enough not to cause any damage to the food product (Bourdoux et al., 2016). This means that organisms capable of food spoilage present before processing can survive and remain in a food product after freeze-drying, especially organisms that can survive harsh environments (i.e., fungal and bacterial spores; Jay et al., 2005). The freeze-drying process works by freezing a food sample and then spreading it

out on thin sheet pans (creating surface area) and then placing the trays into a vacuum chamber (Mellor & Bell, 1993). The entire vacuum chamber will then freeze before the immense vacuum is created (about 100 mTorr). The chamber is then slowly heated, incrementally, from -80^{o} C to 20^{o} C. The water changes phases from solid to vapor while under a vacuum (sublimation) (Assegehegn, 2018). The water vapor is then re-condensed with cooling coils in another section of the vacuum chamber, successfully dehydrating the food product. Once out of the freeze-dryer, the food product must then be packaged. The exposure to the ambient air poses a risk to up moisture and microbial contamination. Just like all processes, there are pitfalls that accompany the technology (Ratti, 2001). The cost of a freeze-dyer and components (i.e., software, computer controls etc.) can be expensive compared to other dehydration processes (Ciurzynska & Lenart, 2011). The freeze-dryer also requires utilities such as chilled water and compressed nitrogen to properly function which can increase the operational costs (Flink, 1977). Not only will a capital investment be required for purchasing the equipment, the components of a freeze-dryer must be regularly serviced for preventative maintenance by qualified personnel as an ongoing expense (Kasper et al., 2013). However, even with the expense of operating a freeze-dryer, the value added can produce a cost-effective product depending on the market. The backpacking and camping industry use this process to create ready-to-eat meals (RTE) for back country exploration. NASA has utilized freeze-drying techniques for food preservation since the space shuttle program (Bourland et al., 1981). The economic model works as these products are sold as a premium product for a substantial higher price.

2.4 ELECTRON BEAM PROCESSING

Principles of Electron Beam Processing

Electron beam (eBeam) processing is a non-thermal process, measured in grays, that can inactivate organisms by direct and indirect interactions of electrons that break DNA bonds, rendering the organism inactive and unable to replicate (Pillai & Shayanfar, 2015). The direct method is the sheer force of the electrons breaking apart the DNA backbone. The indirect effects of inactivation are the interactions the electrons have with surrounding molecules near the DNA strands. The energy from the electrons can break down the molecules and create highly reactive free radicals, which can then react with the phosphodiester backbone of the DNA. The most abundant molecule that can cause indirect damage to the DNA backbone is water, which is a major component in fresh fruits and vegetables. By removing the water from fresh fruit, the critical factors (ie package dimension, food pH, moisture content etc.) change causing a change in inactivation rates of microorganisms. Therefore, it is important to study the inactivation rates of microorganisms for each individual product.

Each product will have unique specifications that must be carefully studied to ensure maximum or minimum doses are achieved during processing. This is referred to as dose mapping and it is a process for modeling the radiation penetration in all sections of the food (Rivadeneira et al., 2007). Similar studies are performed in the thermal processing industry called heat penetration studies (Ali et al., 2005). Measuring the radiation dose of a product is done by placing alanine pellets inside the food product at various depths/positions. There is an initial increase of energy into the product with first contact; however, the energy eventually dissipates and creates a peak dose after initial penetration. This must be accounted for during dose mapping as the highest

dose is not necessarily the first contact point. Density and package thickness influence the dose/depth curve. The aerial density calculation (Eq. 1) is used to determine if a package can be eBeam processed using a 10 MeV linear accelerator. Aerial density that is between 0-3.3 use single beam eBeam processing as long as the dose uniformity ratio (DUR) is below 2. Aerial density between 3.3-8 must utilize dual beam eBeam processing (Brown, 2015). The package must be processed from both directions, either using two beams concurrently or one beam and flipping package to process the other side using the same beam. Aerial densities above 8.3 cannot be processed using electron beam without package reconfiguration.

(1) Aerial Density = (weight in lbs. * 70.4) / (area perpendicular to the beam in inches^2)

There are also interferences with the packaging material and voids due to package configuration that can affect the radiation distribution as studied with the dose mapping of live oysters inside a shell (Praveen et al., 2013). Another concept in dose mapping is the DUR which is the ratio of maximum dose / minimum dose achieved in the processed sample and is critical for determining process efficiency.

The efficiency of eBeam processing is drastically lowered on dried foods as compared to fresh fruits and vegetables due to the lack of availability of free water in such samples (Ic et al., 2006; Farkas, 2007). Assuming a linear inactivation rate on freeze-dried foods, the eBeam process will still achieve the desired log reduction; it will just require a higher dose. Although a higher dose will be needed, the reduced moisture environment could prevent undesirable quality changes to the food matrix (retention of ascorbic acid and color values) in eBeam processing (Wei et al., 2014).

Irradiation Regulation in the United States

New food processing techniques must meet or exceed regulatory compliance requirements for consumer safety (Smith & Pillai, 2004). The Food and Drug Administration (FDA) is the governing body that regulates the use of eBeam processing for the commercial applications in prepared foods and fresh fruits & vegetables. eBeam processing and packaging must follow strict guidelines as stated in the Code of Federal Regulations (CFR) Title 21 part 179 to protect consumers from food processing corporations (FDA, 2017). Currently the FDA has put limits on the maximum allowable doses for eBeam processing (Title 21 part 179.26; Table 01).

Table 1: eBeam Dose Limitations by FDA Title 21 CFR Part 179.26

Use	**Limitations**
For control of *Trichinella spiralis* in pork carcasses or fresh, non-heat-processed cuts of pork carcasses	Minimum dose 0.3 kGy; maximum dose not to exceed 1 kGy.
For growth and maturation inhibition of fresh foods	Not to exceed 1 kGy.
For microbial disinfection of dry or dehydrated enzyme preparations (including immobilized enzymes)	Not to exceed 10 kGy.
For microbial disinfection of the following dry or dehydrated aromatic vegetable substances when used as ingredients in small amounts solely for flavoring or aroma: culinary herbs, seeds, spices, vegetable seasonings that are used to impart flavor but that are not either represented as, or appear to be, a vegetable that is eaten for its own sake, and blends of these aromatic vegetable substances. Turmeric and paprika may also be irradiated when they are to be used as color additives. The blends may contain sodium chloride and minor amounts of dry food ingredients ordinarily used in such blends	Not to exceed 30 kGy.
For control of food-borne pathogens in fresh (refrigerated or unrefrigerated) or frozen, uncooked poultry	Not to exceed 4.5 kGy for non-frozen products; not to exceed 7.0 kGy for frozen products.
For the sterilization of frozen, packaged meats used solely in the National Aeronautics and Space Administration space flight programs	Minimum dose 44 kGy

Table 1: Continued

Use	Limitations
For control of foodborne pathogens in, and extension of the shelf-life of, refrigerated or frozen, uncooked products that are meat within the meaning of 9 CFR 301.2(rr), meat byproducts within the meaning of 9 CFR 301.2(tt), or meat food products within the meaning of 9 CFR 301.2(uu), with or without nonfluid seasoning, that are otherwise composed solely of intact or ground meat, meat byproducts, or both meat and meat byproducts	Not to exceed 4.5 kGy maximum for refrigerated products; not to exceed 7.0 kGy maximum for frozen products.
For control of Salmonella in fresh shell eggs.	Not to exceed 3.0 kGy.
For control of microbial pathogens on seeds for sprouting.	Not to exceed 8.0 kGy.
For the control of Vibrio bacteria and other foodborne microorganisms in or on fresh or frozen molluscan shellfish.	Not to exceed 5.5 kGy.
For control of food-borne pathogens and extension of shelf-life in fresh iceberg lettuce and fresh spinach.	Not to exceed 4.0 kGy.
For control of foodborne pathogens, and extension of shelf-life, in unrefrigerated (as well as refrigerated) uncooked meat, meat byproducts, and certain meat food products	Not to exceed 4.5 kGy.
For control of food-borne pathogens in, and extension of the shelf-life of, chilled or frozen raw, cooked, or partially cooked crustaceans or dried crustaceans (water activity less than 0.85), with or without spices, minerals, inorganic salts, citrates, citric acid, and/or calcium disodium EDTA	Not to exceed 6.0 kGy
FDA, 2018	

These limits are set based on the approval of food additive petitions for industry use primarily for pathogen prevention or other microbial inactivation. Food packaging materials and food contact surfaces that are subject to ionizing radiation must comply with FDA ruling for integrity (Title 21 part 179.45). eBeam processing is considered a food additive and follows the food additive petition (Title 21 CFR 171.1(c)G; Shames, 2010). The first step is to petition to the FDA and define the desired changes. Supporting scientific data must be presented with the petition that the proposed changes are not going to harm the consumer. FDA will then evaluate the petition for the safety of the food and the impacts it can have on the environmental and consumer markets.

An official ruling is required from the FDA before foods can be sold in the United States with eBeam processing of fruits and vegetables above 1 kGy.

Ehlermann (2016) reported that food irradiation is safe for consumption at doses above 10 kGy as long as radiolytic byproducts have dissipated. Although foods are safe to consume when eBeam processed beyond 10 kGy, eBeam processing that utilize high doses (10 kGy or higher) are a concern to the food processing industry due to the formation of undesirable sensory changes to the food product (Feliciano, 2018). Currently, the only approved processes in the United States of America that allow processing above 10 kGy are for the microbial disinfection of spices (not to exceed 30 kGy; Title 21 part 179.26 (b)[5.]) and for the commercial sterility of frozen, packaged meats for the National Aeronautics and Space Administration (minimum dose 44 kGy; Title 21 part 179.26 (b)[7.]). The maximum doses for the control of foodborne pathogens in lettuce, spinach, sprouts, molluscan shellfish, shell eggs, fresh/frozen meats range between 3 kGy and 8 kGy.

Types of Ionizing Radiation and Their Features

Types of ionizing radiation are gamma radiation, beta radiation and x-rays. These sources of radiation have been extensively studied and compared for their utility of microbial inactivation. (Tallentire et al., 2010; Cleland, 2007). The modes of inactivation are the same for beta and gamma radiation, although the sources of the ionizing radiation, energies, penetration depth and dose rates are different (Pillai & Shayanfar, 2015; Praveen et al., 2013; Handayani & Permawati, 2017). Gamma radiation comes from the energy that is emitted from radioactive isotopes of Cobalt (^{60}CO) or Cesium (^{137}CS) where eBeam radiation source from Linear Accelerators (LINAC) powered by commercial electricity (Pillai & Shayanfar, 2017). The eBeam radiation process cannot penetrate

food stuffs as well as a gamma source of radiation or x-ray causing gamma to be more effective for penetration (Jeong & Kang, 2017). eBeam processing, although it has limitation on penetration depth, has a place in the food industry compared to other ionizing radiation due to the efficiency of energy transfer to the food (Smith et al., 2013). Low energy eBeam has been developed for surface sterilization of packaging materials in the aseptic packaging and processing market (Lindell, 2017). eBeam is an on/off technology making it a much safer and cost effect source of ionizing radiation for the food industry. However, just because the radiation can be turned off, that does not mean safety protocols should be relaxed. Facilities must follow all state and federal regulations (Occupational Safety and Health Administration, standard 1910.1096) for the safe practice of ionizing radiation. These radioactive isotopes are constantly emitting radiation and require very strict storage procedures to ensure the right amount of shielding is used to avoid any occupational hazards. Improper storage and handling of radioactive material can be extremely dangerous leading to radiation poisoning (Acute Radiation Syndrome ARS). eBeam technology is considered a green technology that utilizes commercial electricity that can be turned on and off (Lado & Yousef, 2002).

Commercial Applications of Electron Beam Processing

Commercial applications that are currently using electron beam processing for various applications have been processing mangos for phytosanitary purposes (Pillai et al., 2014). The FDA requires that imported mangos from foreign countries be processed for the destruction of insects of imported mangos. The technology is being adapted in the aseptic packaging and processing industry for the sterilization of food packaging material. The small size and minimal shielding requirements make low energy eBeam technology a beneficial addition to an aseptic

filler (Urgiles et al., 2007). Currently, aseptic fillers are utilizing peroxides to sterilize packaging, which is concerning due to the residuals of the peroxides making their way into the final package (Title 21 part 178.1005). Using eBeam technology eliminates the concern of peroxides making their way into the final packaged product and the concern of storing highly reactive peroxide. Currently, aseptic fillers have not been commissioned in the United States for production using eBeam sterilization.

2.5 COMBINATION OF FOOD PROCESSING TECHNIQUES FOR FOOD PRESERVATION

The combination of food processing techniques is not a new concept for the safety of a food product and to extend the shelf life (Mukhopadhyay & Gorris, 2014). The use of two or more processing techniques is called hurdle approach and is commonly used in the food industry for the preservation of food (Leistner, 2013). There is an array of food processing techniques available (conventional and novel) to the food industry and each with their own benefits and pitfalls. Hurdle concepts are to combine the benefits of multiple processing techniques (minimizing pitfalls) to create an environment that promotes food preservation (Mogren et al., 2018; Khan et al., 2017). An industry example of hurdle technology is the combination of thermal processing and lowering pH equal to or below 4.6 (Leistner, 2000). The thermal process reduces the bioburden with a kill step and the acidic manipulation of the food matrix inhibits the growth of various spoilage and pathogenic bacteria (Tucker, 2015). Combining complementary food processes with appropriate packaging, the safety and the quality of the food sample can drastically be increased (Nair & Sharma, 2016). The concept of hurdle technology can create benefits of multiple processing technologies on a food sample by reducing the stressors of one technology to optimize food quality and safety (Degala et al., 2018). Spoilage is not always due to microbial activity; there are

interactions within the food package such as oxidation can cause undesirable changes to the food. To overcome this, the package can be flushed with mixed gasses, producing a modified atmospheric packaging (MAP) (Shayanfar, 2013). MAP has been utilized as a control method for minimizing these adverse effects and when combined with low dose irradiation (<2 kGy), the quality can be preserved (Pillai & Shayanfar, 2015; Fan & Sokorai, 2002). This can be observed with the radiation of sprouts in a modified atmosphere (controlling O_2, CO_2, N_2) at low doses for pathogen reduction (approx. 5-log) and improve shelf life (Shurong et al., 2006). Electron beam processing has been coupled with high pressure processing (HPP) for the complementary inactivation effects of microorganisms in food products (Pillai & Shayanfar, 2015). The HPP utilizes high pressures (100 to 1,000 MPa) to inactivate a microorganism by denaturing proteins and other cellular components for pathogenic and spoilage organisms (Abera, 2019). The eBeam inactivation of microbes has been described in section 2.4. The combination of these two complementary technologies creates a process that approaches microbial inactivation from two directions. This means that the same level of inactivation can be achieve with reduced intensities of each process to preserve quality.

2.6 ORGANOLEPTIC ISSUES WHEN COMBINING FOOD PROCESSING TECHNIQUES

The organoleptic attributes of a food are defined as the flavor, color, texture, and aroma (Civille & Carr, 2016). These organoleptic attributes are closely studied during the developmental stages of a food product and then controlled & monitored as part of a Q/A program (Costa et al., 2000). Color attributes are part of the eating experience and can deter an individual from eating food product if the color is outside expectations (ie brown apples) (Jiang et al., 2016). There is always a chance for undesirable changes to a food product that can compromise the quality after a

food processing method (Ottley, 2000). Therefore, it is important to understand the induced chemical changes of a process that can lead to an unacceptable product. Chemical changes have been observed due to pectin breakdown in strawberries with increasing eBeam processing (Yu et al., 1996). This breakdown of starches can have detrimental effects, such as loss in viscosity and mouthfeel, to the overall quality of the product.

The food industry is always looking to extend the shelf life of foods by incorporating one or more food processing technique (drying, pasteurization, sterilization, formulation etc.) for food safety and economic reasons (Torres et al., 2016). There are two main aspects that must be met when studying food preservation: food safety and food quality (Kilcast, 2000). A food is no longer in shelf life if the food has potential to cause harm when ingested by a consumer or when the food product is outside of organoleptic thresholds (Food Safety Authority of Ireland, 2017).

Accelerated shelf life (ASL) testing is commonly used in the food industry to determine the End-of-Shelf life (EOS) of foods by subjecting the packaged food product to harsh storage conditions and testing using objective equipment (colorimeters, texture analyzers, pH meters etc.) or by putting the product to a trained or untrained sensory panel for overall product acceptance (Perchonok et al., 2003; Catauro & Perchonok, 2012). An example of a subjective method for testing a product's acceptance can be done by using a 9-point hedonic scale on a sensory panel (1 = extremely dislike to 9 = extremely like), where product acceptance is greater than 6 (Cooper et al., 2011). Once the product falls below 6, the product is no longer acceptable, and the EOS can be determined. Food companies will perform accelerated shelf life testing to determine the EOS for a product line, where the EOS can be extrapolated based on accelerated storage conditions to then be printed on the package for the consumer (Hough, 2010). This accelerated method is not without its flaws as the harsh conditions are not always indicative to accelerating the food product

the EOS and are not always accurate due to multiple factors influencing the mode of EOS (Hough, 2006). The best method to determine product acceptance during storage is to store product at regular storage conditions and test for acceptance periodically until product results are out of shelf life. However, when dealing with commercially sterile or freeze-dried products, the projected end of shelf life could be 2+ years and commercial food companies must utilize methods (predictive modeling using empirical data) to determine the EOS faster than the product naturally expires (Kilcast, 2011).

Food packaging goes hand in hand with shelf life studies. There are multiple levels of food packaging (primary, secondary, and tertiary packaging) and all serve a specific function (Robertson, 2010). The function can have promotional benefits for marketing or special barrier properties for shelf life extension (Han, 2014). Packing materials are critical when determining the shelf life of a product because packages have specific barrier properties that create a separation of the food substance and the outside world (i.e., oxygen, microbes) (Siracusa, 2016). Thermostabilized foods utilize cans, jars and pouches that can be hermetically sealed, meaning no gas exchange to cause food spoilage. Besides hermetically sealed packages, there are other barrier properties that can cause concern to the shelf life of a food product: gas, moisture and light permeability (Piergiovanni & Limbo, 2015). Glass jars have great gas barrier properties as the glass is made from inert material, but light can still shine though causing oxidation. There are packaging materials that use a laminate (combination) of plastic materials that serve various functions to preserve the food (Kirwan et al., 2011). Packages that undergo a thermal process must be able to withstand the extreme temperatures of the process without compromising the structure. Therefore, each food product and package must be carefully studied to optimize the food product and process.

2.7 POTENTIAL HAZARDS ASSOCIATED WITH ELECTRON BEAM PROCESSING

Food safety is critical when it comes to food preservation and necessary when designing a food system. The main concern for the high dose irradiation of foods is the potential to induce chemical changes that are known to be human carcinogens (Scholz & Stadler, 2019). Head-Space Solid Phase Micro Extraction (HS-SPME) has been used to extract furans from gamma irradiated and thermally processed orange juice (Fan, 2005). The literature for furan formation due to radiation processing is not as extensively studied as the thermal processing. Therefore, it is important to study the processing effects of eBeam processing on foods (Fan, 2008).

Furans are five membered cyclic rings that have aromatic characteristics and pose potential harm to humans if ingested as high levels (Crews & Castle, 2007). They are extensively studied for their abundance in commercially available thermally processed foods such as soups, sauces, and meal kits (Fan, 2005). Coffee products have also shown to form furans during the roasting processing (Guenther et al., 2010). There have also been findings of furans in commercially available baby formula (Condurso et al., 2018; Tesfai et al., 2014). Fan (2014) reported that it is not uncommon for both thermal and radiation processing of ascorbic acids & sugars will form these toxic chemicals. The levels of furans in thermally processes foods can be as high as 100-200 ng/g for some of those current commercially available food products but as high as 2,000-4,000 ng/g in roasted coffee products (Seok et al., 2015). Thermal processing of foods (roasting, cooking, commercial sterilization etc.) undergoes the Maillard reaction which is widely accepted to be known for furan formation (Yun-Jeong, 2015). The chemical has potential harm to humans, being classified as a possible carcinogen by the International Agency for Research on Cancer (IARC, 1995). Furans have shown to cause liver tumors in mice, however, the toxicity levels of the average human diet are not high enough to make the connection between furans and human cancer (Moro

et al., 2012). Epidemiological studies have been performed on coffee drinkers and non-coffee drinkers to assess the risk of cancer formation in a high furan diet, however, the results are inconclusive and cannot make the connection between a high furan diet and cancer (Bakhiya & Appel, 2010). There have been minimal changes to dried food products after eBeam processing (Condurso et al., 2018).

Studies have used volatile extraction methods for identifying quality and safety attributes to various processing methods. The use of Gas-Chromatography (GC) is a method for separating and quantifying volatile compounds. This process can then be coupled with Mass-Spectrometry (MS) for the identification of the unknown volatiles and quantifying them against a standard curve of concentrations (Sanches-Palomo et al., 2005). This method is great for identifying aromatic compounds but there is potential for food processing methods to create undesirable changes in a process that will go undetected with GC-MS. Another new process used in the scientific community to identify and quantify these non-volatile compounds is high performance size exclusion chromatography (HPSEC) and high-performance liquid chromatography (HPLC) (Brezinski & Gorczyca, 2019). These methods are used for the separate of non-volatile compounds by the use of columns with beads that slow down compounds based on their molecular weights (Snyder et al., 2012). The consumer market has been known to be slow to adopt new technologies, but with the right awareness, consumers are accepting electron beam processing as an alternative food process technique (Finten et al., 2017).

CHAPTER III

IDENTIFICATION OF ELECTRON BEAM RESISTANT ORGANISMS

3.1 ISOLATION OF ELECTRON BEAM RESISTANT FUNGI

Preparation of Freeze-Dried Berry Medley

Strawberries, raspberries, and blackberries were purchased from a local grocery store and transported to National Center for Electron Beam Research (NCEBR) for freeze-drying. A non-sterile production Millrock Lyophilizer (Millrock Technologies, USA) was used to dehydrate the berries. The berries were spread out on a stainless-steel pan in a thin layer and placed into the freeze-dryer vacuum chamber. A two-phase drying cycle with ramp temperatures were programmed into the freeze-dryer as a standard recipe for the Space Food Systems Laboratory production (SFSL NASA). Initial, primary and secondary phase details can be found in Appendix C (FD3 Master.rcp Appendix C). The berries were removed from the freeze-dryer upon completion of the secondary phase and weighted into sterile bags in equal parts; 3.33 g. strawberry, 3.33 g. blackberry, and 3.33 g. raspberry using a scale in a class 100 hood. The medley was then heat sealed in a way to remove as much air as possible, crushed by hand in the bag and stored in a freezer until further processing. This standard process was used for all downstream testing on freeze-dried berries unless otherwise stated.

eBeam Irradiation of Berry Samples

The samples were irradiated at the National Center for Electron Beam Research in College Station, TX. A 15 kW, 10 MeV Linear Accelerator (LINAC) was used for the irradiation process

from one direction. This study was performed on crushed berries for dose uniformity purposes. A commercial application would not use crushed berries but rather whole, intact berries. This would affect the dose mapping that was performed and would need to be re-evaluated to make sure maximum or minimum doses are being achieve based on the desired outcome. For example, if a maximum dose of 2 kGy cannot be exceeded and the measured dose ranges in a food product from 1.5 to 2.0 kGy (DUR = 1.3), then the target processing dose is 1.5 kGy. The package configuration will affect the dose uniformity since food packaging contains voids and uneven product densities in certain areas. These uncertainties were avoided by creating a thin layer of berry medley in the sterile bag.

The samples were taped down to carboard carriers and placed on a conveyor belt as a delivery method for eBeam exposure. L-α-alanine pellets (Gamma-Service Produkbestrahlung GmbH, Germany) traceable to ASTM standards and the E-scan electron paramagnetic resonance spectroscopy (Bruker, BioSpin., Billerica, Mass.) were used to measure the absorbed dose of the berry medley samples. A pellet was placed on the top and bottom of the berry medley to measure the entrance and exit dose. The berry medley was exposed to target doses of 3 kGy, 5 kGy and 10 kGy of eBeam radiation; measured doses were 2.8 kGy, 4.9 kGy, and 9.9 kGy respectively.

Isolation of Fungi and Pure Culture Preparation

The eBeam-processed berries were then diluted with 10 mL of phosphate buffered saline (PBS) solution and plated on Sabouraud Dextrose Agar (SDA) and Dichloran Rose-Bengal Chloramphenicol (DRBC). Then incubated at room temperature (25^{o} C +/- 2^{o} C) for 7 days. Only five (5) different fungi were visible after the 7-day incubation period on the 3 and 5 kGy samples. Those five colonies were chosen based on growing patterns and phenotypical differences. The

fungi were then streaked on SDA and DRBC and incubated at 25° C +/- 2° C for 2 to 7 days (depending on isolate) for pure cultures. A portion of each fungi was then placed in -80°C freezer for long term storage.

3.2 DNA EXTRACTION FROM FUNGAL ISOLATES

Molecular identification was based on the amplification of the Internal Transcribed Spacer (ITS) sequence. Each fungal colony was grown on DRBC for 3 to 7 days. A section of the plate was then cut out and used as the starting sample for each DNA extraction and extracted using the Qiagen AllPrep® Fungal/DNA/RNA/Protein Kit (Qiagen, 2018). The first step was to break open the cell and expose the DNA using the power bead tube. The DNA was then separated from the proteins and other organic debris. The DNA was then isolated and concentrated (Pillai & McKelvey, 2017). Each isolate's DNA was extracted separately and quantified using Qubit 2.0 (Hessen, 2016; Table 2). Extracted DNA was labeled and stored at 0° C until further analysis.

Table 2 Quantification of dsDNA Using Qubit 2.0 of Fungi

Isolate Number	dsDNA Concentration	Units
1	0.06	µg/mL
2	0.07	µg/mL
3	0.06	µg/mL
4	0.06	µg/mL
5	0.06	µg/mL

3.3 FUNGAL IDENTIFICATION USING INTERNAL TRANSCRIBED SPACER (ITS)

The Internal Transcribed Spacer (ITS) was used for identification of eukaryotic cells using forward (5'-TCC GTA GGT GAA CCT GCG G-3') and reverse (5'-TCC TCC GCT TAT TGA TAT GC-3') primers that target a conservative region of ribosomal 18S and 5.8S rRNA genes

(approximately 800 bp; Schoch et al., 2012). The DNA from section 3.2 was amplified using the forward and reverse primers in the Plant Pathology Laboratory (Appendix B for lab protocol). The amplified DNA fragments were shipped to Eton Biosciences INC. (San, Diego California) for sequencing the PCR product. Each isolate's ITS sequence was compared to the online ITS sequence database for identification (Madden, 2002; National Center for Biotechnology Information, Bethesda MD). In this study, the morphological features were used as secondary confirmation for identity.

3.4 RESULTS AND DISCUSSION

The fungi were identified using the ITS sequence amplification, each isolate was identified using a consensus of the amplified sequence from the forward and reverse primers (Table 3). The consensus sequence matched 400 to 500 base pairs using Geneious software (Geneious, 2018). Each isolate sequence was then uploaded to GenBank (National Center for Biotechnology Information, Bethesda MD) and identified to the genus level with 100% coverage.

Five (5) fungal organisms were isolated from freeze-dried berries that were irradiated at 3 and 5 kGy based on their phenotype and growth rates. The radiation doses for this study were chosen based on the natural bioburden of the product and the organism's resistance to eBeam radiation. Fungal growth was observed in the 3 kGy and 5 kGy dose treatments. No growth was observed on the 10 kGy sample.

The fungi that were isolated and identified from the berry medley studied are prevalent in fruits and vegetables and can vary in concentration from grower to grower (Ribes, 2018). The average contamination of fungi in a berry sample can range from 10^2 to 10^4 CFU/g of sample (Verde et al., 2013). Typical fungal contaminates found on berries are *Botrytis cinerea*, *Rhizopus*,

Alternaria, Penicillium, Cladosporium and *Fusarium*; these can colonize at any step from harvest to consumers (Tournas & Katsoudas, 2005). Similar fungal contaminants have been isolated on dried grains: *Alternaria, Aspergillus, Cladosporium, Eurotium, Fusarium, Mucor, Penicillium,* and *Rhizopus* (Aziz et al., 2007). These fungal contaminants on dried berries is a concern to the public as some of these drying processes do not have a microbial inactivation step, especially since the occurrence of fugal mycotoxin production from organisms such as *Aspergillus* and *Penicillium* can occur (Ic et al., 2006; Adeyeye & Yildiz, 2016). This exposes the consumer to the toxin or the fungal contaminant. The purpose of this study was to eliminate the fungal populations in berry medley based on what is naturally present in berries using eBeam technology. These results showed that berry medley (strawberry, raspberry, and blackberry) used in these studies harbored 10^4 CFU/g fungi. The main fungal genera that were resistant equal to or greater than 3 kGy were *Aspergillus* spp. and *Penicillium* sp. and *Cladosporium* spp.

Table 3: Fungal Identification Using Internal Transcribed Spacer (ITS) Sequence

Unknown Fungal Number	Fungal ID	Morphological Characteristics	Incubation Period	Extraction Dose
1	*Aspergillus* sp.	White/yellow with spherical green conidia	2 to 3 Days	2.8 kGy
2	*Penicillium* sp.	White – light green with spherical conidia	2 to 3 Days	2.8 kGy
3	*Aspergillus* sp.	White yellow stipe with spherical green conidia	2 to 3 Days	2.8 kGy
4	*Cladosporium* sp.	Dark black/brown to green with non-spherical conidia	5 to 7 Days	4.9 kGy
5	*Cladosporium* sp.	Dark black/brown to green with non-spherical conidia	7 to 10 Days	4.9 kGy

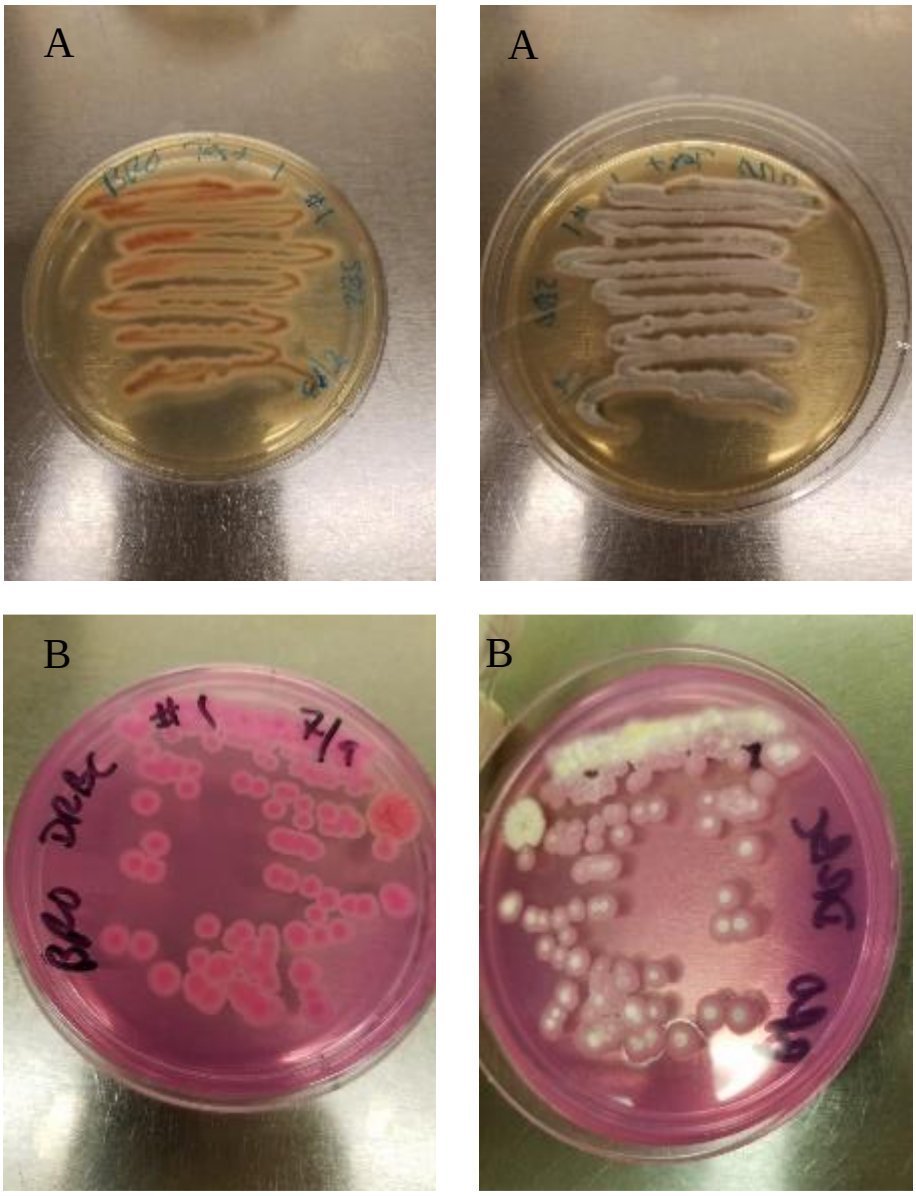

Figure 1: Isolate #1 (*Aspergillus* sp.) when isolated from a berry medley at an eBeam dose of 2.8 kGy and plated on (A) Sabouraud Dextrose Agar (SDA), and (B) Dichloran Rose-Bengal Chloramphenicol (DRBC).

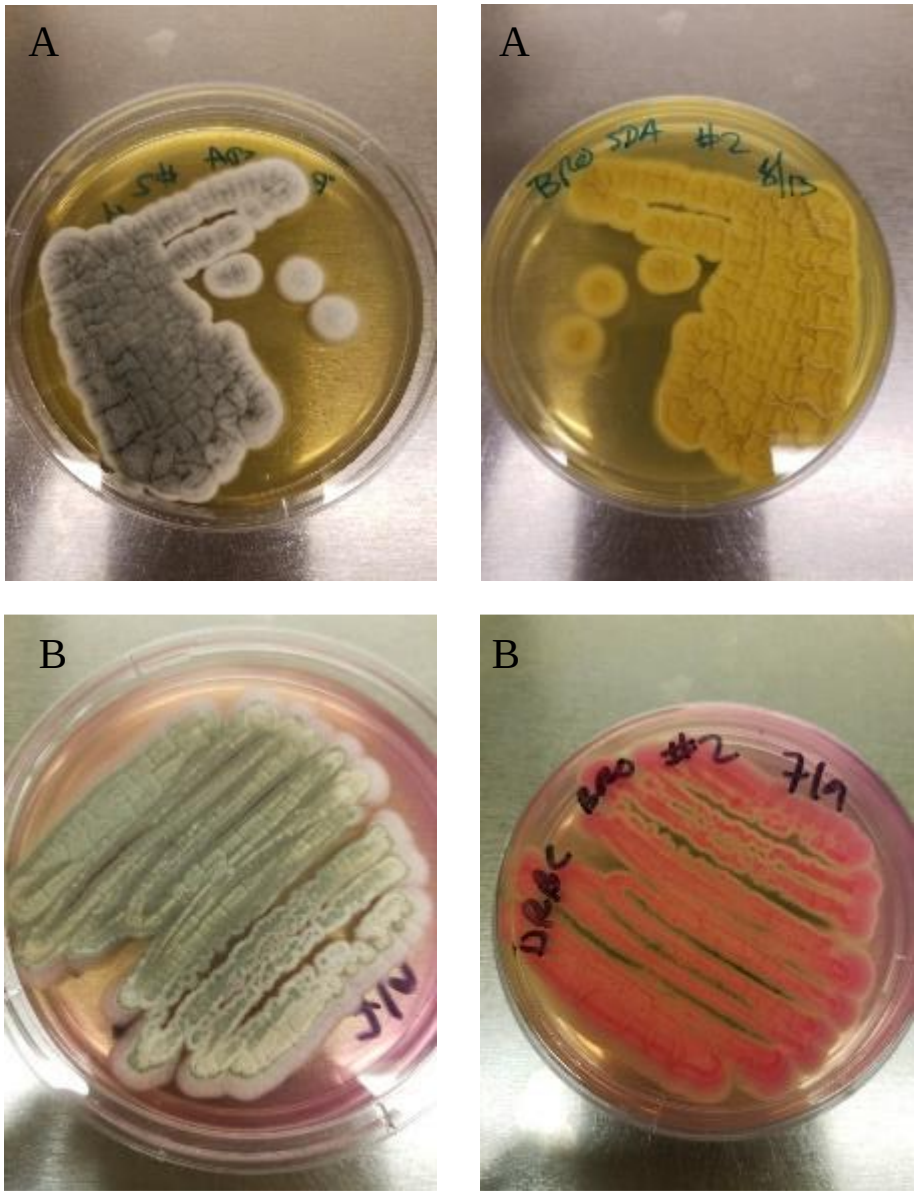

Figure 2: Isolate #2 (*Penicillium* sp.) when isolated from a berry medley at an eBeam dose of 2.8 kGy and plated on (A) Sabouraud Dextrose Agar (SDA), and (B) Dichloran Rose-Bengal Chloramphenicol (DRBC).

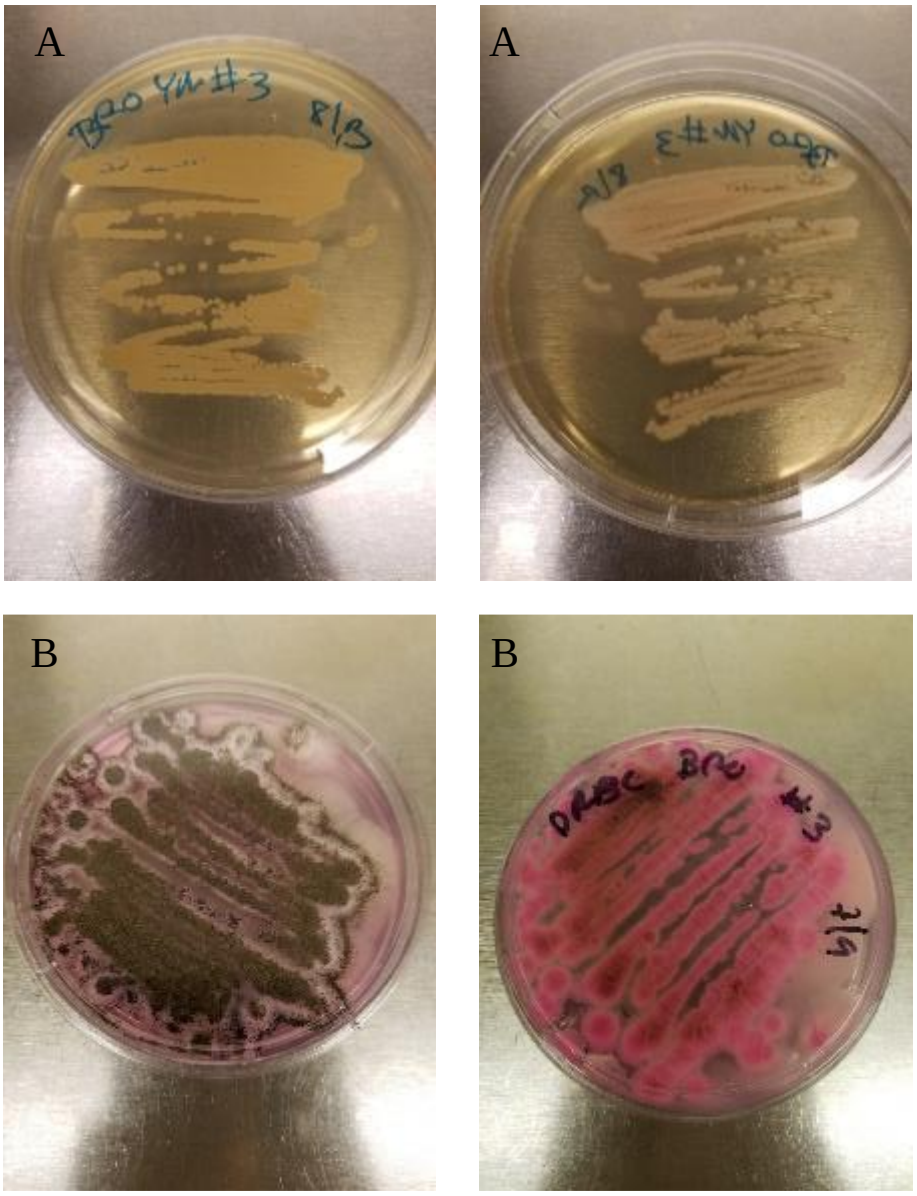

Figure 3: Isolate #3 (*Aspergillus* sp.) when isolated from a berry medley at an eBeam dose of 2.8 kGy and plated on (A) Sabouraud Dextrose Agar (SDA), and (B) Dichloran Rose-Bengal Chloramphenicol (DRBC).

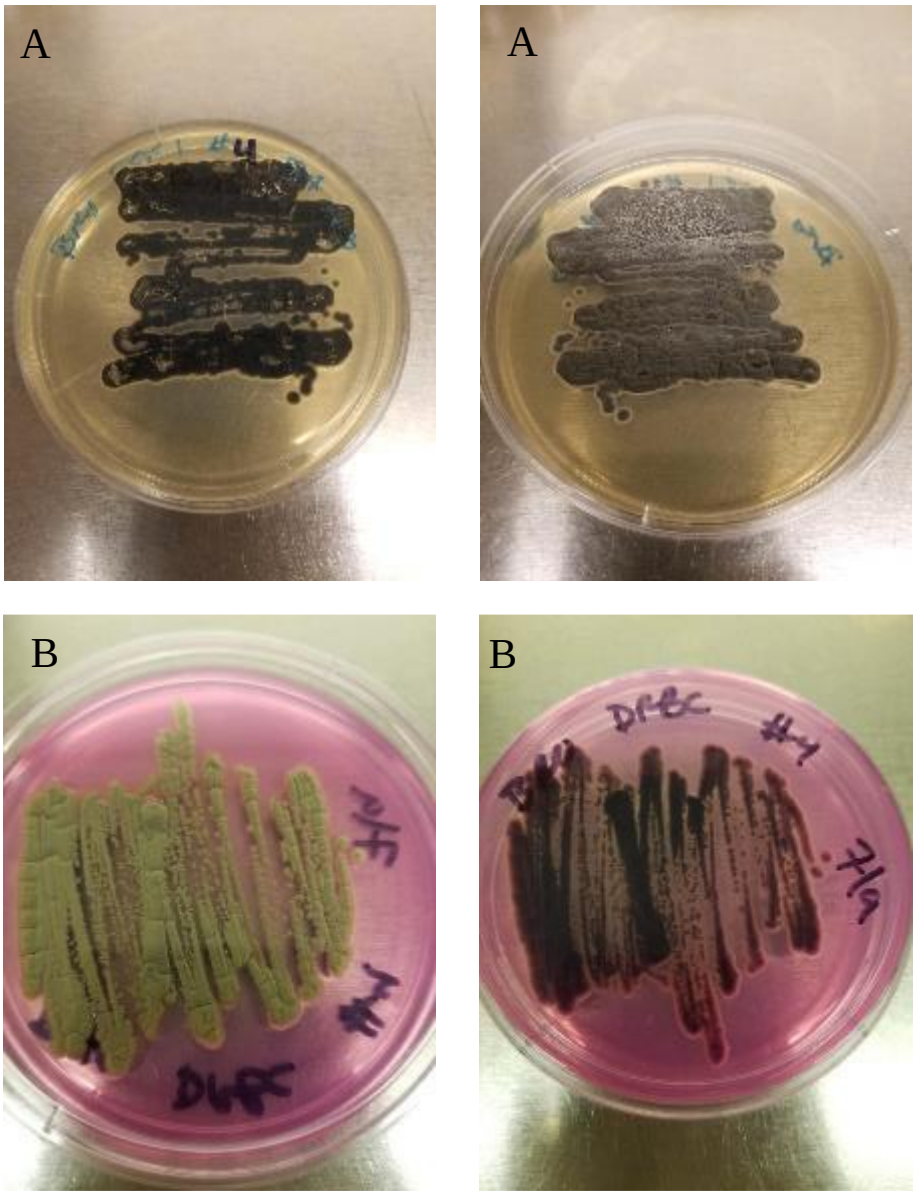

Figure 4: Isolate #4 (*Cladosporium* sp.) when isolated from a berry medley at an eBeam dose of 4.9 kGy and plated on (A) Sabouraud Dextrose Agar (SDA), and (B) Dichloran Rose-Bengal Chloramphenicol (DRBC).

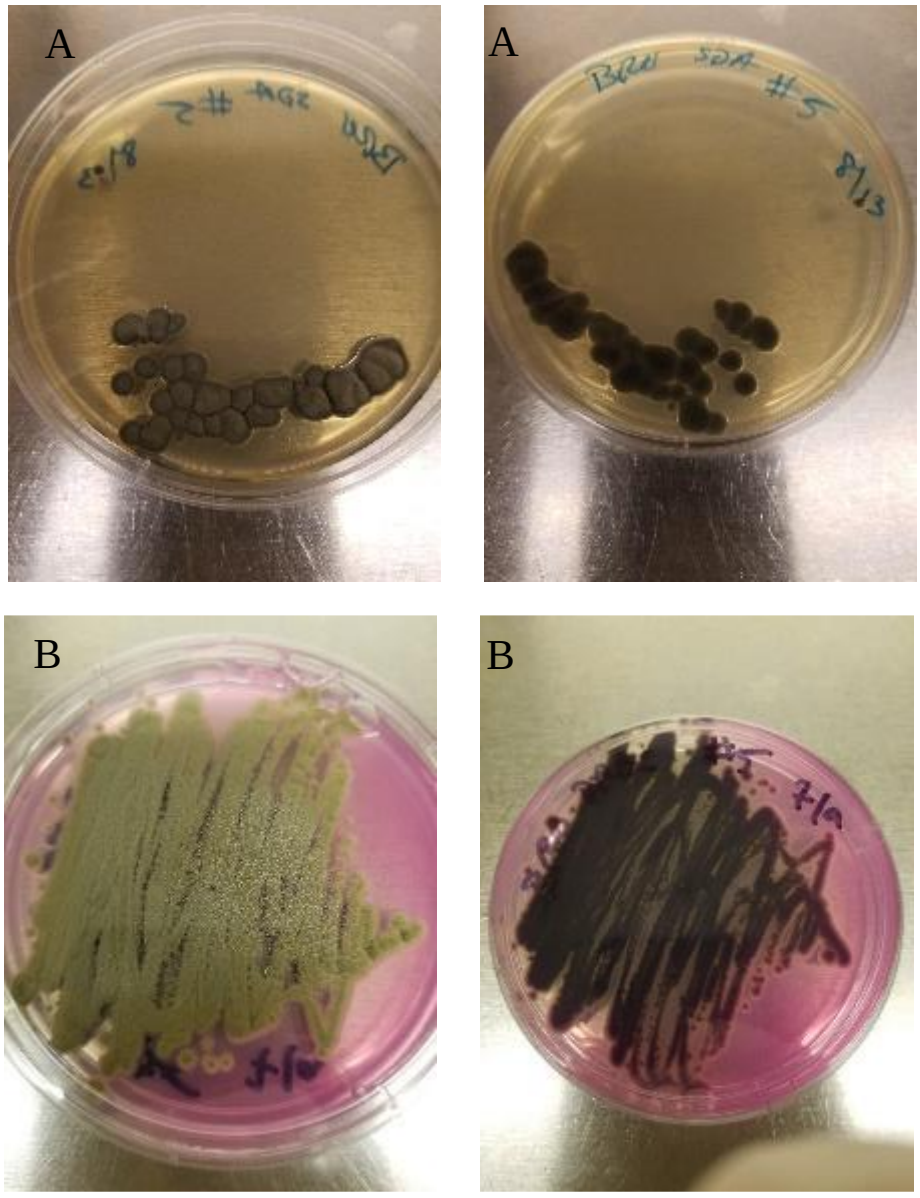

Figure 5: Isolate #5 (*Cladosporium* sp.) when isolated from a berry medley at an eBeam dose of 4.9 kGy and plated on (A) Sabouraud Dextrose Agar (SDA), and (B) Dichloran Rose-Bengal Chloramphenicol (DRBC).

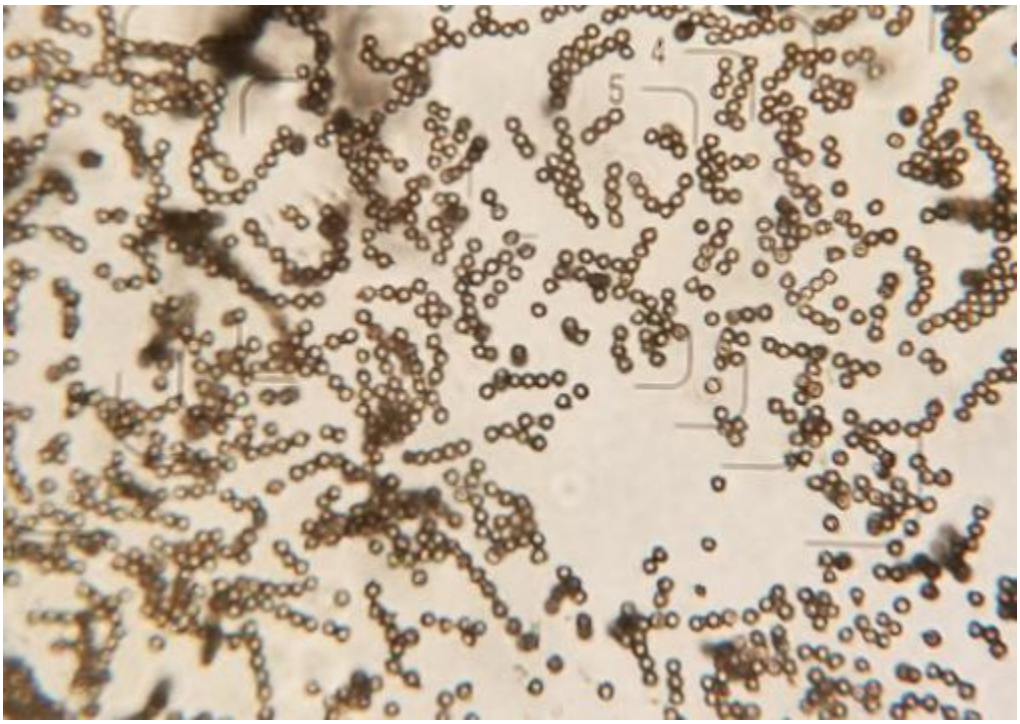

Figure 6: Isolate #1 (*Aspergillus* sp.) as viewed using the scotch tape method (Harris, 2000). 60X magnification.

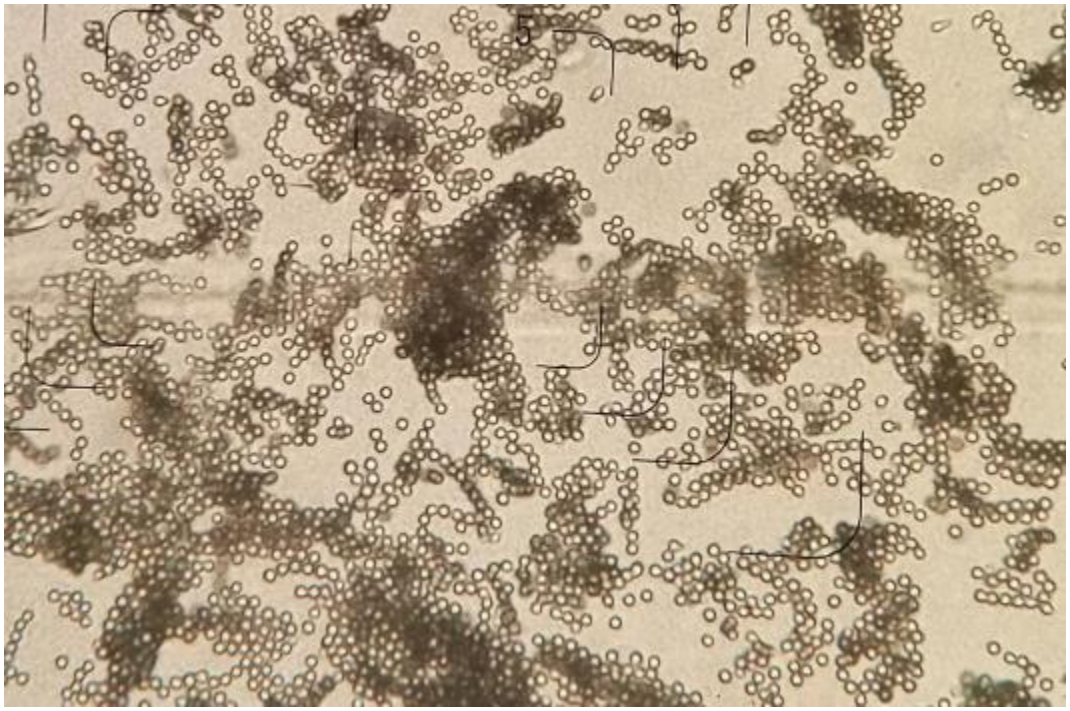

Figure 7: Isolate #2 (*Penicillium* sp.) as viewed using the scotch tape method (Harris, 2000). 60X magnification.

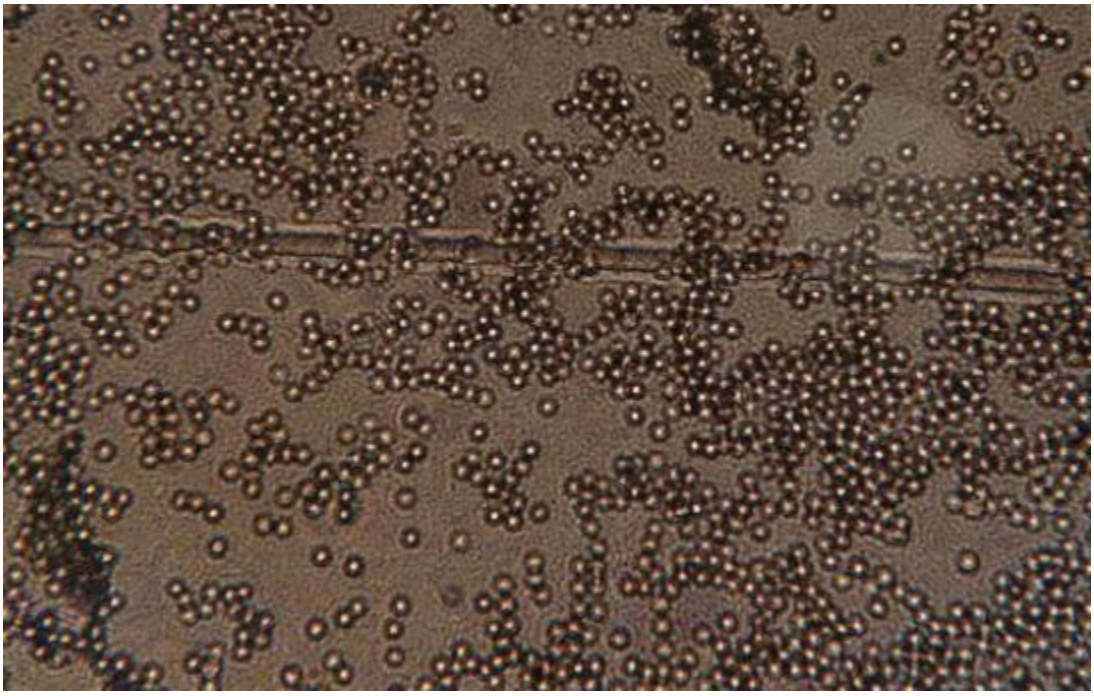

Figure 8: Isolate #3 (*Aspergillus* sp.) as viewed using the scotch tape method (Harris, 2000). 60X magnification.

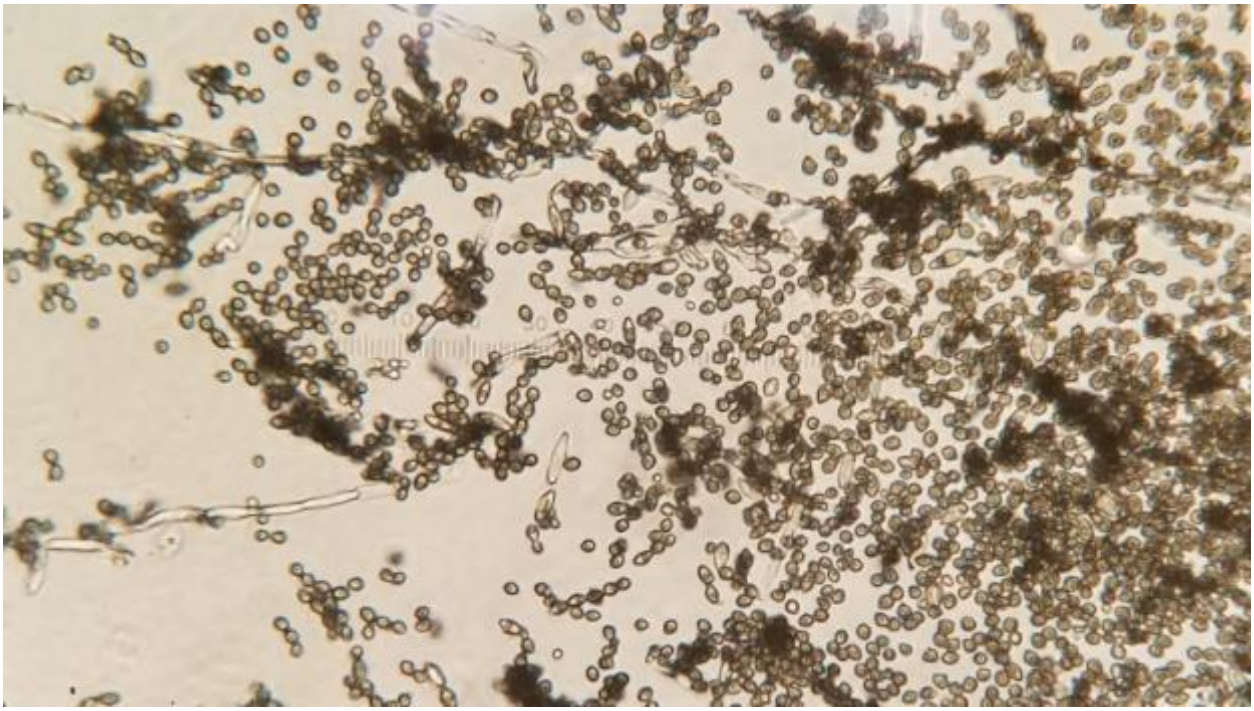

Figure 9: Isolate #4 (*Cladosporium* sp.) as viewed using the scotch tape method (Harris, 2000). 60X magnification.

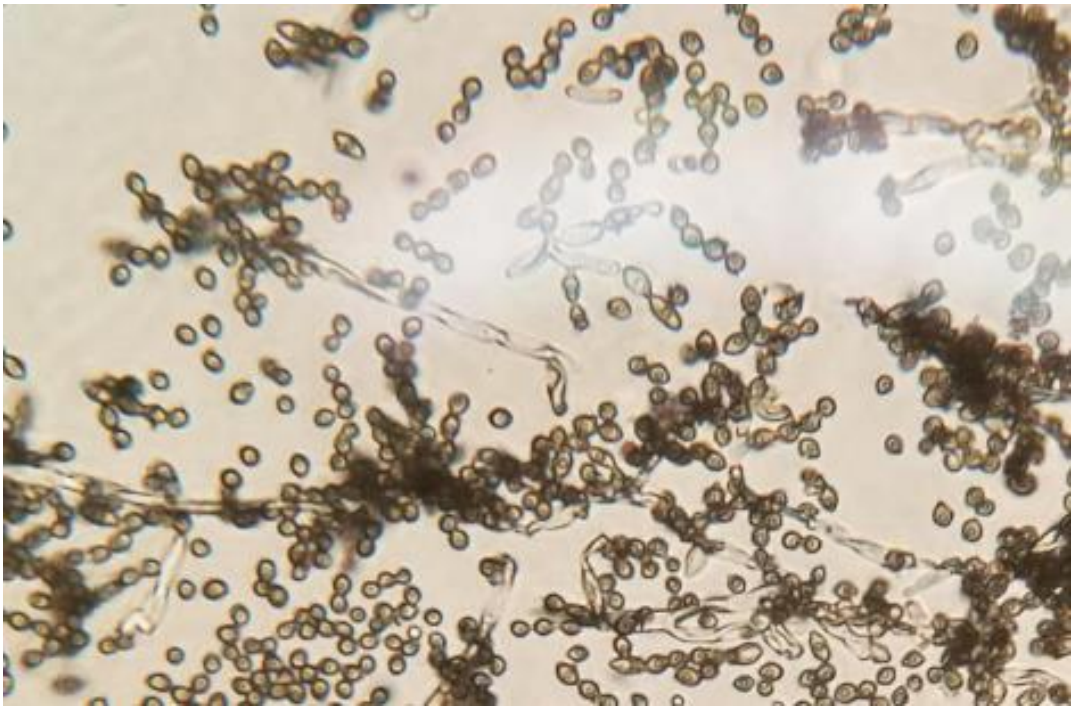

Figure 10: Isolate #5 (*Cladosporium* sp.) as viewed using the scotch tape method (Harris, 2000). 60X magnification.

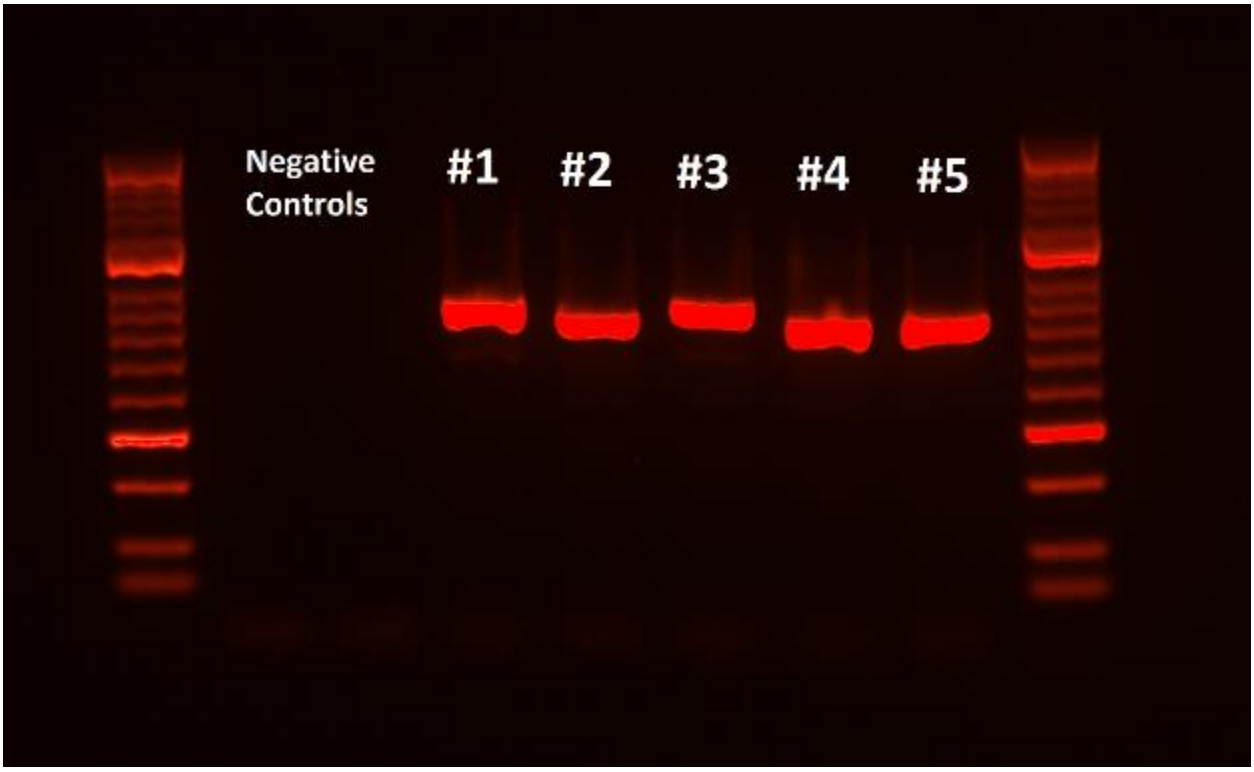

Figure 11: Gel electrophoresis of the PCR product from the ITS sequence amplification from fungal isolate (#1, #2, #3, #4 and #5). Two negative control lanes and two DNA ladders are also shown. The gel was stained using GelRed.

CHAPTER IV

ELECTRON BEAM INACTIVATION KINETICS OF ELECTRON BEAM RADIATION RESISTANT FUNGAL ISOLATES

4.1 PREPARATION OF FREEZE-DRIED BERRY MEDLEY

The berries were freeze-dried as described previously and using the recipe in Appendix C. The packaged freeze-dried berry medley was then processed using a 15 kGy eBeam dose to eliminate the background microorganisms in the berry medley. The bags were then stored at room temperature until further processing.

4.2 CULTURE METHODS FOR FUNGAL ISOLATES

The fungi (isolated as described in Chapter 3) were grown on Dichloran Rose-Bengal Chloramphenicol (DRBC) for 3 to 7 days (depending on fungal isolate needs) at room temperature (25^{o} C +/- 2^{o} C).

4.3 PREPARATION OF FUNGAL ISOLATES FOR EBEAM RADIATION EXPOSURE

The fungi were not filtered for spores or mycelia. The entire culture was used to inoculate the freeze-dried berry matrix. A sterile beaker and scoop were used inside the laminar flow hood to inoculate the fungus into the freeze-dried berry matrix directly mixing the two together. Ten (10) grams of crushed freeze-dried berries were mixed with each individual fungal culture and then placed into a sterile beaker. The berries were then mixed to evenly distribute the fungal cells in the berry medium. Aliquots of the berry medley (0.5 to 1 gram) were then divided into sterile bags all with approximately the same weights and heat sealed in a way to remove as much air as

possible. The berries were then spread into a very thin layer to achieve uniform eBeam dosing. Individual weights were measured and recorded. Five eBeam dose points were used for the inactivation studies. Three replicate samples were prepared for each dose point for each fungus. The inoculated bags were then double-bagged in sterile bags for transport to the National Center for Electron Beam Research.

4.4 EBEAM DOSING AND DOSIMETRY METHODS

The eBeam preparation and procedures were performed as described in chapter 3.1 with the following exceptions. The belt speeds were set as follows for each dose point (in kGy and feet/min); 0 – (not processed), 1 – 60.00, 2 – 31.70, 3 – 23.87, 4 – 17.98. The measured doses were; 0, 1.20, 2.27, 2.99, 3.99 kGy, respectively.

4.5 POST EBEAM EXPOSURE CULTURING METHODS

Microbial analysis was performed no more than 5 hours after eBeam exposure. The berry samples were diluted using PBS solution to 10 mL and stomached for 2 minutes on high. Each sample was then serially diluted in PBS, plated on Dichloran Rose-Bengal Chloramphenicol (DRBC) agar and incubated at room temperature (25^oC +/- 2^oC) for 3 to 7 days (depending on isolate growing rates). The fungi on the plates were then counted and recorded.

4.6 D_{10} VALUE CALCULATION

The inactivation rates were calculated using the equation below (Eq. 2) where N_o is the initial unirradiated fungal sample (control) and N is the surviving organisms at the respective radiation dose (Farkas, 2007).

(2) $D_{10} = \frac{absorbed\ radiation\ dose}{logNo - logN}$

The D_{10} calculation can also be calculated by plotting the log CFU/g survivors vs. the absorbed radiation dose. The D_{10} value is the negative reciprocal of the linear regression relationship of the Log CFU/g survivor vs. absorbed radiation dose.

4.7 RESULTS AND DISCUSSION

Table 4. below compares the D_{10} values of the studied fungi under dry and wet conditions and the eBeam dose at which individual isolate were obtained. The dry D_{10} values were between 0.8 kGy and 2.7 kGy, which are much higher when compared to the D_{10} values of the same organisms in an aqueous solution. The D_{10} values for *Aspergillus* spp., *Penicillium* sp., and *Cladosporium* spp. were 0.17 to 0.25 kGy, 0.17 to 0.25 kGy and 0.60 to 0.65, respectively in an aqueous solution (Saleh et al., 1988). The higher D_{10} values seen in a dry environment were expected in eBeam processing due to the lower water activity minimizing the indirect methods for microbial inactivation (Pillai, 2004). As reported by Ic and colleagues (2006), the D_{10} values obtained in this study are comparable to the results for total fungus bioburden testing on dried fruits and nuts. The reduced water environment restricts the formation of free radicals which minimizes DNA breaks (Farkas, 2007). A higher radiation dose is required to achieve the same level of inactivation on a food sample with a lower moisture content. Increasing the dose can pose a risk of undesirable changes in eBeam processing (i.e., off flavors, color changes). The lowered moisture content could benefit the process as reduced free radicals could restrict these radicals from making undesirable changes to the food product during processing (Shayanfar et al., 2016).

The *Aspergillus* spp. #1 and #3 showed inactivation rates of 0.9 and 1.1 kGy, respectively and *Cladosporium* spp. #4 and #5 showed inactivation rates of 2.7 and 1.8 respectively as seen by the Table 4 below. Figures 12 to 16 are the inactivation curves of the 5 fugal isolates using GraphPad Prism 5.0 (GraphPad Software INC., California). The inactivation rates differed slightly most possibly due to the inherent genetic and metabolic differences in the fungi. The isolates varied phenotypically and their growth rates on agar media under the same incubation conditions. The difference in inactivation could result from to different strains of the same genus or stress response mechanisms when exposed to a harsh environment of sublethal kills. eBeam radiation is known to metabolically injure cells which in turn can induce a repair response (Jay et al., 2005). This could be the observed outcome of the same fungi genera having different growth patterns after eBeam exposure.

Table 4: D_{10} Values for Individual Isolates

Isolate Number	Isolate	D_{10} value	Isolation Dose	Linear Regression
1	*Aspergillus* sp.	0.90 ± 0.21	2.8 kGy	y = -1.12x + 6.65
2	*Penicillium* sp.	0.87 ± 0.05	2.8 kGy	y = -1.12x + 6.50
3	*Aspergillus* sp.	1.10 ± 0.48	2.8 kGy	y = -0.95x + 7.72
4	*Cladosporium* sp.	2.70 ± 0.39	4.9 kGy	y = -0.37x + 5.58
5	*Cladosporium* sp.	1.79 ± 0.49	4.9 kGy	y = -0.57x + 5.65

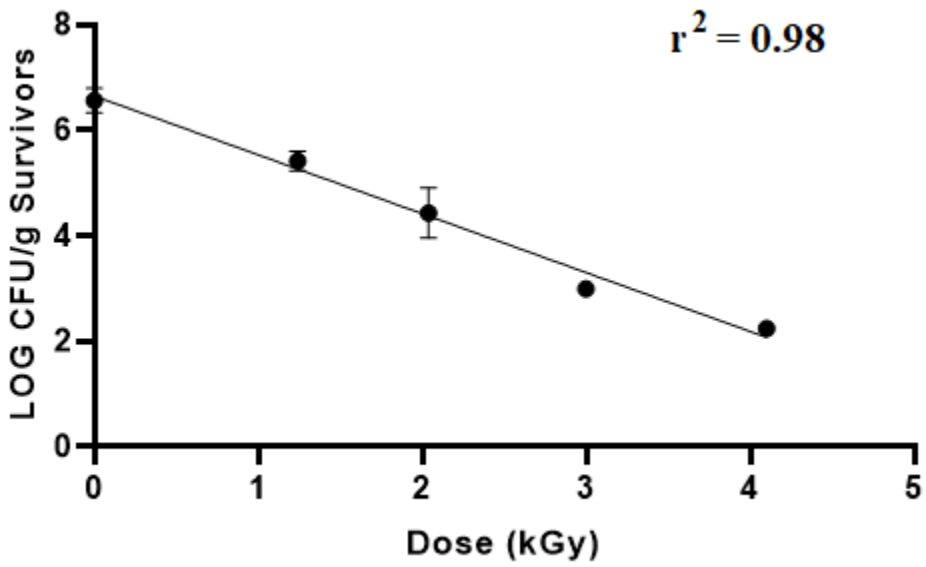

Figure 12: Inactivation curve of fungal isolate #1 (*Aspergillus* sp.) in freeze-dried berry medley matrix.

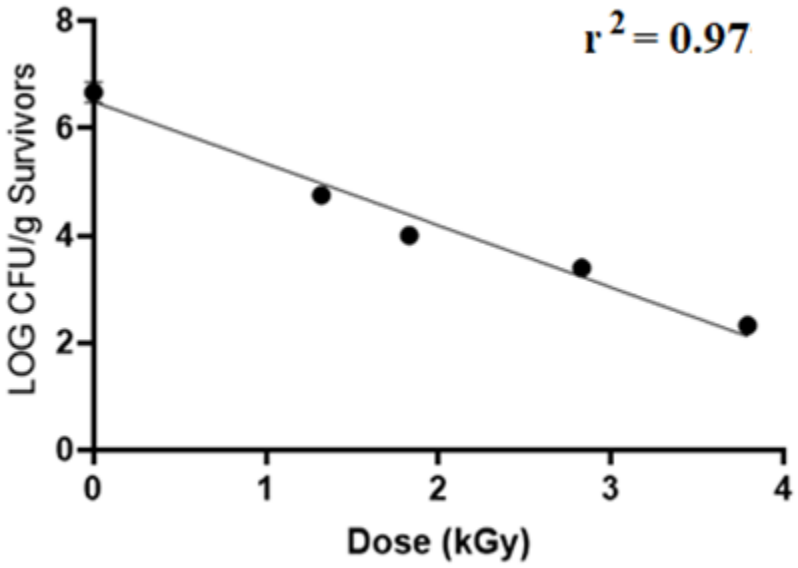

Figure 13: Inactivation curve of fungal isolate #2 (*Penicillium* sp.) in freeze-dried berry medley matrix.

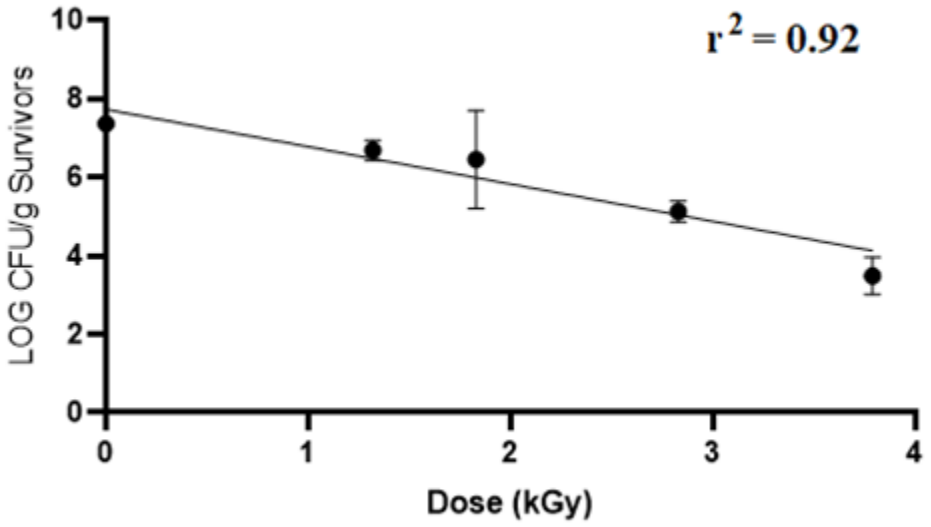

Figure 14: Inactivation curve of fungal isolate #3 (*Aspergillus* sp.) in freeze-dried berry medley matrix.

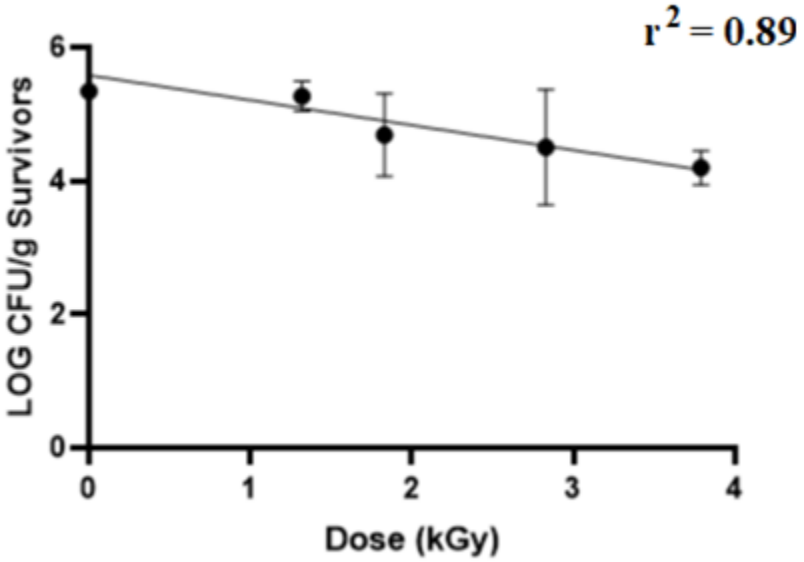

Figure 15: Inactivation curve of fungal isolate #4 (*Cladosporium* sp.) in freeze-dried berry medley matrix.

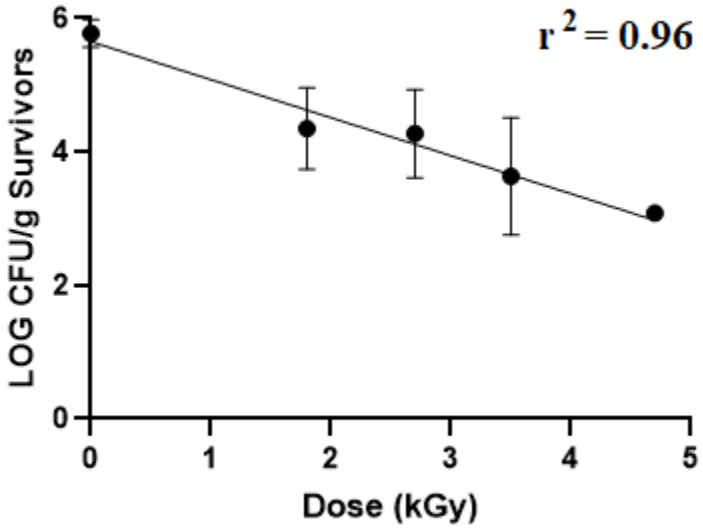

Figure 16: Inactivation curve of fungal isolate #5 (*Cladosporium* sp.) in freeze-dried berry medley matrix.

CHAPTER V

VALIDATING THE ELECTRON BEAM DOSE REQUIRED FOR INACTIVATING FUNGAL POPULATIONS IN BERRY MEDLEY

5.1 DETERMINING THE INACTIVATION OF FUNGAL POPULATIONS IN BERRY MEDLEY SAMPLES

All fruits and vegetables have a level of fungal contamination from the point of harvest to the market. The goal was to eliminate the organisms that can cause spoilage or organisms that can cause harm in terms of mycotoxin production or other health ailments. The average bioburden of fruits is approximately 10^2 to 10^4 Colony Forming Units/g (CFU/g) and are primarily consisting of fungi (79 to 98 %; Verde et al., 2013; Tournas et al., 2015). This means that a 4-log reduction (on average) is required to eliminate the fungal population in the berry sample. Using the $_{D10}$ values of the most resistant fungi (*Cladosporium* sp.; D_{10} value: 2.7 ± 0.393 kGy) inactivation rates of the most resistant fungi from Chapter 4, a 15 kGy dose was hypothesized to be sufficient for a 5-log reduction of the fungal contaminants in the berry medley.

5.2 PREPARATION OF FREEZE-DRIED BERRY MEDLEY SAMPLES

The berries were freeze-dried as described previously and using the recipe in Appendix C.

5.3 EBEAM DOSING AND DOSIMETRY METHODS

The freeze-dried berries used the same dosimetry methods as described earlier. Three biological replicates were prepared and processed. The target dose was set to 15 kGy and the belt speed was set to 14.1 fpm. The final measured (absorbed) dose was 15.26 kGy.

5.4 POST EBEAM CULTURING METHODS

The samples were analyzed in a laminar flow biosafety food (to prevent contamination). One gram of processed freeze-dried berry from each biological replicate was placed in a stomacher bag (with filter) and 20 mL of phosphate saline buffer (PBS) solution was added. The sample was then stomached for 2 minutes on high and 1 mL of the mixture was plated on both SDA and DRBC. Five (5) technical replicates were performed for each biological replicate on each media. The plates were then sealed in sterile bags using a heat sealer. After about 30 minutes (allowing the plates to dry), the plates were then inverted and incubated at room temperature (25^{o} C +/- 2^{o} C). The plates were checked periodically for visual growth: 24 hours, 7 days, and 30 days.

5.5 RESULTS AND DISCUSSION

The validation of 15 kGy eBeam dose for the elimination of fungal colonies on freeze-dried berries was confirmed. No growth was observed on eBeam-treated plates for fungi. The 15 kGy dose was chosen based on the log reduction required to inactivate fungi in a freeze-dried berry medley. Cobalt 60 studies have been performed on the inactivation of various yeast and molds from environment sources (Shathele, 2009). The results of that study showed the two molds, *Aspergillus* sp. and *Penicillium* sp., were eliminated with a dose of 6 kGy using gamma radiation. Farkas (2007) has reported that the reduction or inactivation of microbial populations in dry food ingredients was achieved in the 3.0 to 10 kGy range. However, the food samples they studied were starches and spices, which were used as parts of a larger recipe.

The worst-case scenario must be identified when designing a process to eliminate a fungal population. An organism that is high in numbers with a low D_{10} value can be just as concerning as an organism that is present in low numbers with a high (resistant) D_{10} value. The *Cladosporium*

sp. is the most resistant organism studied but could be present in the berry sample in low numbers. *Cladosporium* sp. can therefore be used as an indicator organism to determine the validity of a sterilization dose for fungal decontamination in freeze-dried berries. The dose can be determined by the log reduction desired, the known contamination levels of fungal populations and finally the inactivation rates of fungal cultures typically found in berry medleys. The starting inoculum of the *Cladosporium* sp. was between 5.3 and 5.6 logs. The D_{10} value was between 1.8 and 2.7 kGy. A 12 to 15 kGy dose is required to fully eliminate the organism from the inoculated sample.

Although there was no growth on the 15 kGy samples, there is a possibility that the fungi have gone into a metabolically active yet not culturable (mAyNC) state. This is a state of injury to the DNA from eBeam processing that prevents the organism from multiplying while DNA repair mechanisms are activated (Smith & Pillai, 2004). The organism is unable to replicate but still metabolically active which has been reported by measuring the ATP levels of bacteria after delivering a lethal dose of eBeam radiation (Hieke & Pillai, 2018). As for future studies, it would be recommended that several media are incubated for longer period of time to allow for the recovery and growth of these injured organisms.

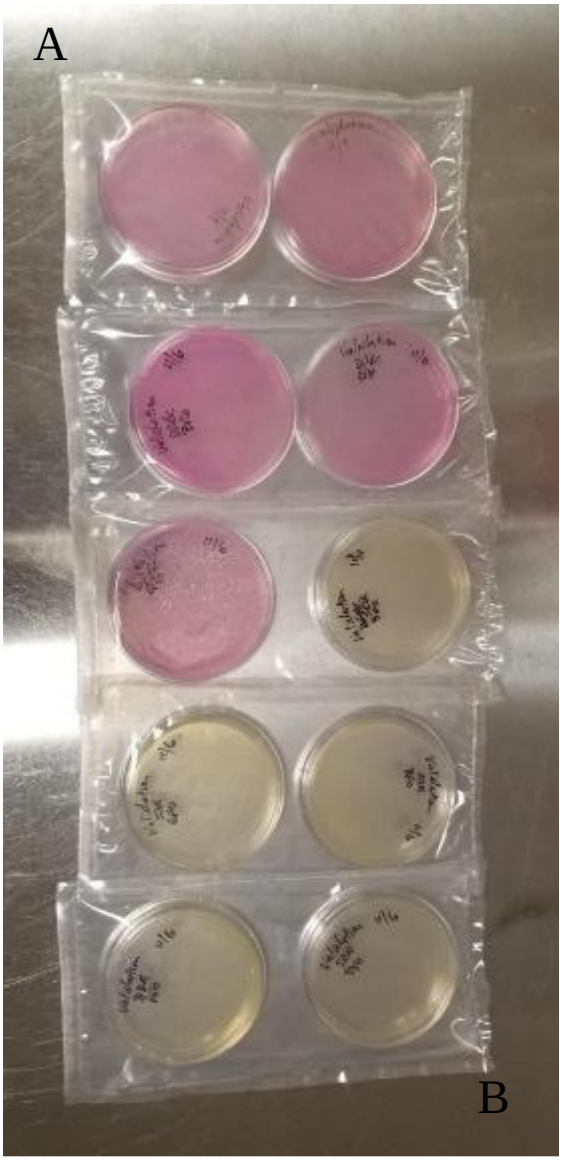

Figure 17: Validation of 15 kGy eBeam dose on eBeam resistant fungal populations in freeze-dried berry medley. Plated on (A) Dichloran Rose-Bengal Chloramphenicol (DRBC) and (B) Sabouraud Dextrose Agar (SDA). Replicate 1 incubated for 24 hours.

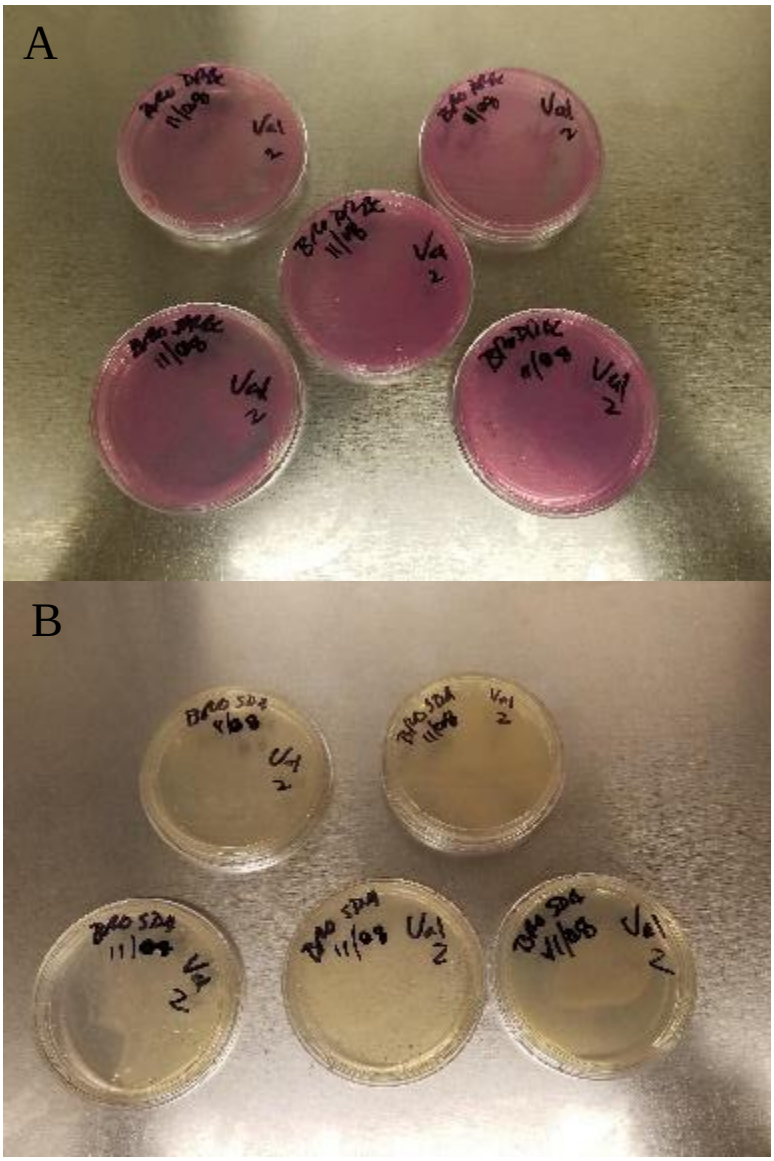

Figure 18: Validation of 15 kGy eBeam dose on eBeam resistant fungal populations in freeze-dried berry medley. Plated on (A) Dichloran Rose-Bengal Chloramphenicol (DRBC) and (B) Sabouraud Dextrose Agar (SDA). Replicate 2 incubated for 24 hours.

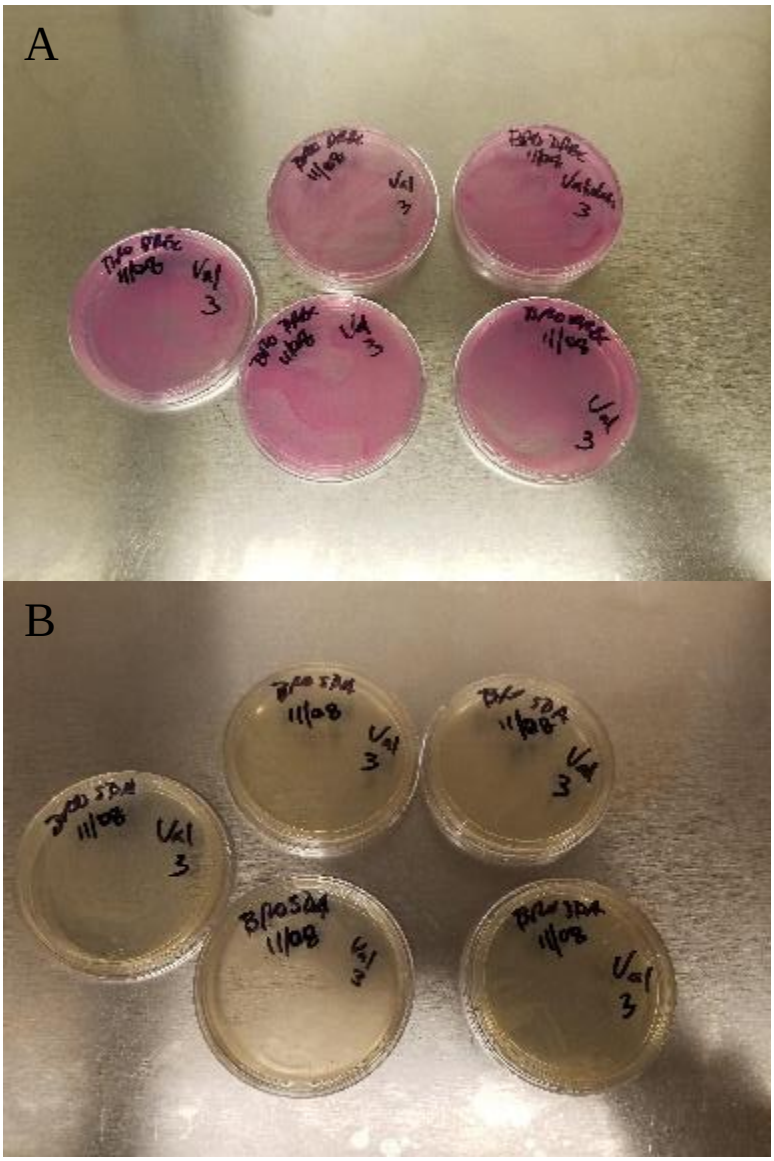

Figure 19: Validation of 15 kGy eBeam dose on eBeam resistant fungal populations in freeze-dried berry medley. Plated on (A) Dichloran Rose-Bengal Chloramphenicol (DRBC) and (B) Sabouraud Dextrose Agar (SDA). Replicate 3 incubated for 24 hours.

Figure 20: Validation of 15 kGy eBeam dose on eBeam resistant fungal populations in freeze-dried berry medley. Plated on (A) Dichloran Rose-Bengal Chloramphenicol (DRBC) and (B) Sabouraud Dextrose Agar (SDA). Replicate 1 incubated for 7 Days.

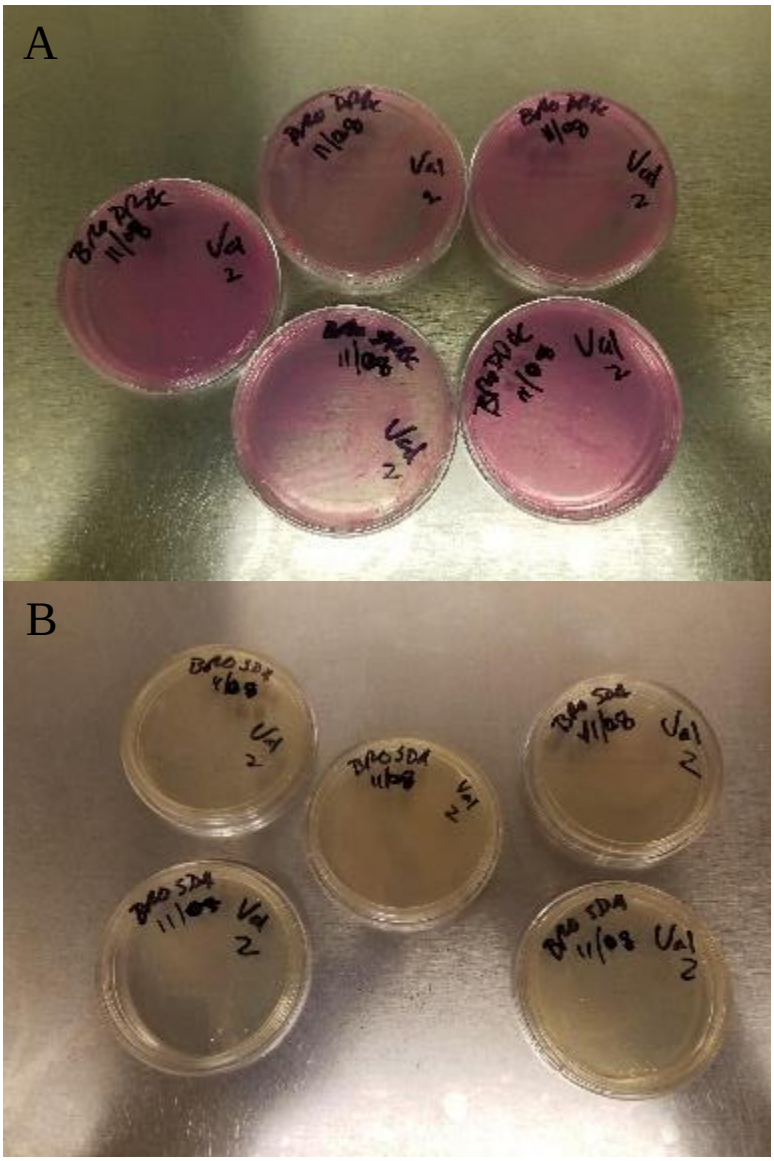

Figure 21: Validation of 15 kGy eBeam dose on eBeam resistant fungal populations in freeze-dried berry medley. Plated on (A) Dichloran Rose-Bengal Chloramphenicol (DRBC) and (B) Sabouraud Dextrose Agar (SDA). Replicate 2 incubated for 7 Days.

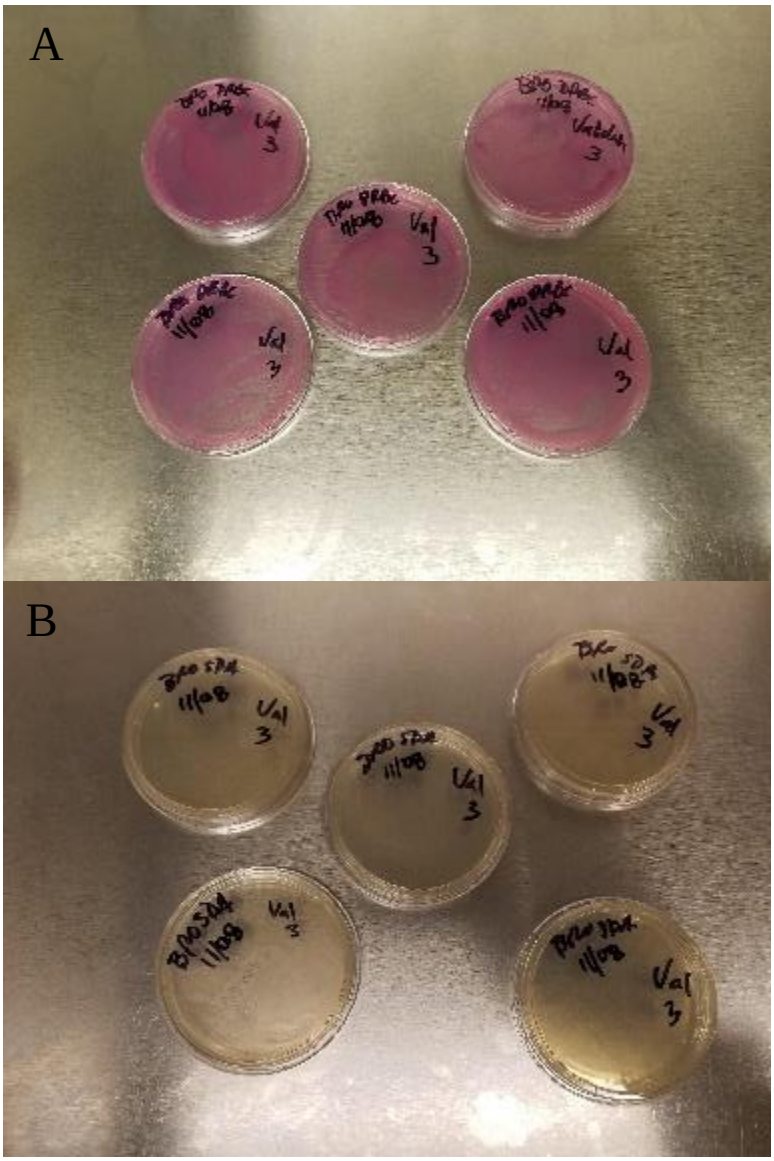

Figure 22: Validation of 15 kGy eBeam dose on eBeam resistant fungal populations in freeze-dried berry medley. Plated on (A) Dichloran Rose-Bengal Chloramphenicol (DRBC) and (B) Sabouraud Dextrose Agar (SDA). Replicate 3 incubated for 7 Days.

Figure 23: Validation of 15 kGy eBeam dose on eBeam resistant fungal populations in freeze-dried berry medley. Plated on (A) Dichloran Rose-Bengal Chloramphenicol (DRBC) and (B) Sabouraud Dextrose Agar (SDA). Replicate 1 incubated for 30 Days.

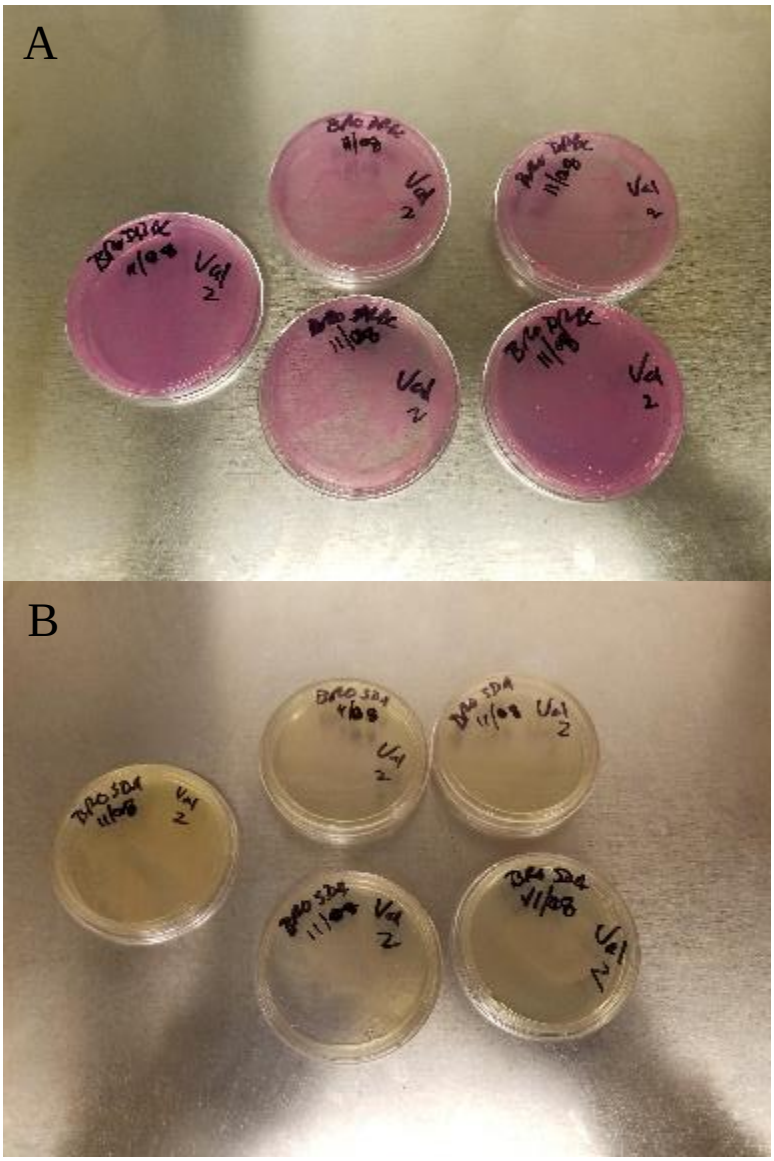

Figure 24: Validation of 15 kGy eBeam dose on eBeam resistant fungal populations in freeze-dried berry medley. Plated on (A) Dichloran Rose-Bengal Chloramphenicol (DRBC) and (B) Sabouraud Dextrose Agar (SDA). Replicate 2 incubated for 30 Days.

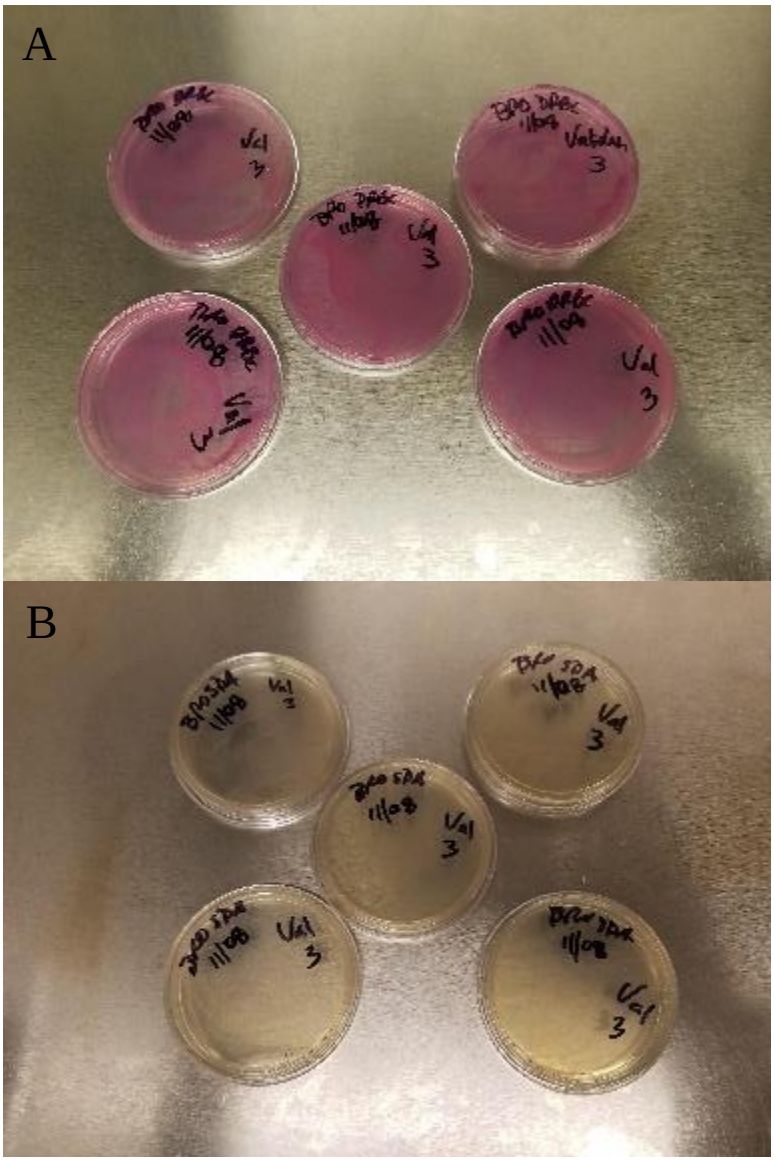

Figure 25: Validation of 15 kGy eBeam dose on eBeam resistant fungal populations in freeze-dried berry medley. Plated on (A) Dichloran Rose-Bengal Chloramphenicol (DRBC) and (B) Sabouraud Dextrose Agar (SDA). Replicate 3 incubated for 30 Days.

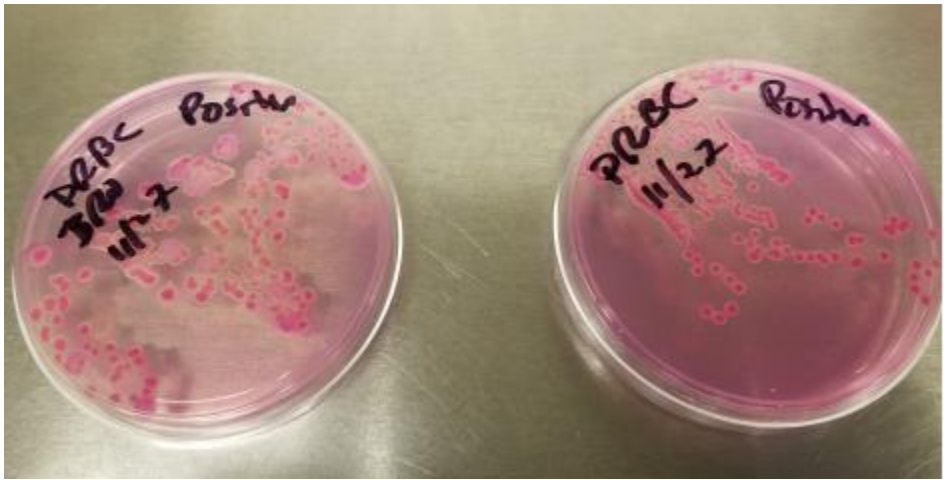

Figure 26: Validation of 15 kGy eBeam dose on eBeam resistant fungal populations in freeze-dried berry medley positive control.

CHAPTER VI

ELECTRON BEAM INDUCED CHANGES IN COLOR AND VOLATILE COMPOUNDS IN BERRY MEDLEY

6.1 INTRODUCTION

A significant part of shelf life studies is the examination of organoleptic attributes of a food sample that could change during the storage of the food product. That means the food product must be studied for any color or volatile changes to the chemical structure after the food processing method. Typically, sensory panels are used to determine subjective measures of consumer acceptance which are then used as predictive model to determine shelf life of a food product (Freitas et al., 2004). However, there are challenges associated with trained and untrained sensory panels. The challenges of using sensory panels for scientific measurements are the reliability of responses. This is because human senses are difficult calibrate and standardize. Multiple factors can influence the outcome of sensory panels such as sample preparation and testing environment (Meilgaard et al., 2016). The use of analytical instruments such as GC-MS and colorimeters can be used to objectively measure these attributes, removing the need for human sensory (Sparkman et al., 2011; Varela et al., 2005).

The eBeam dose points chosen for this study were outside of FDA available maximum dose of 1 kGy for use on fruits and vegetables for human consumption (Title 21, part 179). Hence, using human subjects was not possible. Gas chromatography (GC) and mass spectrometry (MS) were vital to this study as a means for identifying the unknown volatile compounds that were being produced by eBeam processing. The method has been accepted as a protocol for the identification of unknown compounds, but it can be difficult to relate back to concentration (Hu et al., 2015).

The ability for aromatic compounds to be detected via GC-MS removes the need for human trials because the compounds can be identified and then analyzed against a known library for toxicology and aromatic characteristics (Burock, 2010).

Multidimensional gas-chromatography – olfactometry – mass spectrometry (MDGC-O-MS) was used to extract and identify volatiles in a sample. Some of these volatiles can influence the overall quality of the product (i.e., ethyl acetate – fruity attributes) and some can have an adverse effect on human health (ie furan, benzene; Wegener & López-Sánchez, 2010). The use of head space solid phase microextraction (HS-SPME) has been applied for the detection of volatiles in foods (Condurso et al., 2017; Bhatia et al., 2017). This research study used the same HS-SPME with MDGC-O-MS methods to study any significant changes in furans or other organoleptic characteristics that were directly produced by eBeam processing.

Alternative methods were utilized for measuring the organoleptic attributes of the freeze-dried & eBeam processed berries. A colorimeter was used to objectively measure the color of berries at various eBeam doses (Yagiz et al., 2009). A colorimeter measured the light waves in the visible length of the electromagnetic spectrum and the output of a colorimeter are L*, a*, and b* values (Leon et al., 2006). The three values were then used to identify a spot on a three-dimensional plane where the L* value measures the light to dark ratio between 0 to 100, where lighter samples have values closer to 100. The a* value measures the red to green ratio, where positive values indicate red color and negative values indicate green. The b* value measures the blue to yellow ratio, where positive values are indicated yellow and negative values indicate blue (Trusell et al., 2005).

The use of a colorimeter is used in the research and quality control industry for comparing color changes due to processing or for process control (Wu & Sun, 2013). The measurements of

L*, a*, and b* values can be observed and recorded within each treatment group and statistically analyzed for significant differences removing the need for human color analysis (Barrett, 2010). Colorimeters were used as a control method in food processing for quality as a requirement for specifications. Each food product will have an unacceptable threshold of color change. Some products require a color change (browning on breads) and then some processes do not want color change (eBeam processing of berries). If the desired outcome is no color change, then parameters must be set to determine an unacceptable threshold of L*, a*, and b* values if color changes are possible.

6.2 MULTIDIMENSIONAL GAS – CHROMATOGRAPHY – OLFACTOMETRY – MASS SPECTROMETRY METHODS

Preparation of Freeze-Dried Berry Medley

The berries were freeze-dried as described previously and using the recipe in appendix C.

Dosing and Dosimetry

eBeam dosing protocol and dose mapping was performed as described previously. The target dose points and belt speeds (kGy and f/m) were; 0 – (no processing), 5 – 14.28, 10 – 7.00, and 15 – 4.76. The measured doses were 0, 5.33, 10.20, and 14.88, respectively.

Gas-Chromatography & Mass-Spectrometry Methods

The irradiated berry samples were then analyzed using multidimensional gas-chromatography – olfactometry – mass spectrometry (MDGC-O-MS) for quality attributes of

foods (Bhatia et al., 2017). The berry samples were placed into individual 200 mL modified glass jars with Teflon screw top lids and rehydrated at a ratio of 10 mL purified water to 1 gram of dried berry prior to extraction. The jars were heated to 60^{o} C (water bath) for 20 minutes for each treatment. The Solid Phase Microextraction (SPME) Portable Field Sampler (Supelco 504831, 75 µm Carboxen/polydimethylsiloxane, Sigma-Aldrich, St. Louis, MO) was fed through a septum at the top the jar to prevent contamination of off aromas and collected the headspace of the jar for 2 hours.

After collection, the SPME fiber was removed from the jar and inserted into the injection port of a gas chromatograph (Agilent Technologies 7820A GC, Santa Clara, CA) where it was desorbed at 280^{o}C for 3 minutes. The sample was then loaded onto a multidimensional gas chromatograph and into the first column (30 m x 0.53 mm ID/ BPX 5 [5% phenyl polysilphenylene-siloxane] x 0.50 µm, SGE Analytical Sciences, Austin, TX). The column was split three ways: (1) valve went to the mass spectrometer (Agilent Technologies, 5975 series MSD, Santa Clara, CA), (2) & (3) went to two separate sniff ports, which were heated to 115^{o} C and fitted with nose pieces. The sniff ports and accompanying software for analyzing volatile aroma are a part of the AromaTrax program (MicroAnalytics-Aromatrax, Round Rock, TX). Once a significant difference was found, the data was then analyzed for increase/decrease of each compound with increasing eBeam dose.

6.3 COLOR METHODS

Preparation of Freeze-Dried Berry Medley

Preparation of freeze-dried berry medley was performed as described previously with the exception that the berries were separated into individual berry type for dosing, dosimetry and color analysis.

Dosing and Dosimetry

eBeam dosing protocol and dose mapping was performed as described previously. The berries were processed at 5 kGy increments at 34^{o} F by keeping the belt speed constant and conducting multiple passes through the LINAC using accumulative dosing. The speed of the belt was set to 14.9 f/m. The target doses and measured doses were; 0 – (no eBeam process), 5 kGy – 4.69 kGy, 10 kGy – 9.64 kGy, 15 kGy – 14.46 kGy, 30 kGy – 29.68 kGy, and 45 kGy – 43.57 kGy.

Color Measurements

The color was measured using a Konica Colorimeter (Chroma Meter CR-400, Minolta, Tokyo, Japan) for L*, a*, and b* values. Each berry sample was measured for L*, a*, and b* values in triplicate while in the bag. The colorimeter used in this project was calibrated using color standards with the instrument. A sterile bag was tested on a white background and processed with the berries as a control sample.

6.4 STASTICAL ANALYSIS

Data were analyzed using JMP (SAS, Inc., Cary, NC) to determine any statistical differences with each treatment group for color and MDGC-O-MS. The students t-test was performed on the color analysis to determine any significant changes in L*, a*, and b* values by berry type over each dose point; alpha was set to 0.05. MDGC-O-MS data were performed using a least squares fit model to determine significant changes in volatile compounds to the berry medley as a function of dose; alpha was set to 0.05.

6.5 RESULTS AND DISCUSSION

Sixty-nine (69) volatile compounds were identified in the berry medley with the MDGC-O-MS (Appendix A). Of these, only 5 compounds showed to be significantly different with respect to eBeam processing ($P < 0.05$) measured in total ion counts (Table 5). Each compound that showed significance was then studied for their known organoleptic attributes (Burdock, 2010). The chemical 2-butenal is naturally derived from various sources including fruits and known for apple & strawberry aromas (Figure 27; CFR 172.525). This chemical has been identified to increase in concentration in various foods during storage (Wang et al., 2019). 3-methyl-butenal can be extracted from 180 sources apple juices and have apple and fruity organoleptic characteristics (Figure 29; CFR 172.515). This chemical has also been commercially used in food products such as beer, cheese, coffee, and olive oil (Cserháti & Forgács, 2003). Ethyl acetate is the acetate ester that is formed from ethanol and acetic acid. The chemical has fruity attributes and can naturally be extracted from raspberries (Figure 31; CFR 73.1, 182.60, 177.560, 173.228, 582.60, 172.372, 172.560, 172.695, 182.60, 584.200). These chemicals showed an increase in total

ion count (increased in chemical formation) with an increase in eBeam dosing; 0 to 15 kGy. These chemicals are of no hazard to humans when used as flavorings (Burdock, 2010).

Alpha pinene has been extracted from various sources including raspberries, blackberries and strawberries and have organoleptic attributes of cedarwood and pine. This chemical showed a decrease in total ion count with an increase in eBeam dosing; 0 to 15 kGy (Figure 28). This chemical is not a concern to humans when used as a flavoring agent (Burdock, 2010; CFR 175.105).

Furans are one of the most abundant and extracted chemicals found in fresh and processed blackberries, giving them their aroma and flavor profiles (Jimenez-Garcia et al., 2013). The chemical 2-furancarboxaldehyde (furfural) is a chemical that is can be naturally extracted from berries. The chemical has organoleptic attributes of sweet, woody, bready, nutty, caramellike with a burnt astringent nuance and are not of human concern when used as a flavoring agent (CFR 175.105). Furans have been produced and sold in the meat and beverage industry as flavor ingredients (Weerasinghe & Sucan, 2005). Furans are produced from various precursors such as ascorbic acid, and berries have a very high ascorbic acid content (Morehouse et al., 2018). 2-furancarboxaldehyde only showed significant increase in formation from 10 to 15 kGy.

Appendix A is a list of all the compounds that were extracted and identified using (MDGC-O-MS). These volatiles were extracted from the berries at doses ranging from 0 to 15 kGy. There was no significant compound formation or compound decomposition with respect to eBeam processing with these volatiles unless stated. The purpose of this study was to determine the effects of eBeam processing on freeze-dried berry medley, making the compounds in Appendix A that show no statistical changes ($P > 0.05$) to be inconclusively affected by eBeam processing. The

unaffected volatiles are either stable with respect to eBeam processing or anomalies from uncertain process deviations.

Exposing the berries to eBeam radiation have minimal effects on the color of each berry type (Table 6). The berries showed no change in L*, a*, and b* values within treatments and control ($P > 0.05$; Table 5; Figures 33-35) except for one treatment; strawberry a* value (Table 7). The strawberry a* value treatment shows decreasing a* value with increasing dose. Significant changes can be seen between 0, 15, and 45 kGy (Table 7). The a* value measures the red-green relationship (Pathare, 2012), where an increase in a* value would be an increase in the intensity of red color and a negative value would be an increase in the intensity of green color. The a* value is inversely proportional to the absorbed dose of eBeam processing. With increasing eBeam dose, the strawberry changes to less red. Strawberries are full of anthocyanins that give the strawberry their red color (Lopes da Silva et al., 2007). The drop in red color is due to the degradation of the of the anthocyanins by oxidation (Pantras et al., 2010). The same trend was observed from control and eBeam samples of fresh strawberries irradiated at 0 and 1 kGy (Smith et al., 2013). All other L*, a*, and b* values showed no significance difference up to 45 kGy of eBeam processing ($P > 0.05$).

The L*, a*, and b* values are very useful tools for determine changes in color, however, the human sensory perspective might not be able to detect changes in just one value (Lee et al., 2013). The three values pick out a point on a three-dimensional plane that make all three values important for determining human perceptions (Indow & Uchizono, 1960). The a* value and b* value was used in combination to determine the hue angle (Mclellan et al., 1995). The hue angle can be used to determine changes due to the processing by using the equation below (Eq. 3).

(3) $\Theta = \text{ArcTan}(b/a)$

There were no changes to the hue from 0 to 45 kGy on freeze-dried berry medley. This signifies that there are no perceivable changes to the color. A human sensory study needs to be performed for color acceptance due to freeze-drying and eBeam berry processing.

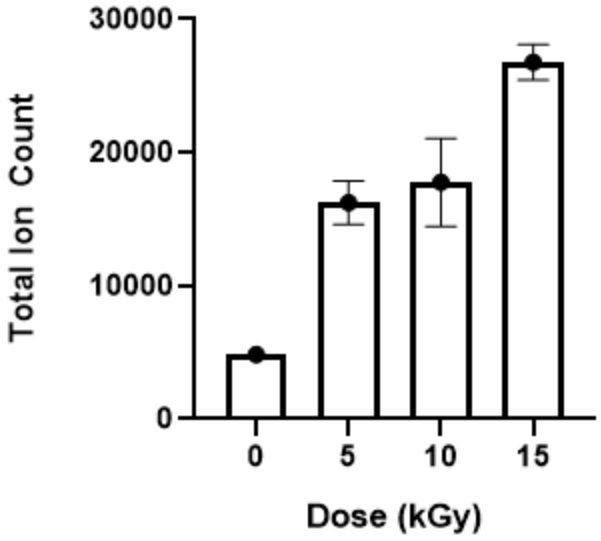

Figure 27: Ion concentration of 2-butenal accumulating in the freeze-dried berry medley at varying eBeam doses as detected using multidimensional gas-chromatography – olfactometry – mass spectrometry (MDGC-O-MS). Data were analyzed using least squares fit model ($P < 0.05$).

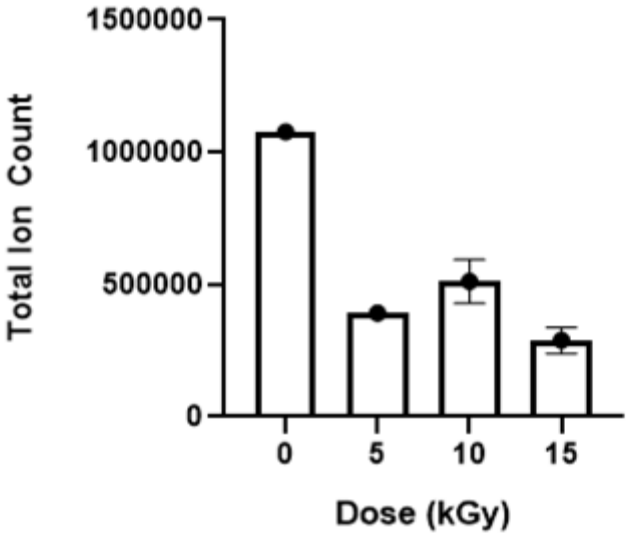

Figure 28: Ion concentration of alpha pinene accumulating in the freeze-dried berry medley at varying eBeam doses as detected using multidimensional gas-chromatography – olfactometry – mass spectrometry (MDGC-O-MS). Data were analyzed using least squares fit model ($P < 0.05$).

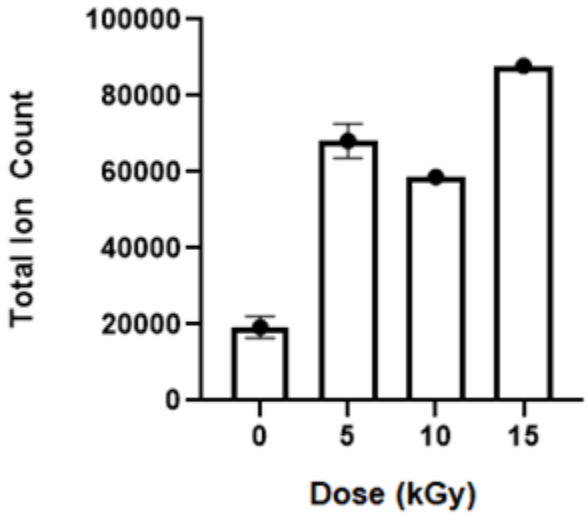

Figure 29: Ion concentration of 3-methyl-butanal accumulating in the freeze-dried berry medley at varying eBeam doses as detected using multidimensional gas-chromatography – olfactometry – mass spectrometry (MDGC-O-MS). Data were analyzed using least squares fit model ($P < .05$).

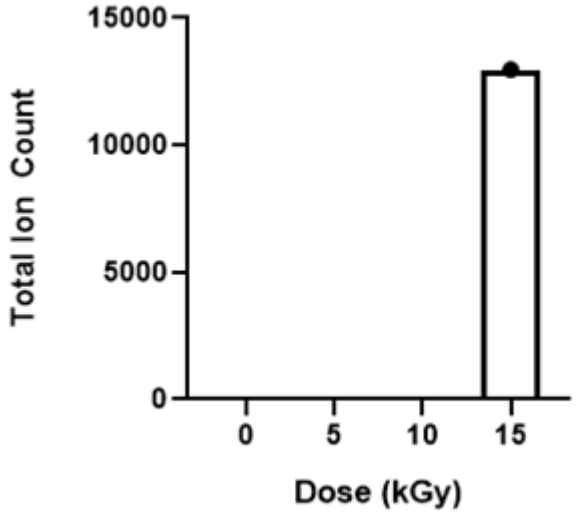

Figure 30: Ion concentration of 2-furancarboxaldehyde accumulating in the freeze-dried berry medley at varying eBeam doses as detected using multidimensional gas-chromatography – olfactometry – mass spectrometry (MDGC-O-MS). Data were analyzed using least squares fit model ($P < 0.05$).

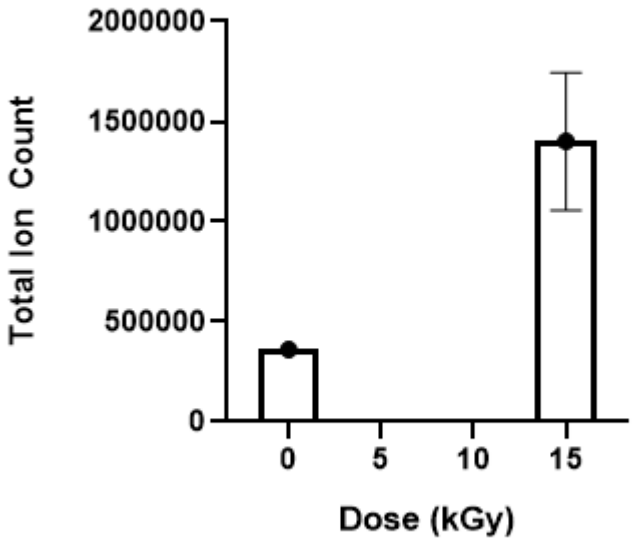

Figure 31: Ion concentration of ethyl acetate accumulating in the freeze-dried berry medley at varying eBeam doses as detected using multidimensional gas-chromatography – olfactometry – mass spectrometry (MDGC-O-MS). Data were analyzed using least squares fit model ($P < 0.05$).

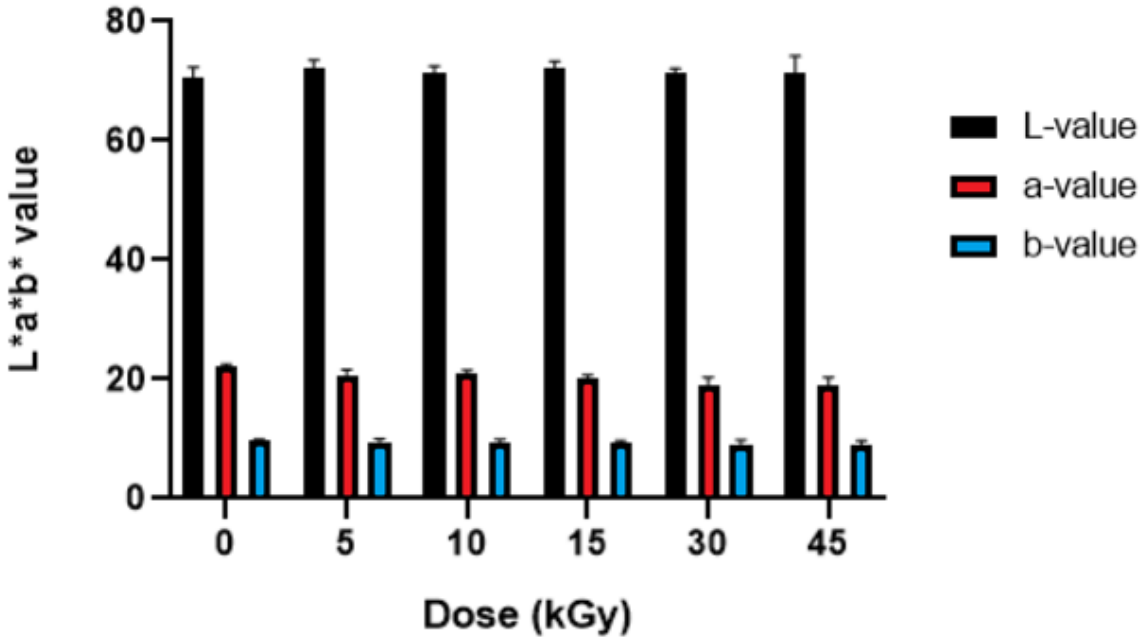

Figure 32: Color (L*, a*, and b* values) of the freeze-dried strawberry at varying eBeam doses as measured using Konica Colorimeter.

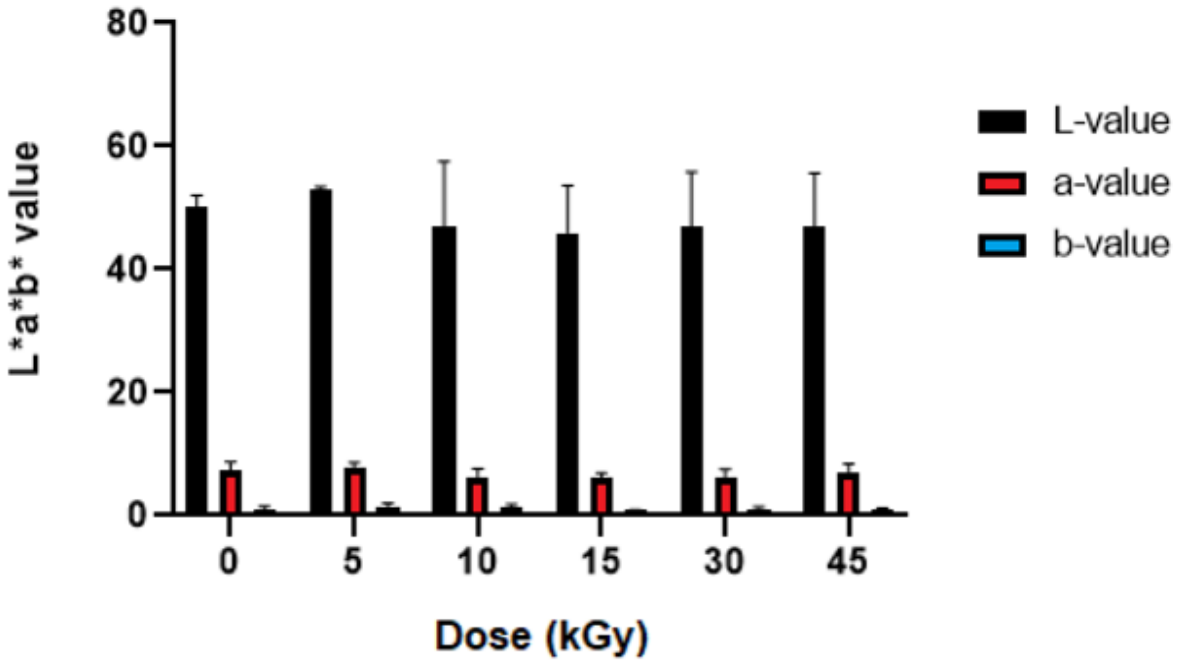

Figure 33: Color (L*, a*, and b* values) of the freeze-dried blackberry at varying eBeam doses as measured using Konica Colorimeter.

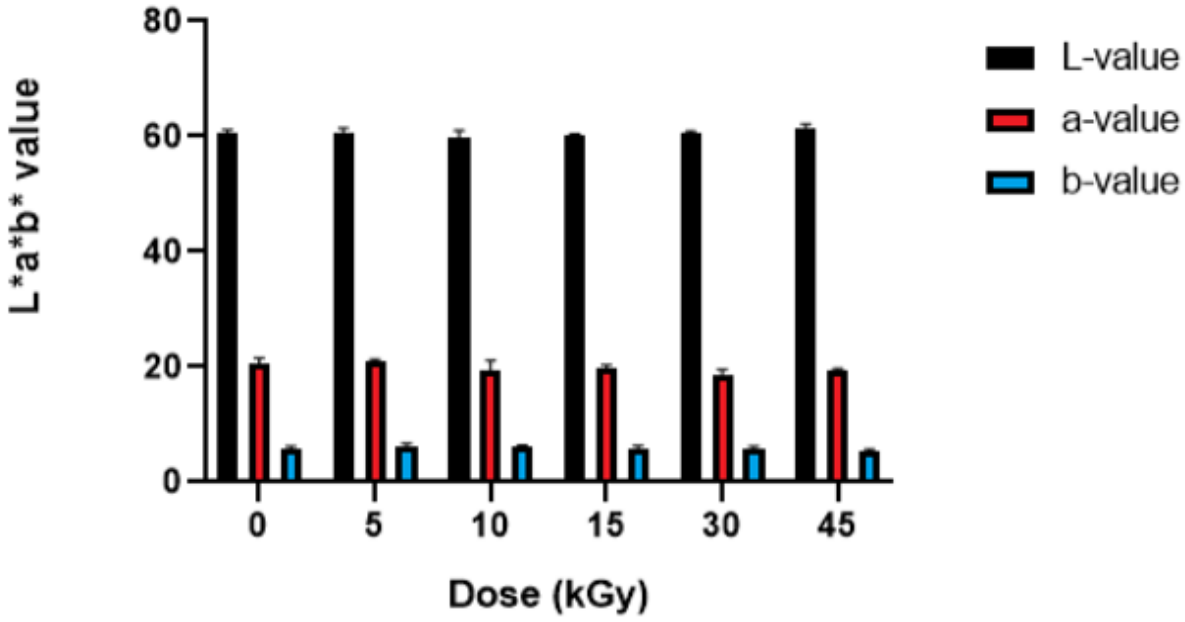

Figure 34: Color (L*, a*, and b* values) of the freeze-dried raspberry at varying eBeam doses as measured using Konica Colorimeter.

Table 5: Chemical changes due to eBeam processing.

Increasing with eBeam Dose

			Total ION Count Dose			
Compound	p-value	Descriptors	0	5	10	15
2-butenal	0.0338	Flower	4844[a]	16235[ab]	17767[b]	26766.5[b]
3-methyl-butanal	0.0321	Apple, Fruitty	19304.5[a]	68219[b]	58699.5[b]	87903.5[b]
ethyl acetate	0.0447	Fruity attributes	360693[a]	0[a]	0[a]	1400176.5[b]
2-furancarboxaldehyde	<0.0001	Brown, sweet, woody, bready, nutty, caramel like	0[a]	0[a]	0[a]	12965[b]

Decreasing with eBeam Dose

			Total ION Count Dose			
Compound	p-value	Descriptors	0	5	10	15
alpha pinene	0.0046	Cedarwood, pine, sharp	1152210.5[a]	284493[b]	512242.5[b]	288850[b]

Table 6: Effect of eBeam processing on L*, a*, and b* values for strawberry, blackberry, and raspberry and control from 0 – 45 kGy

Berry Type	L*, a*, and b* value	Average					
	L* value	Dose (kGy)					
		0	5	10	15	30	45
Strawberry		70.65[a]	72.32[a]	71.27[a]	72.09[a]	71.31[a]	71.52[a]
Blackberry		50.20[a]	52.90[a]	53.03[a]	50.46[a]	51.86[a]	52.27[a]
Raspberry		60.68[a]	60.80[a]	59.91[a]	60.15[a]	60.48[a]	61.59[a]
Control (bag)		87.49[a]	NM*	NM*	87.39[a]	87.87[a]	87.70[a]
	a* value	Dose (kGy)					
		0	5	10	15	30	45
Strawberry		22.21[a]	20.79[a,b]	20.83[a,b]	20.32[b,c]	19.16[c]	18.86[c]
Blackberry		7.44[a]	7.91[a]	7.04[a]	6.70[a]	7.20[a]	7.82[a]
Raspberry		20.52[a]	21.04[a]	19.25[a]	19.88[a]	18.75[a]	19.57[a]
Control (bag)		0.45[a]	NM*	NM*	0.44[a]	0.53[a]	0.42[a]
	b* value	Dose (kGy)					
		0	5	10	15	30	45
Strawberry		9.72[a]	9.56[a]	9.56[a]	9.54[a]	9.19[a]	8.89[a]
Blackberry		0.93[a]	1.46[a]	1.33[a]	0.90[a]	1.21[a]	1.04[a]
Raspberry		5.64[a]	6.14[a]	6.03[a]	5.93[a]	5.77[a]	5.43[a]
Control (bag)		-1.33[a]	NM*	NM*	-1.49[a]	-1.36[a]	-1.41[a]

*NM – value was not measured

CHAPTER VII

SUMMARY AND CONCLUSIONS

7.1 SUMMARY AND CONCLUSIONS

The study successfully isolated and identified eBeam resistant fungi: *Aspergillus* spp., *Penicillium* sp., *Cladosporium* spp. The isolation of these fungi was important to provide a worst-case scenario for electron beam processing of freeze-dried berries for fungal decontamination. This study shows that fungal isolates in freeze-dried berry medley are susceptible to eBeam processing with D_{10} values ranging from 0.87 to 2.70 kGy. The elimination of fungi from a freeze-dried sample was seen at 15.26 kGy. At this dose, a 5.6 log reduction of the fungi in a freeze-fried berry medley can be achieved based on the most resistant organism studied. This is enough to eliminate an average fungal bioburden of 10^4 (Verde et al., 2013; Tournas et al., 2015).

eBeam processing at 15 kGy has minimal to no effect on color. Only the a* value for strawberries showed significant changes from 0 to 45 kGy. All other berries showed no changes in L*, a*, and b* values. There were sixty-nine (69) volatile compounds that were successfully extracted from the berry medley across 0 to 15 kGy. Of these sixty-nine (69) compounds, only five (5) were significantly affected by the eBeam process ($P < 0.05$). 2-butenal, 3methyl-butanal, 2-furancarboxaldehyde and ethyl acetate showed significant increase in total ion count with increasing eBeam dose from 0 to 15 kGy. The attributes for these compounds include descriptors such as flower, apple, fruity, bready, sweet and nutty. The increase of these organoleptic attributes could have multiple benefits in the flavor industry. The crushing and eBeam processing of the dehydrated berries could be utilized as a flavor enhancer as a smaller portion of a bigger formulation in the food industry. This higher concentration of flavor ingredients would benefit a

beverage manufacturer by achieving the same flavor thresholds while using fewer starting materials.

The volatile alpha pinene showed a decrease in total ion count with increasing eBeam dose from 0 to 15 kGy. The attributes for this compound include descriptors such as cedarwood and pine. All other volatiles that were detected cannot be scientifically linked to eBeam processing at the doses studied.

The study design was to evaluate volatiles production and color changes in the freeze-dried berries from eBeam processing by measuring these attributes less than a week after eBeam processing. There is a potential for volatile changes in the berry medley from longer storage (Bhatia et al., 2017). The end user for this product will not be eating this product directly after eBeam processing which is why it is important to design a study to include storage time and conditions as additional variables for the berry medley.

Freeze-drying is an extensive and costly process when compared to a conventional dehydration process. Higher temperatures are used for a conventional dehydration process compared to freeze-drying which can have potential changes in the volatile and color profiles to the berry medley (Berk, 2013). Another look at this study would be to determine if there are any benefits to using a freeze-dried process vs. a conventional process to reduce costs.

eBeam technology is a tool in the food processing industry that can be coupled with another food preservation technology such as freeze-drying. It should not be considered the silver bullet or a cleanup technology. This means that a comprehensive food safety program should be implemented with eBeam processing to improve food safety.

7.2 RECOMMENDED FUTURE RESEARCH

(1) The nutritional content of the eBeam processed freeze-dried berries were not studied. Berries have health benefits as stated in Chapter 2, but we are unsure of the phytochemicals and micronutrients stability with increasing eBeam processing.

(2) The volatiles were not quantified in this study. Future studies need to quantify the increases and decreases of the volatiles that were related to eBeam processing.

(3) Human sensory studies with appropriate IRB approval need to be performed on the eBeam freeze-dried berries. Objective studies were performed on the color changes and volatiles produced from eBeam processing but a true test for consumer acceptance are sensory panels.

(4) Only volatiles were extracted and identified in this study. The effects of eBeam processing on non-volatile chemicals need to be studied as well.

(5) Study the chemical changes of freeze-dried and eBeam processed foods over time.

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APPENDIX A

ALL COMPOUNDS EXTRACTED FROM BERRIES

Compound	Compound
Alpha pinene	(E) – 2-heptenal
3-carene delta	4-methyl-1-(1-methylethyl) 3-cyclohexen-1-ol
2(3H)- dihydro furanone	6-Methyl-5-hepten-2-one
2-3-hydroxy-butanone	Acetic acid
2-furancarboxaldehyde	Acetic acid, hexyl ester
2-heptanol	Acetic acid, methyl ester
2-hexenal	1,3-dimethyl-benzene
2-1-propoxy-propanol	1-methyl-2-(1-methylethyl)- benzene
2-propanone	3-methyl- butanal
Benzaldehyde	Butanoic acid, methyl ester
Butanoic acid	Caryophyllene
Butanoic acid, butyl ester	Ethyl acetate
Decanoic acid, ethyl ester	Tetrahydro-furan
Heptanal	Hexanoic acid, ethyl ester
Hexanal	Hexanoic acid, methyl ester
dl-Limonene	Octanal
Thiobis-methane	2,3-Dihydrofuran
Nonanal	2-beta pinene
Pentanal	Butanoic acid, ethyl ester
Sabinene	2,3-Butanediol
Acetic acid, ethyl ester	Butanal
Decanal	Decane
Dimethyl-disulfide	Butyrolactone
Longicyclene	3-methyl-3buten-2-one
l-Phellandrene	1-methyl-4-(1-methylethyl)-benzene
Trans-caryophyllene	Xylene
Dimethyl-trisulfide	1,8-Cineole
Terpinolene	2-methyl-furan
Gamma terpinene	3-methyl-2butenal
1-Butanol	2-Propenal
2,3-Butanedione	Furan
2,4-Hexadienal	Furfural
2-Butanone	2-methyl-propanal
2-Butenal	Toluene
2-Heptanone	

APPENDIX B

INTERNAL TRANSCRIBED SPACER SEQUENCE PROTOCOL

Document Control Number WI	TEXAS PLANT DISEASE DIAGNOSTIC LAB Texas AgriLife Extension Service 1500 Research Parkway, Ste 130A College Station, TX 77845	Revision Number 1
Effective Date: 02/15/17	Fungal ID using ITS primers with Phusion Polymerase 20µL	Page 1 of 2

Note: With every run, make sure to run positive and negative control.

PCR procedure:

1. Remove all reagents and samples from freezer and thaw, but keep on ice.
2. Vortex and centrifuge reagents before adding to master mix (just lightly invert the polymerase tube several times instead of vortexing).
3. Vortex and centrifuge extracted DNA samples.
4. Prepare sufficient amount of master mix for the number of samples and controls (n) plus 1 extra→ n+1

PCR Master Mix:

a. 48 µl Water (Sterile distilled water – Fisher)(6 µl x (n+1))
b. 80 µl Polymerase (Phusion polymerase – Fisher)(10 µl x (n+1))
c. 8 µl ITS1** primer (1 µl x (n+1))
d. 8 µl ITS4** primer (1 µl x (n+1))

** ITS1F may be used instead of ITS1 for 38 more bp→ does not change annealing temp
**primer conc. 10µM working concentration for both primer sets - see below for sequences

5. Vortex prepared master mix well and centrifuge.
6. Label a set of 0.2ml PCR tubes.
7. Add to each 0.2ml reaction tube (20µl reaction):
   a. 18µl PCR Master Mix
   b. 2µl DNA template (50ng/ µl)
8. Vortex reaction tubes lightly (or just invert once or twice), and pulse centrifuge.
9. Run PCR program "FAST-ITS"

i. Lid Temp 101°C Preheated
ii. 98°C – 10 sec.
iii. 98°C – 01 sec.
iv. 52°C – 05 sec.
v. 72°C – 15 sec.
vi. Repeat iii – v (35 cycles)
vii. 72°C – 1 min.
viii. 4°C - ∞

**Store products at -20°C C until ready to run on gel.

10. Run PCR product out on 2% agarose gel (TAE buffer).
Mini gel recipe: **10ml** 3X GelRed; **600µl** 50X TAE; **19.4ml** Water; **0.6g** Agarose
Run at 80V for 60minutes.
Expected Band is ~800bp

**ITS primer set
use a working concentration of 10µM

ITS1 (5'-TCC GTA GGT GAA CCT GCG G -3')
ITS4 (5'-TCC TCC GCT TAT TGA TAT GC- 3')
ITS1F (5'-CTT GGT CAT TTA GAG GAA GTA A-3')

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Status (Original/Revision/Cancelled)	Document Revision Number	Effective Date	Description
Original	Original	02/15/2017	JU-Fungal ID using ITS primers

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Date:

Approved By: Quality Manager

APPENDIX C

FREEZE DRYING PROTOCOL