Effect of ethanol vapor fumigation on survival of *Salmonella enterica* biofilms on whole black peppercorns

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ABSTRACT

Salmonella embedded biofilms were formed on whole black peppercorns and treated with ethanol vapor under atmospheric pressure conditions and under vacuum assisted pressure conditions. The effect of ethanol vapor, heat and vacuum pressure on the survival of multiple *Salmonella enterica* serovars within a biofilm contained on low moisture food products (A_w 0.30-0.40) was observed in this study. Samples were exposed to zero, one, five and ten minute ethanol vapor treatments at atmospheric boiling temperature (Atmospheric Pressure Boiling Method, AB), and at reduced temperature and pressure conditions, -20 inHg (Vacuum-Assisted Boiling Method, VB). The AB treatments showed 4.0 log CFU/g reductions on nonselective media that included native microbiota, and 6.0 log CFU/g reductions on *Salmonella* selective media. The VB treatments showed 2.69 log reductions on Tryptic Soy Agar and 4.55 log reductions on Xylose Lysine Tergitol-4 agar. Ethanol vapor treatments should be further investigated as an alternative to ethylene oxide or ionizing radiation processes to treat dry spices to control *Salmonella*.

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LIST OF ABBREVIATIONS

AB- Atmospheric Pressure Boiling

CFU- Colony Forming Unit

CLSM- Confocal Laser Scanning Microscopy

FDA- United States Food and Drug Administration.

ESEM- Environmental Scanning Electron Microscopy

GC-MS- Gas Chromatograph Mass Spectrometer

SPME- Solid Phase Microextraction

TSA- Tryptic Soy Agar

TSB- Tryptic Soy Broth

VB- Vacuum Assisted Boiling

XLT4- Xylose Lysine Tergitol-4 agar.

ATTRIBUTION

There are many people who contributed to the completion of the research described here. Those individuals as well as their contributions are described here.

Robert Williams – Ph.D. (Department of Food Science and Technology, Virginia Tech) Primary advisor and committee chair. Provided guidance and advice for the implementation of this research project.

Monica Ponder – Ph. D. (Department of Food Science and Technology, Virginia Tech) Committee member. Performed laboratory safety training and provided feedback throughout the progression of the project.

Kumar Mallikarjunan – Ph.D. (Biological Systems Engineering, Virginia Tech) Committee member. Provided feedback and support throughout the progression of the project.

Ken Hurley – (Department of Food Science and Technology, Virginia Tech) Provided advice on glassware design and selection, working with compressed gasses, and working with ethanol.

Ershad Sheibani – (Department of Food Science and Technology, Virginia Tech) Performed the Solid Phase Microextraction tests on the samples provided.

Hengjian Wang – Ph. D (Department of Food Science and Technology, Virginia Tech) Provided advice on statistical analyses and performed Weibull Regression.

Chapter I

INTRODUCTION

Salmonella enterica is estimated to cause the greatest number of bacterial foodborne infections and hospitalizations in the United States *(23)*. *Salmonella* can be introduced into the food supply by a number of means, but recently spices have been identified as an important source of this foodborne pathogen *(72)*. Spices may become contaminated during any stage of their production, and are primarily produced in warm, humid regions. Many spices undergo a drying step as an attempt to increase the shelf life of the product via decreased water activity, but this drying step alone may not adequately eliminate pathogens prior to packaging and distribution across the world. The FDA has reported that nearly seven percent of tested, imported spice products were found to be contaminated with *Salmonella (99)*. As one of the largest importers of spices, safety of spices is of upmost concern for the food industry, food safety scientists, and regulators in the United States.

Spices are widely consumed in foods in the United States and are often added to foods that will receive no further processing. Spices are grown and harvested in various parts of the world where food safety management practices may be lacking. There is evidence that spices may become contaminated with foodborne pathogens during any phase of their production. There have been several reported *Salmonella* outbreaks traced back to the consumption of contaminated spices *(100)*. It would be to the benefit of the food industry to apply good food safety management practices to improve the safety of spices. The current processes used to treat spices are lacking due to the nature of the product: spices are dried products that contain flavor and aromatic compounds that must be preserved. Many validated processes have been shown to alter the color, brightness, and flavor characteristics of the spices being treated. Some approaches have not been properly evaluated as a means to treat spices, one of which is an ethanol fumigation treatment. Unlike other fumigation treatments, ethanol is a Generally Recognized as Safe and is non-deleterious to health in residual quantities. This project aims to provide useful data to the industry to make decisions about the possible utilization of ethanol vapor as a processing step to control the foodborne pathogen *Salmonella enterica*.

OBJECTIVES

- To design an apparatus for the effective treatment of *Salmonella* embedded biofilm covered peppercorns using ethanol vapor.
- To measure the effectiveness of one, five, and ten minute ethanol vapor treatments on mixed serovar *Salmonella* embedded biofilm populations on whole black peppercorns.
- To observe the differences in population reduction when ethanol boiling treatments are applied under atmospheric and vacuum assisted pressure conditions.
- To determine the effect these treatments would have on volatile content using Solid Phase Microextraction.

LITERATURE REVIEW

Properties of *Salmonella enterica*

Salmonellosis

Salmonella is a leading cause of acute human gastroenteritis caused by bacteria *(55)*. According to the Centers for Disease Control and Prevention, there were over 42,000 reported cases of salmonellosis in 2009, with estimates of unreported cases being almost 29 times as many *(23)*. As of 2006, there was an estimated 644,000-1.6 million cases of salmonellosis in the United States, but it is believed most people with mild symptoms do not always seek medical assistance. Estimating the total impact of foodborne illness is difficult due to the nature of the illness, the host factors, and limited laboratory test reports*(84)*. Symptoms of salmonellosis include diarrhea, fever, headache, and abdominal cramps that often begin from 12 to 72 hours after infection *(23)*. *Salmonella* virulence varies among serovars, but the average infectious dose for most outbreaks has been reported as 10^5 to 10^{10} organisms. However, under certain circumstances, such as low-moisture, high-fat environments, the infectious dose may be less than 100 organisms. The attack rate is estimated to be between 16% and 50% *(55)*. The illness itself can last from 4 to 7 days.

Most people recover without treatment, but in some cases hospitalization is required. Certain *Salmonella* infections have the ability to go systemic, causing a host of long term problems which require prompt treatment with antibiotics *(23)*. Infections that have gone systemic have been shown to result in conditions such as reactive arthritis, cardiac inflammation and certain neural disorders*(27)*. Joint pain, irritation of the eyes and painful urination for several months have been reported as long term consequences of salmonellosis. These symptoms are usually mild, but salmonellosis is more likely to occur in infants and the elderly, or those otherwise immunocompromised *(55)*. Children are the most at risk of developing salmonellosis. It is estimated that 400 persons die each year due to acute salmonellosis *(23)*. Food-Net does active surveillance for *Salmonella* spp., but lab based surveillance data is only available if the ill person sought medical help *(84)*. The vast majority of sporadic cases are caused by contaminated food, the most frequently implicated include undercooked poultry, eggs, pork and contaminated ready-to-eat products. In recent years, there has been an increase in the amount of plant based

Salmonella outbreaks, including fruits, vegetables and spices *(71, 111)*, most likely noted due to improved detection techniques.

Resistance

Salmonella can adapt to a range of temperature conditions; *Salmonella* growth has been observed at temperatures as low as 2°C and as high as 54°C, and survive in conditions outside of that range *(72)*. Optimum pH for growth of *Salmonella* is between 6.5 and 7.5, but the organism has been known to survive pH conditions as little as 3.8 and as great as 9.5 *(72)*. Other factors that affect the growth and survival of *Salmonella* include pH, temperature, and redox potential of the environment *(77)*. Low-moisture foods do not support the growth of *Salmonella*, but have been implicated in many salmonellosis outbreaks due to resilience and persistence strategies that the organisms possess *(111)*. Whereas *Salmonella* can survive for weeks in water systems, it may survive for years in soil, depending on the conditions.

Salmonella has been a concern for the food industry due to heat and drying resistance, as well as the ability of *Salmonella* to form biofilms on stainless steel equipment. The ability of *Salmonella* to form biofilms can affect the survival of bacteria under dry or otherwise harsh conditions*(43)*. *Salmonella* has been shown to increase its ethanol resistance under desiccation conditions; in one study, *Salmonella* was shown to have resisted up to 30% ethanol solution treatments after desiccation *(37)*. When exposed to water activity conditions below 0.94, *Salmonella* have been shown to elongate and retain a filamentous morphology *(49)*. *Salmonella* strains also show strong resistance to dryness under refrigeration conditions. One study shows improved effectiveness of ethanol and acid treatments on population reduction at ambient temperatures *(21)*, and consequently the lowered effectiveness of treatments at more extreme temperatures.

Ampicillin, chloramphenicol and trimethoprim-sulfamethoxazole are the primary antibiotics used to combat salmonellosis, but strains have been appearing with resistances to one or more of these antibiotics. In a study of ground meat products in the Washington, DC area, 41% of samples taken tested positive for *Salmonella* strains that were resistant to at least one antibiotic*(104)*. There have been successful clinical results with different drugs, quinolones, but there have already been signs of salmonellae that are resistant to these as well *(27)*. *Salmonella*

Newport, Weltevreden, and Lexington strains have been reported to be commonly associated with some degree of antibiotic resistance *(109)*.

Survival in Low Moisture Environments

Reducing the water activity of a food product has been a method of increasing shelf life for thousands of years. The food industry uses drying as a way to suppress bacterial growth in a food product *(37)*. The resilience of *Salmonella* has allowed it to be present in or on a variety of low moisture food products, from chocolate to salami, deer jerky to dried milk *(41)*. Although, the preferred water activity conditions for *Salmonella* are 0.93 and above, some strains have been reported to survive for years in low-moisture foods. It has been reported that when food is dried to water activity readings as low as 0.2, *Salmonella* populations remain stable and can take anywhere from 248 to 1351 days for a 1-log reduction *(72)*. One study reported *Salmonella* strains maintaining population levels equal to initial post-drying populations for 22 to 24 months *(41)*. A study has shown that *Salmonella* survival after being subjected to a drying step is more closely dependant on storage temperature than storage water activity *(77)*. When storage temperatures are low, the number of surviving cells after desiccation over time is greater when compared to higher storage temperatures.

Foods containing large amounts of sucrose, fatty acids, or accessible proteins *(41, 55, 72)* are thought to have a synergistic effect on not only the survival of *Salmonella* in low moisture conditions, but also in its survival through the human gastrointestinal tract. *Salmonella* heat and stress resistance have been shown to be affected by many factors prior to exposure to that stressor. The growth medium, growth phase, current temperature and previous heat or acid conditions have effects on *Salmonella* stress resistance. After desiccation, *Salmonella* exhibit resistance to extreme heat conditions *(4, 41)*; water activities of 0.65 or lower have a protective effect on *Salmonella* when exposed to 70°C or greater temperatures *(64, 72)*. It is hypothesized that water molecules, when heated in the near vicinity of a *Salmonella* cell, vibrate and transfer heat to the outer membrane proteins, disrupting bonds and folding patterns- in low moisture foods, there is no water to do this disruption. Another theory is that under low water conditions, the dipoles of proteins link to one another in order to provide greater stabilization, thus requiring more energy to unfold the peptide chains and damage the organism. Studies have shown desiccated *Salmonella* cells in the rough, dry and red morphotype that maintain infectivity for 30

months *(3)*. Where sucrose, fatty acids and accessible proteins are believed to increase *Salmonella* survival in harsh conditions, salt and acidity have a detrimental effect on the survival of *Salmonella* under desiccated conditions *(41)*.

Salmonella are able to enter a viable but non culturable state (VBNC). It is unclear whether *Salmonella* in VBNC states retain pathogenicity. *Salmonella* have also been known to modify the outer membrane composition to reduce osmotic stress in such conditions *(72)***.** It is thought that these changes are brought on by the activation of Sigma factors E and S *(67)*, and activation of Sigma factors prior to harsh conditions trigger their advanced survival characteristics, allowing them to survive some food processing measures and storage *(1)*. Some serotypes have been known to show greater levels of filamentation after exposure to marginal growth conditions as a survival mechanism.

How Salmonella *is Introduced*

There has been an increase in prevalence of plant based *Salmonella* outbreaks reported in recent years. The ability of *Salmonella* to adhere to fresh produce depends on the serovar *(15)*, but *Salmonella* contamination can happen at any stage of the pre-harvest, postharvest, shipping, retail and home consumption processes. The containment of *Salmonella* is vital and in order to prevent it from entering the food chain, Good Agricultural Practices and Current Good Manufacturing Practice should be followed *(77)*. Contamination can occur due to differing and often inadequate agricultural and manufacturing practices *(111)*, however, *Salmonella* has been shown to be able to enter a plant through the root system and have been found in the leaves and fruit of a plant through the plant water uptake functions *(71)*.

Contaminated manure or improperly treated compost, wild and domestic animals on farmland, improper irrigation systems and wash water are all possible pathways for *Salmonella* to enter the food supply. Cross contamination is thought to be a major cause of salmonellosis, especially in restaurants and catered functions *(69)*. Restricting the movement of plant operators, regulating airflow, and having properly designed equipment specifications are all necessary steps towards reducing cross contamination of *Salmonella(86)*. Stainless steel has a transfer rate of 20-100% for at least four days after being moderately contaminated *(72)*, it is critical to have effective sanitation protocols in place that can adequately remove *Salmonella* from food contact surfaces.

Salmonella *and Biofilms*

Biofilms are structured communities of bacterial cells that have encased themselves in a hydrated extracellular polymeric substance (EPS) matrix on living or nonliving surfaces. Most biofilms in nature consist of multiple species which interact synergistically as well as competitively *(39)*. Biofilms formed of multiple species tend to show increased biomass when compared to single species films *(19)*. Aggregative fimbriae of *Salmonella* have been shown to allow for the adherence of organisms to numerous organic and inorganic surfaces such as stainless steel and Teflon*(6)*. Once the organism has adhered, the biofilm forming process begins. Biofilm formation is determined by the interaction of curli, fimbriae *(12)*, BapA, flagella *(14)*, cellulose *(89)*, colonic acid, the O-antigen capsule *(15)*, fatty acid and other substrate availability, *(58, 93)* and quorum sensing.

Bacteria in biofilms are generally well protected against environmental stresses, antibiotics *(29)*, disinfectants and the host immune system, making them very difficult to destroy. The EPS layers contribute to the ability of bacteria to withstand the host immune system and antibiotics *(73)*. The ability to form biofilms within mucosal linings of animals increases the ability of *Salmonella* to persist in the environment. Cholesterol coated surfaces encourage formation of biofilms. It is unknown whether *Salmonella* can form biofilms within plant tissue, but it is theorized that *Salmonella* Typhimurium is a candidate for endophytic biofilm growth *(93)*.

Salmonella outbreaks on plant based products such as sprouted seeds, fruits and spices have been attributed to the presence of biofilms. Various surface sterilization methods are ineffective when attempting to remove biofilms due to the resistance and resilience of the biofilm, and the delicate nature of the food products. Biofilms are often formed as an environmental persistence strategy rather than a virulence strategy, but environmental persistence often leads to *Salmonella* out-competing other bacteria.

Biofilm production is especially important in food processing environments due to the ability to form biofilms on a number of nonliving surfaces such as plastic, rubber, cement, and stainless steel *(50, 72, 93)*. A recent study has noted the ability of *Salmonella* Tennessee to form biofilms on glass beads when placed in a single layer in Tryptic Soy Broth *(7)*. *Salmonella* can form biofilms at solid-liquid interfaces as well as liquid-air interfaces *(43)*. Pellicle formation in LB

broth is a widely used laboratory manifestation of *Salmonella* embedded biofilms, but biofilms grown in lab culture conditions differ by thirty percent of the functional genome when compared to those found in nature *(93)*. *Salmonella* originating from different sources can yield different strengths of biofilms; *Salmonella* isolated from produce have shown greater strength biofilm formation on polystyrene, polycarbonate and stainless steel than *Salmonella* isolated from poultry *(71)*. Biofilm strength and composition depend on the characteristics and demands of the environment, as well as the medium on which the biofilm persists *(74)*. Biofilms have different compositions based on what they are attached to; cellulose is the largest polysaccharide component of biofilms and is critical when attaching to certain surfaces, but is not necessary when attaching to others *(74, 93)*.

Visualizing bacterial biofilms requires that powerful microscopy techniques be performed. Scanning electron microscopy (SEM) requires biofilm specimens to be stained, dried and coated before imaging under vacuum. Each of these processes has the ability to disrupt a bacterial biofilm*(73)*. Transmission electron microscopy preparation stabilizes the EPS matrix, but typically the resolution of this method is not great enough to adequately assess surface-associated growth *(32)*. Environmental SEM preserves native morphology, including surface structures, and allows for external polymers to be seen more clearly than with SEM. The EPS is less visible with ESEM without the use of a concentrated metal stain and a binder. Ruthenium red and Alcian blue at low concentrations are two common metal stains used in visualizing bacterial biofilms *(32, 73)*. Low voltage SEM can be used to directly visualize the glycocalyx in bacteria that have been treated with cationic probes.

Spices

Spice Properties

The term “spice” is applied to food products of plant origin that are sold either whole, in pieces, or ground that have mostly no nutritional value, but are instead added to improve the organoleptic properties of a food*(68)*. The United States is one of the largest importers of spices, with over 80% of retail spices and seasonings provided by imports *(99)*. Spices are considered to be a Category II food by the Food and Drug Administration, as listed in the Bacteriological Analytical Manual. Spices are used all over the world for their aromatic and flavor properties. As

far back as antiquity people have valued spices for their uses in food, medicine and perfume. Spices were so coveted that they were used as currency for years in many parts of the world *(66)*.

An important step in the production of commercially sold spices is drying. Drying has been shown to extend the shelf life of many spices and prevent bacterial spoilage, but it is known to significantly reduce the amount of aroma compounds in many herbs and spices *(110)*. In addition to a drying step, many spices such as clove, mustard, oregano, garlic and paprika*(24)* have been shown to contain some level of antimicrobial properties. Unfortunately, the concentrations of antimicrobial compounds in spices are generally very low. In order to have the desired antimicrobial effect, extracts or oils would have to be isolated and used in large quantities, which would severely alter the flavor profile of the food product *(68)*. One study shows the antimicrobial compounds found in these spices to be more effective at deterring Gram positive organisms when compared to Gram negative organisms *(24)*. The growing conditions of a spice heavily dictate its level of bacterial contamination, and spices are potential carriers of pathogens and a host of native yeasts, fungi and other bacteria.

Spices and Contamination

Spices are grown and harvested in warm, humid areas where microorganism growth is readily supported. After the spices are harvested, they may be spread out on an open field or tarp to dry, in an area that is not sanitary or of Good Agricultural Practices minimum level of quality *(81)*. These practices may lead to greater levels of contamination *(68)*. In developing countries where spices are grown, the primary defense of a spice product against contamination is its drying step in which initial populations are lowered significantly. The drying step that most spices undergo will provide some line of defense against microbial growth, provided the spices remain dry and kept under low water activity conditions. In addition to large initial bacterial loads, spices are able to be contaminated at any step along the food production pathway, from harvest to import, and processing to retail *(48)*. After drying, spices are often bundled and sold without additional processing *(10, 68)*. Spices that are contaminated can become a significant microbial hazard when applied to ready to eat foods *(81)*.

When spices are improperly stored, the population of microorganisms that survived the drying process may multiply to the maximum population density within 24 hours *(48)*. When stored at

room temperature for 14 days, there were no significant changes to cell counts of an organism commonly found on black pepper, *Bacillus cereus,* nor were significant changes in the level of enterotoxins present *(11)*. Spices have been reported to be the primary source of spore-forming bacteria in large volumes of food such as soups, casseroles, stews and gravies produced by catering establishments *(10)*.

Proper precautions, such as those outlined in Good Agricultural and Manufacturing Practices, will significantly reduce the risk of foodborne illness related to contaminated spices. Dry or steam heat treatment processes may be applied to spices, but it is not mandated by law in many countries *(81)*. Many spices possess antimicrobial compounds, but it is not known whether the antimicrobial properties of spices have full effect under storage conditions *(48)*.

Microbiological Quality of Global Spices

Aerobic microorganism population can vary widely across the world in terms of spice contamination. Factors that affect these counts include the microorganism genus or species, strain, the type of spice, and the climate or environmental conditions where it is grown, as well as the handling practices of the people tasked with handling them *(66)*. A majority (51%) of samples taken in retail level stores in India were of unacceptably large microbial populations whereas yeasts and molds were found to be at unacceptably large levels in 4.5% of samples of samples taken *(10)*. Total bacterial numbers of ground black and white pepper in Brazil range from 6 to 8 log CFU per gram in Brazil, but yeast and mold populations are significantly less *(34)*. Spice shipments imported into the United States were reported to be 1.9 times more likely to test positive for microbial contamination than every other imported food combined, excluding exotic meats *(99)*. One study in the United Kingdom shows that allspice, cayenne, garam marsala, turmeric, and black pepper were more likely to be of unacceptable microbiological quality when compared other spices *(81)*. In a study of microbiological quality of spices in Mexico, samples of black pepper, garlic powder and cumin seed showed great levels of contamination, up to 10^7 CFU/gram *(35)*. Mesophilic aerobic organisms were found in lower levels on bay leaves and oregano.

Bacillus cereus is of especially great prevalence in spices around the world. A study of over 1900 food products showed that 2% of ready-to-eat foods with added spices or spice ingredients

were of unacceptably large levels of *Bacillus cereus (62)*. Studies in India have shown chili powder and cumin to house bacterial populations up to 8 logs, and *Bacillus cereus* was among the samples taken for chili powder *(66)*. *Salmonella* has been isolated from paprika in South Africa. Also in South Africa, studies show contamination levels between several hundred and several million organisms per gram depending on the spice; black pepper, coriander, paprika, mace, pimento and white pepper were all reported to contain an average of 10^6 CFU/gram or greater*(13)*. A study found a total of 160 samples of different spices in Vienna such as ginger and curry contained 5 log CFU/g *Bacillus cereus* counts *(53)*. Another notable pathogen in the global spice marketplace is *Clostridium perfringens*, which has been detected in a number of retail spices in the Netherlands. Spice blends have been reported to contain greater numbers of *C. perfringens* than in the individual spices by themselves *(66)*. Curry, paprika, ginger, basil, dill, thyme and coriander all have had high reported aerobic populations. In Spain, 26% of herbs sampled showed unacceptably large populations of mesophilic aerobic organisms and *Enterobacteriaceae (91)*.

Outbreaks Linked to Spices and Herbs

Spices have become a suspect when observing foodborne illness outbreaks. Paprika powdered potato chips were involved in an outbreak in Germany in 1993- this incident demonstrated to the public that minor ingredients such as spices are equally capable of being responsible for large scale foodborne illnesses, even at low water activity and contamination levels *(48, 59)*. Also in Germany, *Salmonella* Agona was isolated from samples of aniseed-containing herbal teas between October 2002 and July 2003- the majority of the cases were persons between the ages of 3 and 20 years old *(54)*.

In March 1999, there were 41 cases of *Salmonella* Thompson that resulted in 3 hospitalizations. The *Salmonella* Thompson organisms were traced back to a restaurant that was making and holding salsa using contaminated cilantro *(20)*. During 2007, from May through October, 1.6% of samples of fresh herbs from the UK were found to be of unacceptable microbiological quality- these herbs were linked to 32 cases of *Salmonella* Senftenberg in Whales, Denmark, the Netherlands and the United States *(30)*. *Salmonella* Senftenberg was recently isolated from basil in the United Kingdom *(14)*. An outbreak at a high-school dinner in Denmark was traced back to spices used in a pesto sauce in November 2006 *(70)*.

Salmonella *in Spices*

Spices are important global commodities that are available year round in developed and developing countries, and thus can become a vehicle for *Salmonella* through these worldwide distribution chains. Between 1970 and 2003 in the United States, there were 21 major recalls of multiple spices, all but one of the recalls were contaminated with *Salmonella (101)*. *Salmonella* contamination was the cause of 95% of US food recalls related to spices between 1980 and 2000 *(101)*.

A sample of spices is considered acceptable if it contains no *Salmonella* in 25g of spice product. Of the spices imported and examined by the FDA, over 6% of spices in the fruits category, over 7% of root spices, and over 11% of leaf spices tested positive for *Salmonella* presence *(99)*. Imported spices claiming to be treated with a pathogenic organism removal process still had recorded *Salmonella* presence in 3% of shipments, indicating either an extremely large initial *Salmonella* population, inadequate processing, or post-processing contamination as a major issue *(48)*. *Salmonella* Senftenberg is a common isolate found in spices, as well as Saintpaul, Montevideo and Agona *(81)*. *Salmonella* Thompson was able to reach large populations on surface contaminated, fresh cilantro leaves when stored at temperatures over 22°C and relative humidity of 60% or greater *(16)*.

Paprika and paprika-powdered potato chips contaminated with *Salmonella* affected over 1000 individuals in Germany in 1993, many of which were 14 years of age or younger. The infective does was between 0.04 and 0.45 organisms per gram, and had a reported attack rate of 1 in 10,000 people *(59)*. It is believed that the fat content of the potato chips helped to protect the various *Salmonella* strains survive the increased salt, decreased water activity environment of the chips as well as from the gastric acidity of those individuals that consumed the food product. 94 different *Salmonella* serovars were isolated from patients, with the most common serovars being Saintpaul, Rubislaw and Javiana *(59)*. One year after an outbreak, a potato chip factory in Germany noted that *Salmonella* recoverable populations of 0.4-11 organisms per gram in their product *(59)*. *Salmonella* was present in most microbiologically contaminated spice shipments that arrived at the production facility. 12% of *Salmonella*-contaminated shipments contained multiple serotypes, showing over 94 unique serotypes. No single serotype constituted more than

7% of the isolates, which is unusual when compared to outbreaks in other industries *(99)*, which would typically contain significantly greater numbers of single serotype isolates.

A review of spice-attributed foodborne illness outbreaks reported that between 1973 and 2010, nearly 71% of reported outbreaks were linked to the organism *Salmonella enterica* subspecies *enterica*, these outbreaks were responsible for 87% of reported illnesses related to spices during this time period. *Bacillus* spp. were reported accountable for 13% of these illnesses *(100)*. This study included outbreak reports from Canada, the United States, England, Wales, France, Germany, New Zealand, Norway, and Serbia.

Black Pepper

Defining Black Pepper

Black peppercorns are defined as dried and cracked mature berries of the woody climbing plant *Piper nigrum.* Black pepper is widely renowned for its characteristic pungency. It has been referred to as the "King of Spices." The earliest recorded use of black pepper as a spice was 1550 B.C. in an ancient scroll known as Eber's papyrus. By the first century A.D., the Roman Empire took control of the pepper trade and popularized it further across the western world. In *Natural History* (23-79 A.D.) by Pliny the Elder, he mentions: "It is surprising that the use of pepper has come so much into fashion. Commodities as a rule attract us by their appearance or utility, but the only desirable quality of pepper is its pungency and yet it is for this very undesirable element that we import it in very huge quantities from the first emporium of India." With the fall of the Roman Empire, the spice trade then was reestablished by the Arabic people, where it largely maintained between the Arabic people and China until the age of exploration by the Europeans in which its popularity exploded once more *(75)*. Today, black pepper is one of the Earth's most widely used spices and it is important to maintain its safety for consumption for years to come.

In the United States, black pepper is often heat treated, fumigated, or irradiated as part of safe handling practices. Typical black pepper production is as follows: harvest, dressing, drying, steam sterilization, metal detection, milling, sieving, packaging and another metal detection to finish the process *(92)*. The chemical specifications for consumer level black pepper is as follows: moisture content: 12% maximum, total ash: 5% max, acid insoluble ash: 0.5% max, volatile oils: 1.5% minimum, piperine: 3.5% minimum *(92)*. Primary headspace volatile oil

content of *Piper nigrum* is includes germacrene D, limonene, beta-pinene and alpha-phellandrene at approximately 10% each *(46)*. P-cymene is another volatile that is found in varieties of black pepper, but the volatile content of each pepper ultimately varies between cultivars *(63)*.

Black pepper has a shelf life of up to 36 months when kept in a cool, dry place away from direct sunlight. It is used at any stage of the cooking process, as well as a condiment. *(102)* Major producers of black pepper include India, Indonesia, Brazil and Vietnam. One study has shown the antimicrobial effect of black pepper extract is not significant in reducing or delaying growth of *Staphylococcus, Klebsiella*, *Morganella*, *Candida*, *Proteus* and *Escherichia* spp *(47)*.

Microbiological Contamination of Black pepper

Black pepper most often contains the greatest microbial loads when compared to other spices on a global scale *(66)*. The microbial specifications for retail black pepper are as follows: total viable count must not exceed 50,000 CFU per gram; coliforms must be present in numbers less than 50 CFU per gram; *Enterobacteriaceae* must be present in less than 100 CFU per gram; *Salmonella* must be absent in 25 grams of product; *E. coli* must be absent in 1 gram of product; yeast and molds, *Bacillus cereus* and *Staphylococcus aureus* are to be kept under 100 CFU per gram; *Clostridium perfringens* must be kept under 10 CFU per gram *(92)*. Black pepper is often contaminated with large numbers of mold, yeast and bacteria, which can lead to rapid spoilage or illness. Aflatoxin is a prevalent hazard in the production of black pepper, in addition to microbial contamination *(102)*. In retail outlets in India, black pepper was shown to contain an average microbial load of 8.0×10^7 CFU per gram *(10, 68)*. The supply chain for black pepper is typically complicated, and may begin with small farms with out of date agricultural practices where the product is exposed to dust, excrement, insects and other sources of pathogens. *(48)*

Contamination of black pepper in South Africa is prevalent, in some cases microbial counts reached well over 6 log CFU/g. Black pepper in the Netherlands was reported to have total aerobic counts over 7 log CFU/gram *(66)*. Samples taken in the United States have been shown to reach total numbers of bacteria between six and eight log CFU per gram of spice. *Escherichia, Staphylococcus, Streptococcus, Bacillus* and *Klebsiella* have all been isolated from spices

imported into the United States and sold at retail level. The average microbial load for black pepper imported from Indonesia, India and Brazil range from 3 to 7 log CFU/gram *(66)*.

Salmonella *in Black Pepper*

Salmonella commonly contaminate ground black pepper *(77)*, regardless of the region of the world in which it is grown and harvested. In Brazil, black pepper showed the greatest contamination rate for *Salmonella (68)*; 4.5% of black pepper shipments imported into the United States are found to be contaminated with *Salmonella.* There is no statistically significant difference in *Salmonella* prevalence between whole peppercorns and those that have been cracked or ground*(99)*, despite the thought that the physical disruption and heat production of the grinding process might have a population lowering effect. *Salmonella* Agona, Typhimurium, Rubislaw, Javiana and Ball have been isolated from black pepper shipments *(99)*. Survival of *Salmonella* on black pepper depends on the strain; one study shows *Salmonella enteritidis* losing viability after 28 days, but *Salmonella* Weltevreden and Senftenberg being viable long past that, and able to grow rapidly on foods at 30°C *(97)*. *Salmonella* Weltevreden and Senftenberg have been isolated from black and red pepper samples in Japanese spice imports *(40)*.

Outbreaks linked to black pepper

In addition to recalls due to pathogenic organisms in black pepper, there have also been outbreaks which have impacted hundreds of people. An outbreak of *Salmonella* Montevideo was linked to a spiced salami product in August and September of 2009. There were 272 cases in 44 states over a period of 9 months *(36)*. The salami products incorporated black and red pepper that had been contaminated with *Salmonella*. The peppers were added after the salami would go though no further kill step, emphasizing the need for prevention of post-processing contamination through adequately sourced and treated spices *(22)*.

One of the earliest known outbreaks linked to spice contamination took place in 1981, took place in Norway when *Salmonella* Oranienburg infections were linked to a supply of black pepper. There were 126 confirmed cases of *Salmonella* Oranienburg infections between November 1981 and August 1982 *(38)*.

<u>Current Treatments of Spices</u>

Ethylene Oxide

Ethylene oxide is a gaseous sterilant that is popular within healthcare facilities, and is known to be a mutagen in bacteria, but also in human lymph cells *(42, 87)*. Ethylene oxide is an alternative treatment for reduction of bacterial populations, though research into its effectiveness has shown that certain thermophilic bacteria can survive its treatment depending on the growth media *(98)*. Ethylene oxide has known carcinogenic properties, and has been banned in many countries *(102)*.

Studies have shown that ethylene oxide is damaging to both the flavor and the color of spices. Ethylene oxide treatments remove on average 10% of the nonvolatile oils and 56% of the volatile oils when treating black pepper. Spices treated with ethylene oxide are reported to have been visibly dulled *(98)*. In a study of retail spice samples in New Zealand, 1% of sampled spices contained detectable levels of ethylene oxide, not exceeding 15 parts per million. The levels of ethylene oxide and its derivatives inside of food products were deemed not hazardous unless the consumer ate at least 2.7 kg of that spice product per year *(33)*.

Ionizing Radiation

Irradiation is a popular method in treating spices within the industry. It is an economically viable alternative to fumigation *(102)*. Many countries allow for irradiation of spices when exposed to an average of 10kGy. The United States allows for spices to be exposed to a 30kGy dose of ionizing radiation. Irradiation has proven effective in destroying bacteria and molds without affecting the quality attributes of the spice in a significant manner. Vadji and Pereira show gamma irradiation having seemingly no effect on volatile oils, but a 10% reduction in nonvolatile oils in black pepper.*(98, 102)*. Ionizing radiation treatments appear to more effectively treat ground spices; whole black peppercorns that had initial populations of approximately 6 log CFU per gram were not completely reduced by X-ray sterilization, where ground black pepper and sage of similar initial populations were both completely sterilized by the treatment. 10 kGy irradiation treatments are effective across the board in black pepper for 6 log CFU reduction of bacterial populations *(66)*. Ionizing radiation has shown to not affect the color content of spices such as paprika *(51)*. One study demonstrates the significant loss of volatile compounds after ionizing radiation treatment of 30 kGy *(80)*.

Historically, the public has not received the idea of irradiated foods well- there is still doubt as to whether the public will accept irradiation as a safe treatment method *(60)*.The United States Food and Drug Administration requires food producers to include the Radura logo as well as the word "irradiated" on food products in which the irradiation causes a change in the food, but there is no requirement that a food product that has an irradiated ingredient be labeled with the Radura symbol. The results of recent consumer attitude studies have shown that consumers are beginning to accept irradiation as a safe means of treating food *(17)*. It is thought that through educational and marketing programs with the help of recognized health authorities paired with higher quality products, irradiation will become a more popular option *(18, 76)*.

Steam Treatment

Steam treatments involve the application of high temperature steam for short periods of time. Traditional steam treatment is usually applied to whole spices prior to grinding for the purposes of reducing total bacterial and fungal populations. Steam treatments are a common method of sterilizing spices *(2)*. Steam treatments are usually viewed favorably by the general populace and are widely considered wholesome. Studies have shown that heated steam treatments degrade spice color and volatile oil content *(26, 51, 102)*.

Temperature and molecular makeup have significant impact on the formations and alterations of volatile compounds *(26)* and should be considered when using a steam treatment. Steam treated spices are recommended to be kept under refrigeration conditions to minimize color loss *(2)*. Steam treatments have also been known to increase the moisture content in spices, leading to decreased shelf life and carries the potential for revitalization of bacterial numbers *(102)*. Studies have observed the effects of steam treatments before drying spices and have met with good results and significant pathogen reductions, but loss of volatiles was still reported *(85)*.

Ethanol

Ethanol Properties

Ethyl alcohol, or ethanol, is a volatile and flammable liquid that is often abbreviated C_2H_5OH. Its molar mass is 46.07 grams per mol and has a boiling point of 78.37°C at sea level. Ethanol poses minimal ingestion hazards to humans due to its low mammalian toxicity *(96)*. When consumed

in large enough quantities, it acts as a depressant on the central nervous system. When excessively consumed over time, addiction, liver, kidney, reproductive and fetal damage can occur. It is a Generally Recognized as Safe (GRAS) substance classified by the Food and Drug Administration. Ethanol is very flammable and is often used in microbiological laboratory settings, though it has the capability of being corrosive on certain types of metal. It can be dissolved in water, is an effective solvent itself, and is incredibly versatile- it can be used in the creation of extracts, dyes, cosmetics, as well as explosives *(28)*. Recently, there was a push to include ethanol as a way to alleviate some of the energy demands from fossil fuels, but there is controversy over subsidies and manufacturing processes that would be required to meet the demands of energy producers from agricultural products.

Ethanol has had a history of being used as a bacteriocidal agent, but it has more properties than that of a disinfectant. Ethanol has been shown to improve shelf life of certain fruits and vegetables; ethanol can ripen some fruits and inhibit the ripening in others, due to its ability to inhibit ethylene production and receptors. It is theorized that ethanol is converted to acetaldehyde in fruit tissue, and that certain fruits lack the enzyme required for this process, causing the disparity. The senescence of non-fruits like oats and carnations can be delayed through the use of ethanol *(78)*. Ethanol can be used as a way to increase shelf life and decrease rot in grapes by eliminating the harmful *Botrytis* organisms primarily responsible for the rotting of the fruit; when dipped in a 33% or greater ethanol solution, berry decay was reduced further than conventional methods *(61)*. Ethanol can be used as a replacement compound for the more potentially harmful sulfur dioxide in the wine industry *(25)*.

Ethanol as a Disinfectant and Deterrent

The mechanism by which ethanol eliminates microbial activity includes acting as a stress agent on cell membranes and outer membrane proteins, partially dissolving membranes, in addition to inhibiting the uptake of nutrients such as glucose *(28, 45)*. Ethanol has a rapid denaturing effect on cell proteins and enzymes critical for growth and function *(65)*. Cell membrane lipid composition has been shown to change in the presence of alcohols *(44)*. The decrease in water activity in the overwhelming presence of ethanol helps lead to microbial death. Alcohols in lower concentrations are often used in conjunction with other biocidal compounds in order to increase the effectiveness of the treatment *(65)*.

One study shows the synergistic effect of temperature, ethanol concentration and water activity on the suppression of *Salmonella enteritidis*, *Staphylococcus aureus* and *Bacillus cereus; S. entiritidis* and *Staph. aureus* were shown to be strongly affected by the same ethanol concentration, temperature and water activity *(57)*. Ethanol is recognized as a mold inhibitor, and has been used in the past as a fungicide. It is widely used as a rapidly evaporating disinfectant, and at concentrations of 95% it can be used as a wet surface disinfectant, at 50% for dry surfaces, and 70% for wet or dry surfaces *(28)*. 95% by volume ethanol solutions have been shown to be effective against *Rotavirus*, *Norovirus* surrogates *(105)*, *Poliovirus*, and other viruses as well *(52, 95)*. Ethanol is not sporicidal, though it has been shown to inhibit sporulation and spore germination *(108)*.

Lactic Acid Bacteria protected by biofilms are damaged less severely than planktonic cells when exposed to ethanol *(56)*. Non-lethal Ethanol exposure has been shown to promote csgD in *Salmonella*, resulting in an increased expression of biofilm forming genes *(93)*. However, studies have shown ethanol in concentrations 40% by volume or greater with one to two minutes of exposure have the ability to inhibit the growth of plaque biofilms *(88)*. Biofilm growth in catheter locks has been completely deterred with the use of 30% ethanol and 4% trisodium citrate, when controls reached populations of up to seven log CFU per gram *(94)*.

Current Applications of Ethanol Vapor

Ethanol vapor has been reported to inhibit ethylene synthesis and action in the ripening of fruits, such as peaches, nectarines, tomatoes, grapes and avocados. Ethanol vapor in these instances was produced via filter paper wicks. Kiwifruit has been shown to maintain good quality for up to 3 weeks after the non vapor treated controls when treated with 300 µl ethanol per liter of container*(103)*. Studies have shown that in wine and grape production, the use of 3.75 mg/kg ethanol vapor to fruit shows effects comparable to that of sulfur dioxide. Containers with 220±80 µl residual headspace ethanol are able to control rot, browning, and senescence in grapes *(25)*. One study shows that tomatoes kept at 5°C that were treated with ethanol vapor exhibited delayed ripening when compared to those held at 20°C that were exposed to the same vapor treatments *(107)*. Tomatoes that have had their ripening delayed by ethanol vapor treatments show no reduction in sensory quality*(83)*. Senescence of broccoli was inhibited with the use of ethanol vapor treatment pads in one study *(5)*.

One study shows the effect of ethanol vapor on the shelf life of apple turnovers by retaining quality for up to 21 days in storage *(90)*. Another study showed ethanol vapor's effect on the shelf life of whole grain rye bread; the shelf life of rye bread increased from 8 to 12 days to 26 days when exposed to 1% by volume ethanol vapor *(82)*. A study of sliced apples showed the ability of ethanol vapor to reduce respiration rates in cut fruit *(8)*. Ethanol vapor has also been shown to enhance aroma volatile production, preserve color, delay ripening, reduce microbial populations as well as kill insects and mites *(9)*. Ethanol vapor treatments on fruits have shown to change the phenolic compound concentrations over time *(96)*. Off flavors have been reported in mangos in a case where ethanol vapor was applied. One study also denotes the breaking of plasma membranes in broccoli and increased ion leakage in tomatoes at levels between four and eight milliliters ethanol per kilogram of produce *(9)*. Tomatoes appear to be especially sensitive to ethanol vapor concentrations *(78)*.

A patent in 1975 describes a method for sterilizing spices using ethanol vapor at 78°C achieving a 6 log reduction in microbial population after 10 minutes of treatment *(106)*. There has been research done into ethanol vapor producing sachets as part of a study of active modified atmosphere packaging, but it has not found widespread acceptance yet *(79)*. One study tested *Bacillus subtillis* biofilm replicas on their ability to be permeated by vapor phase antimicrobials. This study concluded that the vapor was not able to penetrate the biofilm replica surface, demonstrating the defensive capability of *Bacillus subtillis* biofilms *(31)*, though the study is not necessarily reflective of native biofilm defenses of *Bacillus* spp. nor of other organisms.

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Chapter II

Developing Biofilms on Whole Black Peppercorns

INTRODUCTION

Spices are a massive global commodity, and have been sought for their organoleptic properties as well as for their uses in medicines and perfumes. Black pepper (*Piper nigrum*) is reported to be the most widely traded and consumed spice in the world. It has seen enormous worldwide popularity as far back as 1550 B.C. Many organisms have been known to contaminate spice products, potentially due to poor food safety management techniques. Common foodborne pathogens that would be found in spice products include *Bacillus cereus*, *Clostridium perfringens* and *Salmonella enterica*. Due to the comparatively higher occurrence of salmonellosis cases reported within the United States, this project will be focusing on a variety of *Salmonella enterica* serovars that were isolated from low moisture food recalls.

Microorganisms such as *Salmonella* often exist inside biofilms in nature *(8)*. These films significantly increase their defenses against hostile environmental conditions, and pathogenic bacteria residing within these complex biofilm communities require significantly stronger treatments in order to be destroyed *(17)*. Biofilms provide increased resistance to antibiotics, environmental stresses such as temperature and pH changes, as well as physical resistance to forces such as shearing *(4,5)*. It is believed that *Salmonella* within these complex biofilm communities are able to withstand the drying and minimal processing conditions that spice products undergo in many countries where spices are produced. Prior exposure to stresses such as drying or starvation may trigger the virulence and biofilm forming responses in *Salmonellae (1, 18)*. One study has shown that *Salmonella* virulence factors must be activated to form biofilms on plant surfaces *(3)*. *Salmonella* has been shown to change biofilm composition in response to different environmental factors as well as the surface on which they are adhered *(13, 16)*. Studies have shown that different serovars of *Salmonella* will adhere differently and have different biofilm compositions and strengths.

The purpose of this experiment was to select *Salmonella* serovars that have the ability to form strong biofilms when attached to whole black peppercorns, in order to create a heavily

contaminated, *Salmonella* embedded biofilm spice product in order to test the strength of an ethanol vapor treatment.

MATERIALS AND METHODS

Bioscreen C Method

The purpose of the development of this method was to compare the different strains of *Salmonella* that had been isolated from low moisture foods (Table 2.1) using their biofilm forming capacities on polystyrene. A crystal violet biofilm assay was adapted from previously reported methods for use in the Bioscreen C Automated Microbiology Growth Curve Analysis System (Growth Curves USA, Piscataway, NJ).

Ten ml sterile Tryptic Soy Broth was added to each of 15 sterilized test tubes. Using disposable loops, one loop-full of frozen *Salmonella* stock culture was inoculated into its respective tube, and each inoculated tube was vortexed for 1 minute at 2000 RPM. 100 microliters of inoculated TSB was dispensed into each of six wells, as shown in Figure 2.5 below, then incubated statically at 28°C for 48 hours *(9)*. After 48 hours, the honeycomb plate was removed from the incubator and visually inspected for growth and turbidity. Each well of the honeycomb was drained and the inoculums discarded. Each well was then washed twice with distilled water, and the plate was allowed to air dry at room temperature. Once dry, each well was stained with 0.1% crystal violet for 20 minutes. The dye solution was then discarded and each well was washed three times with distilled water and the plate was then again allowed to dry at room temperature. The plate was transferred to the Bioscreen C machine, and one reading (600 nm) at room temperature was recorded for each well. This experiment was replicated once.

Isolated Colony Method

A method for *Salmonella*-embedded-biofilm formation on peppercorns was modified from a previous method used to grow biofilms on silicon beads *(2)* as described here. A loop-full of frozen stock culture was streaked for isolation onto XLT4 followed by incubation at 37°C for 24 h. Whole black peppercorns (approximately 8 g) were placed in a sterile, 125 ml Erlenmeyer flask in a single layer of Tryptic Soy Broth was added to the flask in a 5:2 ml/g ratio of broth to peppercorns where peppercorns were covered in TSB. Three flasks were used for each strain of

Salmonella. One flask was prepared as a non-inoculated control. For each strain, one isolated colony from XLT4 was removed using a sterile loop and placed into the TSB containing peppercorns. Each inoculated flask was swirled for 2 minutes at 90 RPM followed by incubation at 37°C for 48 h. After incubation, the each flask was removed from the incubator, visually checked for growth, and the spent TSB was decanted. The peppercorns were then washed with the equivalent volume of 0.1% peptone-0.1% Tween solution, fresh TSB was added to each flask, and the flasks were incubated at 37°C for 24 h. After this second incubation, the flasks were removed from the incubator, the spent TSB was decanted, and the peppercorns were again washed with the equivalent volume of peptone-Tween solution. After washing was complete, the peppercorns in each flask were poured into separate, sterile Petri dishes in a single layer, and allowed to air-dry in a Biological Safety Cabinet until they achieved a water activity between 0.30-0.40 (approximately 48 h).

Salmonella populations in biofilms on peppercorns were enumerated using the following method. One gram of peppercorns were placed into a sterile stomacher bag, 9.0 ml of 0.1% peptone 0.1% Tween 0.2% cellulase solution (PTC) was added, and the mixture was stomached for 2 min. One ml of the suspension was removed from the stomacher bag, serially diluted in PTC, vortexed for 30 seconds at 2000 RPM, and surface-plated in triplicate onto XLT4 agar. All plates were incubated at 37°C for 48 hours prior to colony counting. The entire study was replicated once, as shown on Figure 2.2.

Strain Selection

Growth rates of four *Salmonella* strains- Abaetetube, Ball, Johannesburg, and Tennessee- chosen for further study, were determined using the Bioscreen C system. Test tubes were filled with 10 ml Tryptic Soy Broth and each was inoculated with a loop-full of frozen stock culture, and vortexed for 1 minute at 2000 RPM. Each strain was added to six wells of a honeycomb plate via 200 microliters aliquots of inoculated Tryptic Soy Broth, for a total of 24 wells. The Bioscreen C was set to record every 15 minutes for 48 hours, and to incubate the microorganisms at 37°C.

Final Biofilm Method

For the purposes of this project, a method to produce a more robust biofilm with higher population recovery was needed. This method was suggested via personal communication with

Dr. Monica Ponder. The base method was designed by Lauren Bowman, a member of the laboratory of Dr. Ponder who was working on cumin seed; the method is modified for use in this project and is described below.

In order to perform this method frozen stock cultures of *Salmonella* Tennessee, Johannesburg, Ball and Abaetetube were gathered. In the Biological Safety Cabinet each strain was streaked for isolation onto XLT4 and incubated for 24 h at 37°C. After 24 h, one isolated colony from each plate was removed and added to the same test tube with 10 ml of Tryptic Soy Broth and vortexed for 30 s at 2000 RPM and transferred to the incubator set to 37°C for 24 h, shaking at 120 RPM. After incubation, 150 ml of Tryptic Soy Broth was added to the flask containing 60 g black peppercorns and 5 ml of the vortexed *Salmonella* cocktail was added then swirled for 2 minutes at 90 RPM. The *Salmonella* cocktail was then serially diluted and enumerated using 0.1% peptone 0.1% Tween blanks and plating in triplicate onto TSA and XLT4. The flask and plates were moved to the incubator set at 37°C for 24 h, statically. After 24 hours, the plates were counted, and the TSB was decanted from the flask. The peppercorns were then washed using 200 ml of the 0.1% peptone-0.1% Tween solution, swirling for 2 minutes at 90 RPM. The peptone-Tween solution was decanted, 150 milliliters of fresh TSB were added back into the peppercorn flask, and then the flask was incubated at 37°C for 24 h. After 24 hours, the TSB was decanted, and the peppercorns were washed with peptone-Tween solution which was then decanted and the peppercorns within the flask were transferred to sterile Petri dishes in a single layer and allowed to air dry for 48 h to water activity 0.30-0.40, with occasional shaking to discourage clumping and moisture retention.

ESEM Images

Five samples of peppercorns were chosen for examination of biofilm components using Environmental Scanning Electron Microscopy (ESEM). Stephen McCartney of ICTAS in the Virginia Tech Corporate Research Center was the operator of the microscope for these images. These images were taken as a way to verify the presence of biofilm production on the surface of black peppercorns. The five samples include one non-inoculated peppercorn sample, one sample peppercorns inoculated using the Final Biofilm forming method with *Salmonella* Tennessee that had not been allowed to dry to storage conditions, one sample of *Salmonella* Tennessee that had been dried, one sample of a mixed *Salmonella* culture that was not allowed to dry, and one

sample of mixed *Salmonella* that had been dried. Each of these samples was stained with Ruthenium Red and osmium as a binding agent, and was held in solution after dying with heavy metals until viewed using the ESEM. When the sample was preparing to be placed within the ESEM, a small application of Carbon Paint was placed on the bottom of the sample chamber well. A peppercorn from the desired sample was removed from the storage liquid and padded dry gently. Once dry, the peppercorn was transferred to the sample chamber well and allowed to dry and adhere to the carbon paint for 10 minutes. Under the ESEM settings "True environmental" at 60.8% humidity in "Wet Mode," the pressure was set to 4 Torr, 5°C, and an accelerating voltage of 10 kV *(15)*, four progressively greater magnification photographs were taken at each region, with 3 to 4 regions selected depending on the run. The lowest magnification setting was 550x, with the greatest being 10,000x.

RESULTS

Figure 2.1 shows the Optical Density reading as a rough estimate of biofilm strength after two days of incubation. Strains that showed comparatively greater biofilm forming capability on polystyrene using this method include *Salmonella* Senftenberg, Johannesburg, Anatum, Newport, Orion, Ball, and Abaetetube and are considered to be moderate biofilm formers *(19)*. *Salmonella* Tennessee, Typhimurium, Infantis, Javiana, Kentucky and Saintpaul were all classified as weak biofilm formers. *Salmonella* Typhimurium readings were affected due to an issue with adherence of the film to the polystyrene plate, as described in the Discussion.

As seen in Figure 2.2, using the Isolated Colony method, populations of *Salmonella* Johannesburg and Tennessee in biofilms on peppercorns were approximately 7.4 log CFU/g. *Salmonella* Ball and Abaetetube also had great recovery with 6.80 and 6.49 log CFU per gram average over two replications, respectively. *Salmonella* Javiana and Orion both showed signs of visible growth within the flask when compared to the non inoculated control, but was not detected in populations above 10^5 CFU/g when plated on XLT4 agar. *Salmonella* Saintpaul showed an average population recovery log CFU/g of 5.15.

Table 2.2 compares the growth phases, exponential growth start time and line of best fit for the four strains selected based on the method described in the Strain Selection section. Figures 2.3

and 2.4 show a selection of ESEM image comparisons at 5,000x magnification and 10,000x magnification, respectively.

DISCUSSION

All methods in this project use biofilms that are formed in laboratory conditions. Laboratory condition biofilms are composed of different materials than those found in nature and on natural products such as spices *(17, 18)*. During the Bioscreen C experiment, the polystyrene plates may have had residual charge or polarity as a result of the manufacturing process on the surface that came into contact with either the *Salmonella* organisms or the crystal violet used to dye the structure. It is believed that this charge could have potentially affected the adhering or staining process in some way that is not necessarily characteristic of a biofilm found in nature. Additionally, the five total distilled water washes could have affected the integrity of the biofilms, as was the case with *Salmonella* Typhimurium. During the wash step after the incubation, every Typhimurium biofilm visibly dislodged from the plate surface and was drawn into the pipette and had to be discarded. This method provided some insight as to the basic biofilm forming capabilities and strengths of the films created by the serovars received from the FDA, after being put through several wash and dry steps. This method also underlined the importance of biofilms when designing food packaging systems, as every strain was able to adhere to the surface of polystyrene, a common food packaging material.

Salmonella serovars that were chosen for use in the Isolated Colony method were chosen using several criteria. Using the data obtained from the Bioscreen C biofilm measurement method, *Salmonella* Typhimurium, Infantis, Senftenburg, Anatum, Newport, Weltevreden, Javiana, Kentucky and Saintpaul were found to be poor biofilm-producers due to low average optical density and great standard error between replicates. *Salmonella* Javiana and Saintpaul were added back into the possible strain selection candidates due to the recalls in which they were associated- *Salmonella* Javiana isolated from white pepper, and Saintpaul was isolated from samples of dried mustard seed. The spices that these serovars were isolated from share similar characteristics to that of *Piper nigrum*, and these spices are part of the fruit portion of the plant rather than from a leaf or a root. These two serovars, the four serovars selected from the

Bioscreen C Biofilm method, *Salmonella* Johannesburg, Orion, Ball and Abaetetube, and *Salmonella* Tennessee are the strains that were tested further by the Isolated Colony method. *Salmonella* Tennessee was selected as a positive control, because of its biofilm forming capacity demonstrated in the Aviles method *(2)*.

Based on the recovery of *Salmonella* Orion, Javiana, and Saintpaul after application of the Isolated Colony method, these strains were considered poor candidates for forming strong, durable biofilms that resist desiccation conditions. The cut-off was selected because of a previously existing patent that achieved a 6 log CFU/g population reduction *(20)*. These four strains, Abaetetube, Ball, Johannesburg, and Tennessee, displayed great recovery and low error reported in the Isolated Colony experiment, which reiterates what was seen in the Bioscreen C Biofilm method, with the one exception of *Salmonella* Tennessee, whose biofilm forming capacity was not well represented.

As was the case with the Bioscreen C method of determining biofilm forming capability of individual strains, the Isolated Colony test was not necessarily representative of biofilms formed in nature. During the growth phase, nutrients were abundant and the conditions were ideal for *Salmonella* and other organisms, which means the inherent biofilm structure and recoverability of the organisms may have been altered significantly when suddenly exposed to harsh drying and starvation conditions. Using this method, the ability of single strains of *Salmonella* to form massive populations and produce biofilms is shown. However, this method would have to be altered if multiple strains were to be used, or if the peppercorn sample size was to be larger. For example, if this were to be tested on a large scale, the size and number of colonies would have to be taken into account when inoculating a large flask, which would lead to large discrepancies in the data.

Due to the differences in growth rate between the strains outlined in Table 2.2, measuring the resistance to ethanol vapor of biofilms comprised of individual serovars using an identical procedure would be not be a fair comparison, and because biofilms occurring in nature are often of multiple species, a mixed culture was deemed necessary for production of the most durable and resilient biofilms. In a "worst-case scenario" type of situation where imported spice products are heavily contaminated with *Salmonella* embedded biofilms, a pathogen reduction treatment needs to be able to significantly reduce the population of potentially harmful organisms. The

Isolated Colony method as described above would prove to be time consuming and inefficient when dealing with multiple serovars, as it was designed to test the differences between individual strains. The Final Biofilm forming method was developed in order to produce robust multi-strain biofilms in an efficient manner. The method is similar to the Isolated Colony method, but shows improvement by having the capacity for multiple strains as well as showing improved recovery on two types of media. Adding multiple strains to a small amount of Tryptic Soy Broth and allowing it to grow to greater initial population is a key difference between the Isolated Colony method and the Final Biofilm forming method, it allows for significantly greater inoculation population when ultimately added to the flask with peppercorns.

Using the Final Biofilm forming method, after staining with Ruthenium Red and osmium and scanned with the ESEM, organisms are undeniably seen in each sample provided, including organisms appearing to either protrude from a mass or appearing to be blanketed in a thick film. Ruthenium Red and osmium aid in the visualization of bacterial biofilms by binding to the carbohydrate layers that are secreted *(6,15)*. By staining with this method, it is possible to increase the resolution of ESEM imaging techniques due to refraction by the heavy metals, it allows the operator to better distinguish between the biofilms, organisms, and the surface on which they are adhered. Looking at the images alone, it is not possible to be absolutely certain that the organisms seen are *Salmonella*, as there are large numbers of bacteria, yeasts and molds recovered on nonselective media using this method. The image in the top right of Figure 2.4 shows what appears to be organisms mid-film formation, with extracellular polymeric substance branching off of the organisms and adhering to the other organisms on the surface, a stark difference from the non-inoculated peppercorns, which do not display substances on the surface. This type of formation is common in the early stages of biofilm development for many bacteria *(7, 15).* Other differences of note include the smoothness the peppercorns appear to display after they have been inoculated as opposed to the roughness of the images in the non-inoculated peppercorns. The images with biofilms described as "fresh" show much softer, less-defined lines- it is theorized that the still-hydrated biofilms produce a less crisp image than those films that have lost their moisture and are more rigid, but it is not possible to say definitively without further testing.

After verifying the presence of *Salmonella* organisms embedded within biofilms using ESEM, the Final Biofilm forming method was chosen as the method for producing biofilms on whole black peppercorns due in part to large initial populations, between 6 and 8 log CFU/g, and larger populations after incubation on whole black peppercorns, between 8 and 10 log CFU/g after drying for 48 hours. Utilizing a method that can simultaneously grow multiple serovars will result in biofilms with greater biomass and increased recovery. Additionally, biofilms with multiple species and serovars more closely mimic those found in nature, and would therefore be a better representation of the strength of the ethanol vapor treatments, as described in the following chapter.

TABLES

Strain	Identification	Isolated from	Year
Salmonella Typhimurium	ARL Se 001	Chili powder	2010
Salmonella Infantis	ARL Se 003	Bone Meal	2010
Salmonella Senftenburg	ARL Se 005	Bone Meal	2010
Salmonella Johannesburg	ARL Se 013	Dried Ginger	2010
Salmonella Anatum	ARL Se 014	Paprika	2010
Salmonella Newport	ARL Se 018	Allspice	2010
Salmonella Abaetetube	ARL Se 021	Chili Powder	2010
Salmonella Saintpaul	ARL Se 029	Mustard Seed	2011
Salmonella Orion	ARL Se 039	Spice Blend	2011
Salmonella Weltevreden	ARL Se 056	Onion and Garlic	2011
Salmonella Ball	ARL Se 085	Black Pepper	2011
Salmonella Javiana	ARL Se 087	White Pepper	2011
Salmonella Kentucky	ARL Se 088	Cumin	2011

Table 2.1 *Salmonella enterica* serovars received from the Food and Drug Administration

Strain	Lag Time (min)	Log Time (min)	Maximum OD	Growth equation	R^2
Salmonella Abaetetube	195	690	1.02	y = 0.0014x - 0.097	0.9601
Salmonella Ball	195	630	1.08	y = 0.0016x - 0.187	0.9721
Salmonella Johannesburg	285	675	0.88	y = 0.001x - 0.099	0.9257
Salmonella Tennessee	135	675	1.11	y = 0.0015x - 0.069	0.9478

Table 2.2 Comparison of the growth rates of *Salmonella enterica* serovars Abaetetube, Ball, Johannesburg and Tennessee by measuring Optical Density (OD) in the Bioscreen C. Each serovar was allotted six wells with 200µl inoculated TSB, OD readings were taken every 15 minutes, incubated at 37°C for 48 hours.

FIGURES

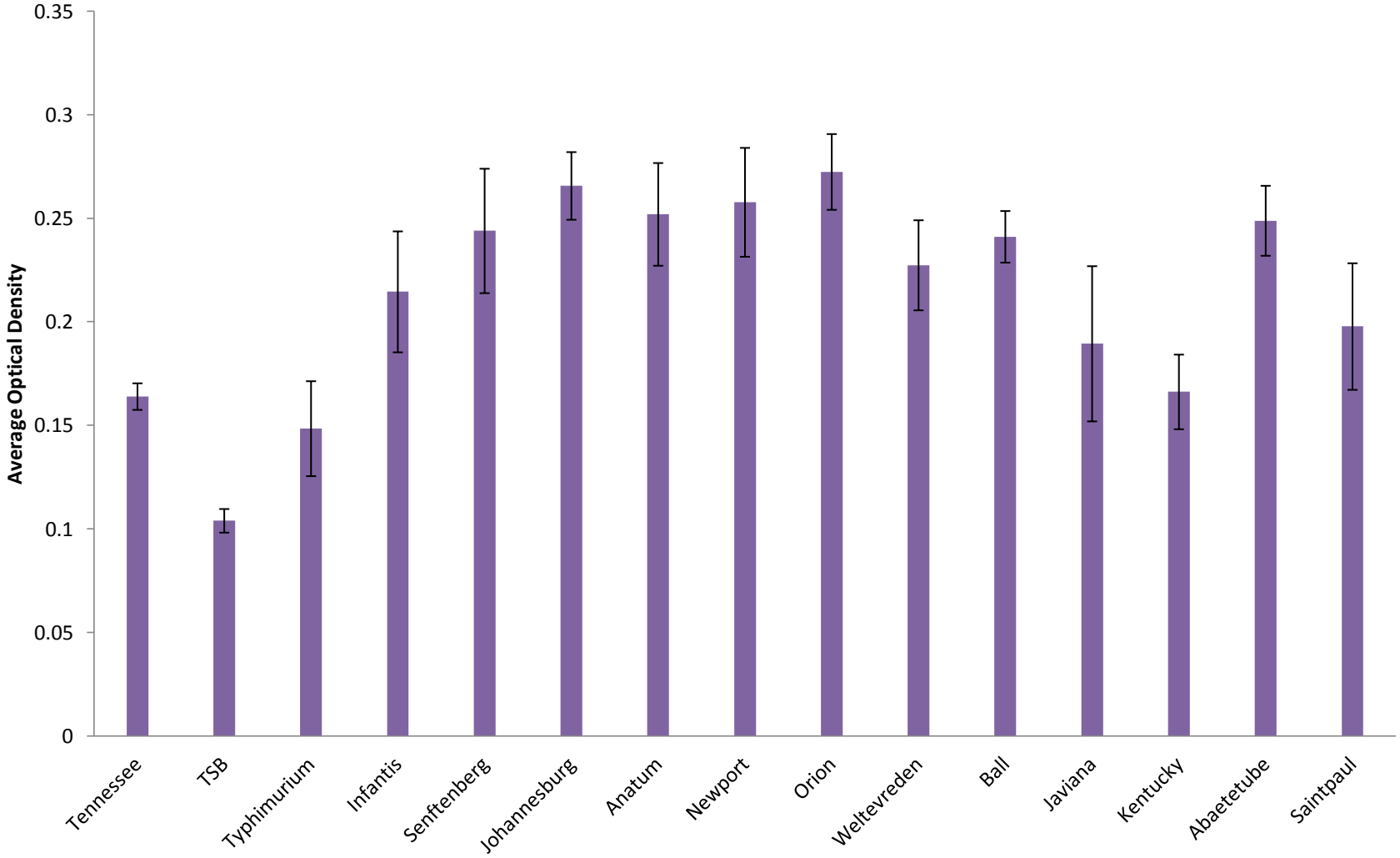

Figure 2.1 Optical Density of single serovar *Salmonella* embedded biofilms stained with Crystal Violet as read by the Bioscreen C machine. This experiment was replicated once, the OD readings are averaged and shown here with standard error bars.
TSB = Tryptic Soy Broth, non-inoculated negative control

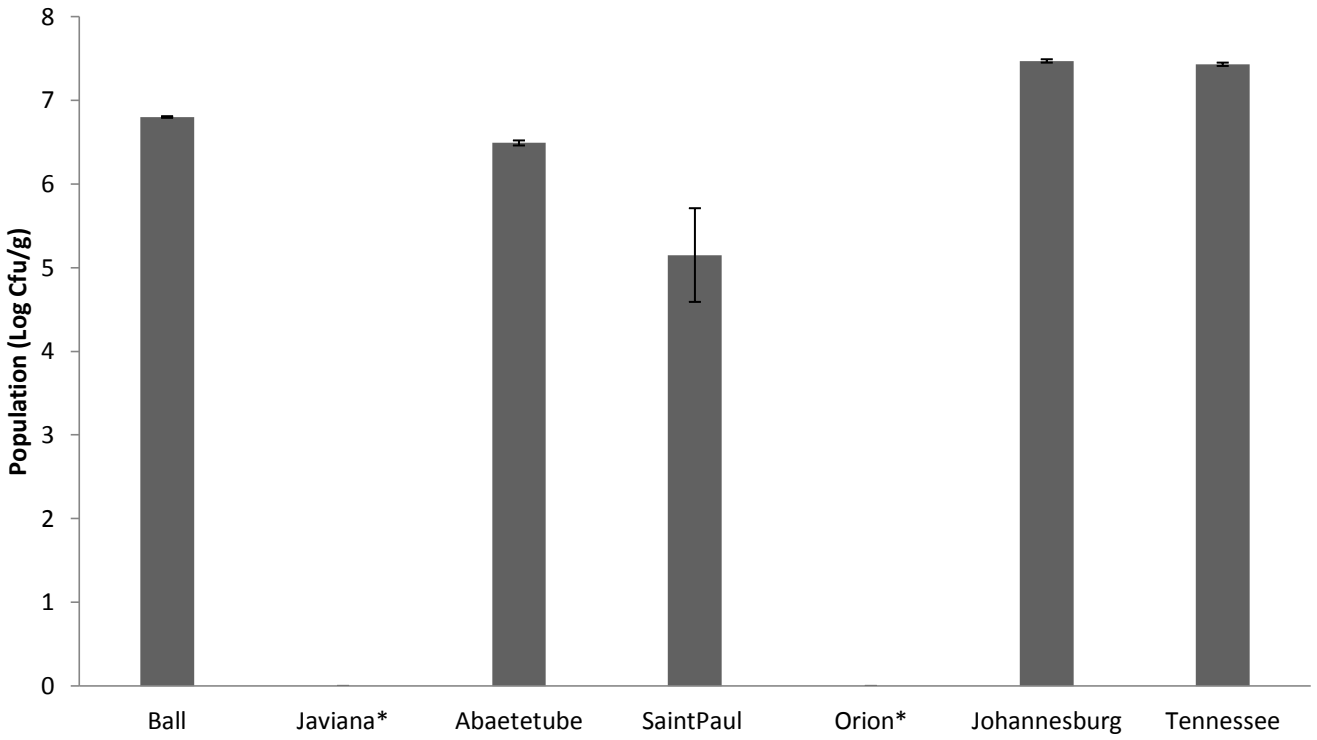

Figure 2.2 Population log recovery of individual *Salmonella* serovars using the Isolated Colony method on whole black peppercorns when plated onto XLT4. Strains denoted by an asterisk showed growth within the flask, but showed no recovery at the lowest level of plating, 10^5 log CFU/g. This experiment was replicated one time, and the results of all plates were averaged and reported here, with standard error bars.

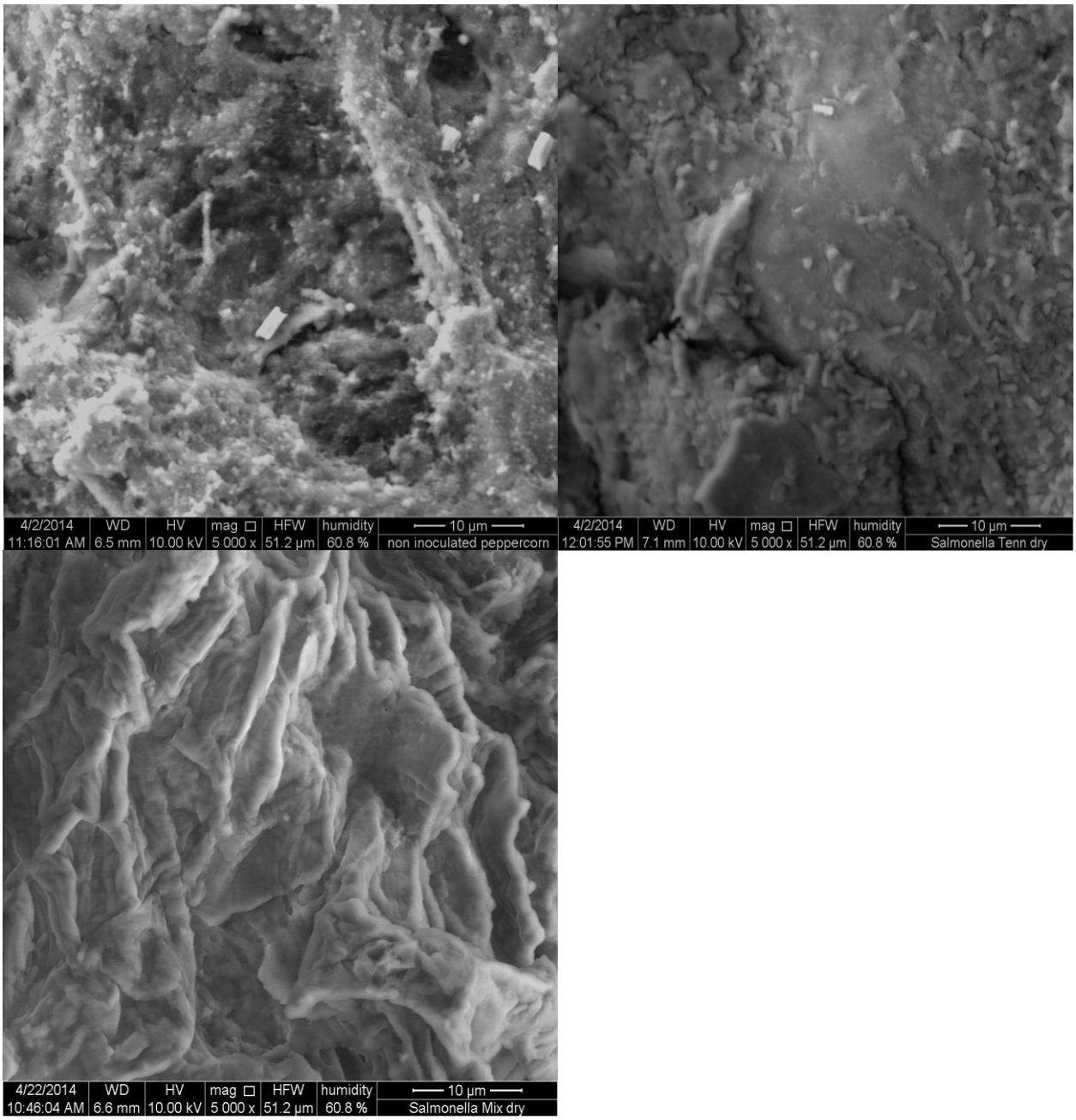

Figure 2.3 Environmental Scanning Electron Microscopy (ESEM) images of the surface of a whole black peppercorn at 5,000x magnification. Top left: an image of the surface of non-inoculated peppercorns. Top right: an image of a single strain biofilm formed by *Salmonella* Tennessee that had been allowed to dry for 48 hours prior to staining with Ruthenium Red and Osmium. Bottom left: an image of the surface of a peppercorn that was inoculated with a mixed culture of Salmonella strains that had been allowed to dry for 48 hours prior to staining. These images were captured by Stephen McCartney of the Virginia Tech Corporate Research Center ICTAS.

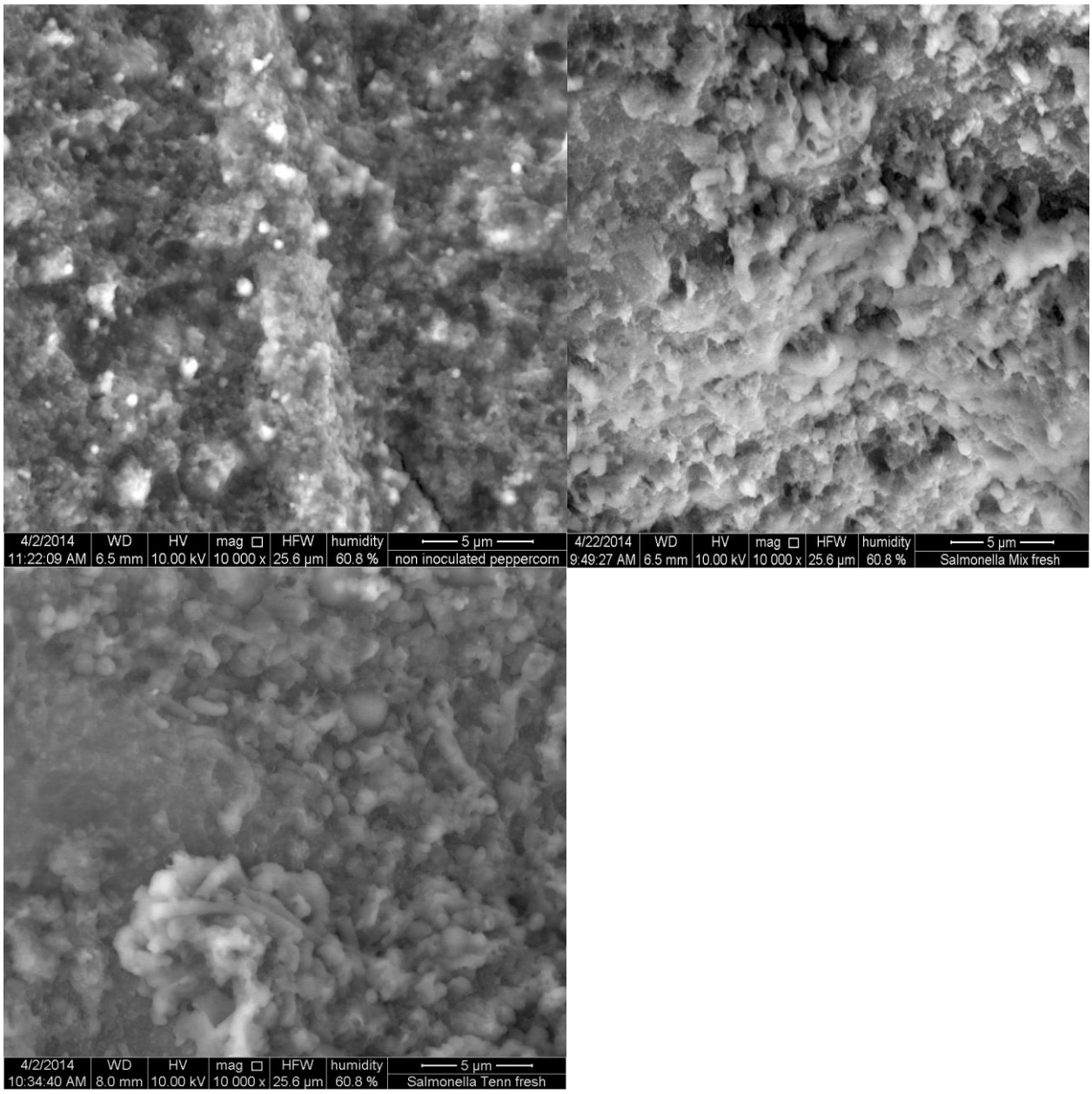

Figure 2.4 Environmental Scanning Electron Microscopy (ESEM) images of the surface of a whole black peppercorn at 10,000x magnification. Top left: an image of the surface of a peppercorn that was not inoculated with *Salmonella*. Top right: an image of the surface of a peppercorn that was inoculated with a mixture of *Salmonella* strains, allowed to grow for two days then stained with Ruthenium Red and Osmium without drying. Bottom left: an image of a peppercorn that was inoculated with *Salmonella* Tennessee and stained with Ruthenium Red and Osimium without drying. These images were captured by Stephen McCartney of the Virginia Tech Corporate Research Center ICTAS.

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Chapter III

Ethanol Vapor Treatments of *Salmonella enterica* Embedded Biofilms Under Atmospheric Pressure Boiling Conditions and Under Vacuum Assisted Boiling Conditions

INTRODUCTION

Ethanol vapor fumigation has been shown to extend the shelf life of fresh produce and processed baked goods *(25, 29).* It is an alternative processing method to those which modern health-conscious consumers might consider dangerous *(4)*. Studies have shown that ethanol treatments can delay the ripening of grapes *(3, 8, 18)*, tomatoes *(30,26)*, broccoli (2), as well as avocado and other climacteric fruit *(23)*. Historically, ethanol has been used as an antimicrobial agent with efficacy against bacteria, including pathogens.

The United States is one of the largest importers of spices, and spices have recently been implicated as the vehicle of infection in foodborne outbreaks. The Food and Drug Administration has reported that over 12% of imported spices in the United States were rated as unacceptable in terms microbiological quality, and 7% of spice imports tested positive for *Salmonella* presence *(33)*. Spices are typically grown in hot, humid climates in countries that do not necessarily abide by effective food safety standards *(33)*. Though spices are often subjected to drying treatments, these steps alone are often not enough to eliminate all human pathogens *(20, 22)*. Historically, it was thought that the low water activity conditions of spice storage would discourage microbial survival. In November of 2009, an outbreak of *Salmonella* Montevideo affected at least 272 individuals across 44 states and the District of Columbia *(7)*. This outbreak was traced back to a company producing salami products containing contaminated imported black and red pepper *(12)*. Common microbial population reduction treatments in the United States include high temperature steam treatments, fumigation by ethylene oxide, and exposure to ionizing radiation.

Salmonellae have been known to utilize biofilms as an environmental persistence strategy. Biofilms often show increased biomass when comprised of multiple species and serovars. It is thought that the vapor form of ethanol has the ability to penetrate dense biofilms and destroy the pathogens within through denaturing of proteins and solving of the bacterial membranes. The purpose of this experiment was to significantly lower *Salmonella* populations within multi-strain

biofilms formed on whole black peppercorns by utilizing ethanol vapor treatments under atmospheric pressure conditions as well as under vacuum assisted pressure conditions.

MATERIALS AND METHODS

Ethanol Vapor Treatment Apparatus

In a previous study, peppercorns were exposed to ethanol vapor in a cabinet-type treatment chamber developed by modifying a bench-top incubator *(21)*. The system did not allow for precise control of temperature, location or volume of ethanol applied. Furthermore, recapture of ethanol vapor was not easily performed, and temperature within the chamber was not reported during treatment times. Therefore, a glassware apparatus was designed and built to allow for greater control of study parameters. Appendix A shows the three components of the ethanol vapor treatment apparatus used for the work reported here. The nitrogen gas input component, the first image in Appendix A, is comprised of a compressed nitrogen tank (D) with attached regulator (A). A brass ball valve (B) is connected to the regulator at the inlet, and a needle valve with barb attachment (C) at the outlet. This arrangement is to regulate the flow of nitrogen into the treatment apparatus in a controlled manner, which is necessary to lower the risk of ethanol ignition. Quarter inch interior surgical tubing is fixed to the barb attachment and a ten foot loop of quarter inch copper coil that is submerged in a water bath set to 80°C (E). The other end of the copper coil is attached to separate piece of surgical tubing which leads to the two-neck flask in the second image of Appendix A. The submerged copper coil increases the temperature of the nitrogen gas in order to warm the apparatus homogeneously to avoid cool pockets where condensation might form.

The central column of the ethanol vapor apparatus as seen in second image of Appendix A is comprised of a two neck flask (G) containing 95% ethanol and distillation beads resting in a stainless steel vessel (B) which contains water and rests on a hot plate as a means to heat the ethanol precisely and evenly. A removable stopper (A) seals the system once the chamber is flushed. The second neck of the flask is attached to a sample chamber (E) which contains silicon wool, the sample, and the end of a thermometer (C). The wool creates a gas permeable barrier to ensure fumigation of the peppercorns, and the thermometer allows the operator to determine the temperature of the samples within the chamber during treatment. The sample chamber connects

with a distillation arm (D) with thermometer port, water jacket and impinger attachments. The water jacket was not utilized in this project. The impinger attachment allows for ethanol vapor traveling through the system to bubble through distilled water contained within a pear-shaped flask in an ice bath (F) to allow for condensation and recapturing of ethanol as well as to block oxygen from re-entering the system through the gas outlet.

Surgical tubing from the impinger barb leads to the gas outlet component of the apparatus as seen in the last image in Appendix A. The vacuum gauge with barb attachment (A) is fitted with the tubing from the impinger and a T-joint (B). The T-joint allows for gas to pass through to the ball valve vent (C) on the bottom and the needle valve (D) on the right. The needle valve is attached to a ball valve with barb attachment (E), and the surgical tubing leading from the barb attachment is fitted to the vacuum port of the 1/8 HP Gast 22D Diaphragm Vacuum Pump (Benton Harbor, MI). The needle and ball valve combination with vacuum gauge set up allows for precise control of the vacuum conditions within the apparatus, and the T-joint allows for the same device to be used for either vacuum or non-vacuum conditions.

Production of *Salmonella* Embedded Biofilms

Biofilms were formed on whole black peppercorns following the final method as described in Chapter Two. In summary: *Salmonella* Abaetetube, Ball, Johannesburg, and Tennessee were isolated from frozen stocks then mixed together and grown in 10 ml Tryptic Soy Broth (TSB). 5 ml inoculated TSB was added to a sterile 2 liter flask containing 60 g whole black peppercorns in a single layer and 150 mL TSB covering the top of the peppercorns, the flask was then statically incubated for 24 hours at 37°C. After incubation, the TSB was decanted and the peppercorns were washed with 200 mL 0.1% peptone- 0.1% Tween solution, swirled for 2 min at 90 RPM, decanted, and fresh 150 mL fresh TSB was added. The flask was then incubated for 24 hours, the spent medium was decanted, and the peppercorns were washed with 200mL peptone-Tween solution for 2 min at 90 RPM, this wash solution was decanted, and the peppercorns were allowed to dry for 48 hours in a biological safety cabinet.

Atmospheric Pressure Boiling Method

Two methods for ethanol vapor treatment were performed; an Atmospheric Pressure Boiling method (AB) and a Vacuum-Assisted Boiling method (VB). The AB method was accomplished

by boiling 95% ethanol at atmospheric conditions whereas the VB method employed a vacuum assist to produce ethanol vapor at a reduce temperature. For both methods, the apparatus was assembled as shown in Figure 2.9. For the AB method, the water bath and stainless steel vessel were filled halfway with distilled water, outfitted with their respective custom lids to allow thermometer and tubing access, turned on and set to 80°C. The hot plate warming the stainless steel vessel was set to 380°C. When the water bath and hot plate reached appropriate temperatures, five grams of biofilm-coated peppercorns were placed into the treatment chamber atop glass wool (0.25 g; Silane treated; Supelco; Bellefonte, PA) that allowed ethanol vapor to pass around peppercorns but did not allow peppercorns to fall into the two-neck flask. 5 ml of distilled water was added to the pear shaped flask to dissolve ethanol for proper disposal. A thermometer was inserted into the treatment chamber to measure vapor temperature near the peppercorns during treatment. Hoses were connected to the warmed nitrogen gas supply. Prior to entering the two-neck flask, nitrogen gas was warmed by passing it through a copper tube coil (1/4 in diameter; 10 feet long) that was submerged in 80°C water. 50 ml of 95.5% ethanol (RICCA; Arlington, TX) was added into the two neck flask, the angled neck of the two neck flask was capped with a glass gas inlet and warmed nitrogen gas was allowed to flow. Specifically, a glassware gas inlet for the nitrogen gas was inserted into the two neck flask, the gas outlet T-valve was opened and the vacuum outlet ball valve was closed to allow for venting of gas. The nitrogen gas ball valve was then opened slowly and nitrogen was allowed to flood the chamber for 30 seconds.

For AB studies, the flow of the nitrogen was stopped, and ethanol was allowed to come to a boil with occasional agitation of the ring stand to prevent superheating and encourage nucleation. When boiling was first visually detected, a timer was started, and the apparatus was left undisturbed for the duration of the treatment time (1, 5, or 10 min). When the treatment time was complete, the distillation arm was removed and the contents of the sample chamber were deposited into a Whirl-Pak bag. The silicon wool was removed using sterile spatula and discarded. Treated peppercorns were subjected to microbiological analysis as described below.

Vacuum-Assisted Ethanol Boiling Method

For VB studies, peppercorns were treated as described for AB studies with the exception that the sample chamber was heated to 50°C and vaporization was achieved through the application of

vacuum (-20 in. Hg) using a vacuum pump 1/8 HP Gast 22D Diaphragm Vacuum Pump (Benton Harbor, MI), immediately after nitrogen flushing and replacing the inlet with a glass stopper. When the treatment time was completed, the vacuum pump was turned off and the pressure in the apparatus was allowed to equalize to atmospheric pressure. Treated peppercorns were collected as described in the AB method.

Microbiological Analysis

Harvested peppercorns were deposited onto a sterile tray in a single layer and placed in a biological safety cabinet for 1 hour in order to release residual ethanol vapor. Five grams of treated peppercorns were placed in a stomacher bag with 45 ml of peptone (0.1%), Tween (0.1%), cellulase (0.2%) diluent (PTC). The samples were stomached for 2 minutes, followed by serial dilutions in PTC. Diluted samples were surface-plated onto XLT4 and TSA agar, followed by incubation at 37°C 24h. 10^3 log CFU/g was chosen as the lower limit of detection due to excessive particulate in the stomached bag providing large amounts of variation at lower levels. In order to account for viable *Salmonella* that may have been present at population densities below the limit of detection via direct plating, enrichment of samples was performed. Enrichments were made by adding one ml of stomacher bag contents into 9 ml of TSB followed by incubation for 24 h at 37°C. After incubation, a loopful of enriched sample was streaked onto XLT4 and incubated for 24 h at 37°C.

Chemical Analysis

Peppercorns were prepared using the AB method as described above with two treatment times: zero minutes and ten minutes. Three 2.0 gram samples of each treatment time were sealed within gas chromatograph vials for 24 hours, to provide enough time to adequately assess the headspace volatile compounds. Solid Phase Micro Extraction (SPME) within the gas chromatograph – mass spectrometer (GC-MS). The raw data from the SPME were analyzed using the Kovat's index as well as chemical structure identifying library within the GC-MS. These structures were double checked with Chemspider, Pherobase.org and Flavornet.org. Area percentages were compared and T-Tests were performed on compounds found in both treated and untreated peppercorn samples to eliminate as much sample variability as possible.

Statistical Analysis

Samples for both AB and VB methods were plated in triplicate on to TSA and XLT4, with three replicates made. Data were analyzed using JMP Pro 10.0.2 software (SAS Institute Inc. 2012) one way ANOVA with Tukey-Kramer HSD means comparisons. Log CFU/g data reported below the limit of detection (3.00 log CFU/g) and positive for enrichment were reported as 2.5 for the purposes of the ANOVA test and Weibull models. For analysis using the Weibull model, the NLIN Procedure (nonlinear regression) in SAS 2005 (Version 9.1.3, Statistical Analysis System Institute Inc. Cary, NC) was used with the Marquardt Computational Method (19).

RESULTS

Preliminary studies showed the no native microbiota were recovered from non-inoculated peppercorns when XLT-4 was used as the plating medium (limit of detection = 3.00 log CFU/g). Effects of ethanol vapor treatment using the AB on recoverable microbial population in non-inoculated peppercorns are shown in Figure 3.5. The one minute treatment of ethanol vapor using AB showed no significant reduction in native microbial population on TSA. The ten minute AB treatment of native peppercorn microbiota showed statistically significant a population reduction of 1.15 log CFU/g on TSA ($p<0.001$).

Effects of ethanol vapor treatment under AB conditions on *Salmonella* populations on inoculated peppercorns are shown in Figure 3.1 and Figure 3.2. Under AB conditions, at time 1, significant population reductions of 2.49 log CFU/g and 3.42 log CFU/g ($p<0.001$) were seen on TSA and XLT-4, respectively in inoculated samples. For both media, significant population differences were seen between all treatment times with the exception of time 5 and time 10 ($p<0.001$). After the ten minute AB treatment, the average total recoverable log CFU/g had been reduced by 3.90 on TSA and 5.95 on XLT-4.

Effects of ethanol vapor treatment under VB conditions on microbial populations in inoculated peppercorns are shown in Figure 3.3 and Figure 3.4. After a 10 minute ethanol vapor treatment under VB conditions, statistically significant differences in population were detected on both TSA and XLT-4 ($p<0.001$): average difference of 2.69 log CFU/g and 4.55 log CFU/g population was detected on TSA and XLT-4, respectively. Temperature within the sample chamber during these runs reached a maximum of 51°C and 72°C during VB and AB trials, respectively.

For the Weibull model calculations, the following equation was used:

$Log(N) = log(N_0) - kt^{\alpha}$

Where N_0 is the initial population, N is the estimated population at the sampling time, "t" is the sampling time, and "k" and "*a*" were determined by regression, displayed in Table 3.2. A pseudo-R^2 value was calculated using the following equation:

$$R^2 = 1 - \frac{\sum_1^n (Y_i - \hat{Y}_i)^2}{\sum_1^n (Y_i - \bar{Y})^2} = \frac{\sum_i^n (\hat{Y}_i - \bar{Y})^2}{\sum_i^n (Y_i - \bar{Y})^2}$$

Where $\boldsymbol{Y_i}$ is the *Salmonella* population (log CFU/g), $\boldsymbol{\hat{Y}_i}$ is the log CFU/g population estimated by the model, $\boldsymbol{\bar{Y}}$ is the average log CFU/g population, and *n* is the number of data points of the inactivation curve.

Effects of a ten minute, AB ethanol vapor treatment on headspace volatile content in whole black peppercorns via Solid Phase Micro Extraction are shown in Table 3.1 and Appendix C. T-Test results with an alpha level of 0.99 indicate several statistically significant differences in headspace volatile content between ten minute ethanol vapor treated and untreated whole black peppercorns.

DISCUSSION

This experiment suggested that the native microbiota are not as susceptible to the ethanol vapor treatment when compared to the laboratory-introduced *Salmonella* strains, despite increased resistance to stressors by drying the spices to 0.30-0.40 A_w. The Weibull distribution model displays an upwardly concave graph with definite rightward tailing, indicating an increasing failure rate over time *(32)*. The tailing is likely due to the ability of certain organisms within the native microbiota of whole black peppercorns, such as *Bacillus cereus*, to form spores to withstand the ethanol vapor treatments; *Salmonella enterica* is not able to form spores, thus less significant tailing was observed on *Salmonella* selective media. The primary mode of action of ethanol is solving bacterial membranes and denaturing outer membrane proteins; ethanol would not affect a spore coat in the same way that it would affect a lipid membrane. Prior exposure of the native microbiota to stress agents may have influenced the failure rate of the ethanol vapor treatments as well. The additional heat under AB condition impacted recoverable populations

with a difference of 1.04 log CFU/g on TSA ($p < 0.001$) and 0.96 log CFU/g on XLT-4 ($p = 0.0323$), implying the synergistic effect of ethanol, heat and pressure provided the six log CFU/g reductions reported in the Wistreich patent *(35)*, and not the effect of ethanol vapor alone. The population reduction reported for XLT-4 is comparable with the 6-log reduction offered by ionizing radiation *(20)* and high-temperature-short-time steam treatment *(1)*, and this treatment does not carry the public stigma and fear of radiation *(27)*. The Ionizing Radiation and high-temperature-short-time steam treatment population recoveries were carried out on nonselective media, making the Ethanol Vapor treatment comparable when eliminating *Salmonella* spp., but not necessarily comparable to the elimination of other organisms.

The results of the SPME indicated significant decreases of many compounds that have been characterized by having a spice-like or herbaceous aroma, while also showing increases in compounds that are characterized by pine-like or mint aromas. According to previous studies, four of the primary compounds that comprise the primary odor profile of black pepper include Germacrene, Limonene, beta-Pinene and alpha-Phellandrene *(14)*. While Germacrene levels were not significantly affected by the ten minute AB ethanol vapor treatment, levels of limonene, beta-Pinene and alpha-Phellandrene were all reported to have statistically significant increases as a result of the ten minute treatment. In the future, the design of this experiment would benefit from running headspace volatile analysis on the same set of samples before and after ethanol vapor treatments and comparing that data, as well as analysis of total volatile and nonvolatile oil content.

It is unclear at this time whether the results of this experiment are directly comparable to other works when considering the differences in levels of contamination, as well as the state in which the microorganisms were present. The previous ionizing radiation, steam, and heated, pressurized ethanol treatments all contained a starting population of approximately six log CFU/g with no steps taken to ensure the production of biofilms *(1,9,15, 31, 35)*, there is no way to definitively claim how these factors impacted the results without further investigation. Previous studies also describe total losses of volatile compounds, but only one study shows increases in specific volatile content *(21)*.

The results of the AB and VB methods suggest that ethanol vapor is a good option for treating spices that have mild to moderate *Salmonella* contamination, if it were able to be determined that

Salmonella were the only pathogens present. Considering the presence of *Bacillus cereus*, *Staphylococcus aureus* and *Clostridium perfringens* in spice products *(20)* it would be potentially unsafe to rely solely on an ethanol vapor treatment, unless it is coupled with heat, pressure, and time, due to the chance that the spices have reached heavily contaminated and biofilm coated population levels, as described in this experiment. An alternative use for this treatment would be using ethanol vapor as a sterilant prior to the drying process, to reduce the amount of external stresses that the organisms have been exposed to prior to the primary pathogen reduction strategy, as was suggested by one study involving post harvest heat treatments *(27)*. Due to the inherent hazards of working ethanol and compressed gases, this strategy may not be able to be implemented in countries that do not have access to such machinery and education.

Future experiments that characterize the synergistic effects of ethanol, heat and pressure on spore-forming organisms both contained biofilms and not would prove beneficial to understanding the full scale applications of this work. Larger peppercorn batch sizes would also benefit the project, by observing the effect that increased biofilm-to-biofilm contact surfaces has on recoverability of pathogenic populations. Additionally, the apparatus could be redesigned to allow for additional ethanol capacity, thus allowing for treatment times greater than ten minutes. The two water bath system could be changed for a single, large water bath with enough space for the copper coil and modified ethanol flask, and a larger ethanol vapor recollection flask would be beneficial for improving the recovery of ethanol which had been passed over the peppercorns.

TABLES

Compound	Characteristic Odor	Content Change	T-Test
Alpha Thujene	Wood, Green, Herbaceous[A]	Increase	$P = 3.50*10^{-3}$
Alpha Pinene, (D)-	Pine, Turpentine[A]	Increase	$P = 4.92*10^{-6}$
Camphene	Camphor[A]	Increase	$P = 3.60*10^{-5}$
Beta Phellandrene	Mint, Turpentine[A]	Increase	$P = 4.07*10^{-3}$
Beta Pinene	Pine, Resin, Turpentine[A]	Increase	$P = 2.55*10^{-5}$
Alpha Phellandrene	Turpentine, Mint, Spice[A]	Increase	$P = 7.81*10^{-3}$
o-Cymene	Gasoline, Citrus[A]	Decrease	$P = 5.33*10^{-5}$
Limonene, (D)-	Citrus, Lemon, Orange[A]	Increase	$P = 9.64*10^{-4}$
p-Cymen-8-ol	Citrus, Must[A]	Decrease	$P = 3.48*10^{-4}$
Alpha Terpineol	Oil, Anise, Mint[A]	Decrease	$P = 4.43*10^{-5}$
Cuminaldehyde	Acid, Sharp[A]	Decrease	$P = 1.21*10^{-6}$
2-Undecanone	Orange, Fresh, Green[A]	Decrease	$P = 3.88*10^{-4}$
Alpha Cubebene	Herbaceous, Wax[A]	Decrease	$P = 1.75*10^{-3}$
Copaene	Wood, Spice[A]	Decrease	$P = 1.60*10^{-5}$
Alpha Gurjunene	Wood, Balsamic[A]	Increase	$P = 1.99*10^{-3}$
Caryophyllene	Wood, Spice[A]	Decrease	$P = 3.96*10^{-6}$ $P = 2.19*10^{-3}$ [B]
Beta Copaene	N/A	Decrease	$P = 3.13*10^{-3}$
1,4,7-Cycloundecatriene, 1,5,9,9-tetramethyl-, Z,Z,Z-	N/A	Decrease	$P = 4.60*10^{-5}$
Gamma Muurolene	Herbaceous, Wood, Spice[A]	Decrease	$P = 3.34*10^{-3}$
Beta Selinene	Herbaceous[A]	Decrease	$P = 3.85*10^{-3}$
Delta Cadinene	Thyme, Medicinal, Wood[A]	Decrease	$P = 1.99*10^{-5}$
Caryophyllene Oxide	Herbaceous, Sweet, Spice[A]	Decrease	$P = 1.32*10^{-3}$ $P = 2.79*10^{-6}$ [B]
Tetracyclo[6.3.2.0(2,5).0(1,8)]tridecan-9-ol, 4,4-dimethyl-	N/A	Decrease	$P = 5.39*10^{-3}$

A: www.flavornet.org
B: Compounds appeared twice during run

Table 3.1 Headspace volatiles as identified by Solid Phase Micro Extraction (SPME) that were significantly different (a = 0.99) between untreated peppercorns and peppercorns that had been treated with a ten minute ethanol vapor treatment at atmospheric pressure.

Treatment	Media	K Value	Alpha	Fitting Parameter (B)	Psuedo-R^2
AB	TSA	2.13±0.23	0.29±0.05	-0.87	0.99
AB	XLT-4	3.22±0.40	0.34±0.06	-0.90	0.98
AB Native	TSA	0.26±0.18	0.69±0.33	-0.97	0.99
VB	TSA	1.79±0.21	0.23±0.06	-0.79	0.99
VB	XLT-4	3.20±0.55	0.24±0.09	-0.80	0.95

Table 3.2 Weibull model nonlinear regression values with standard errors shown. AB refers to the Atmospheric Pressure Boiling Method, and VB refers to the Vacuum Assisted Boiling Method. Tryptic Soy Agar is displayed as TSA, and Xylose Lysine Tergitol-4 agar is displayed as XLT-4. Treatments were plated in triplicate with three replicates.

FIGURES

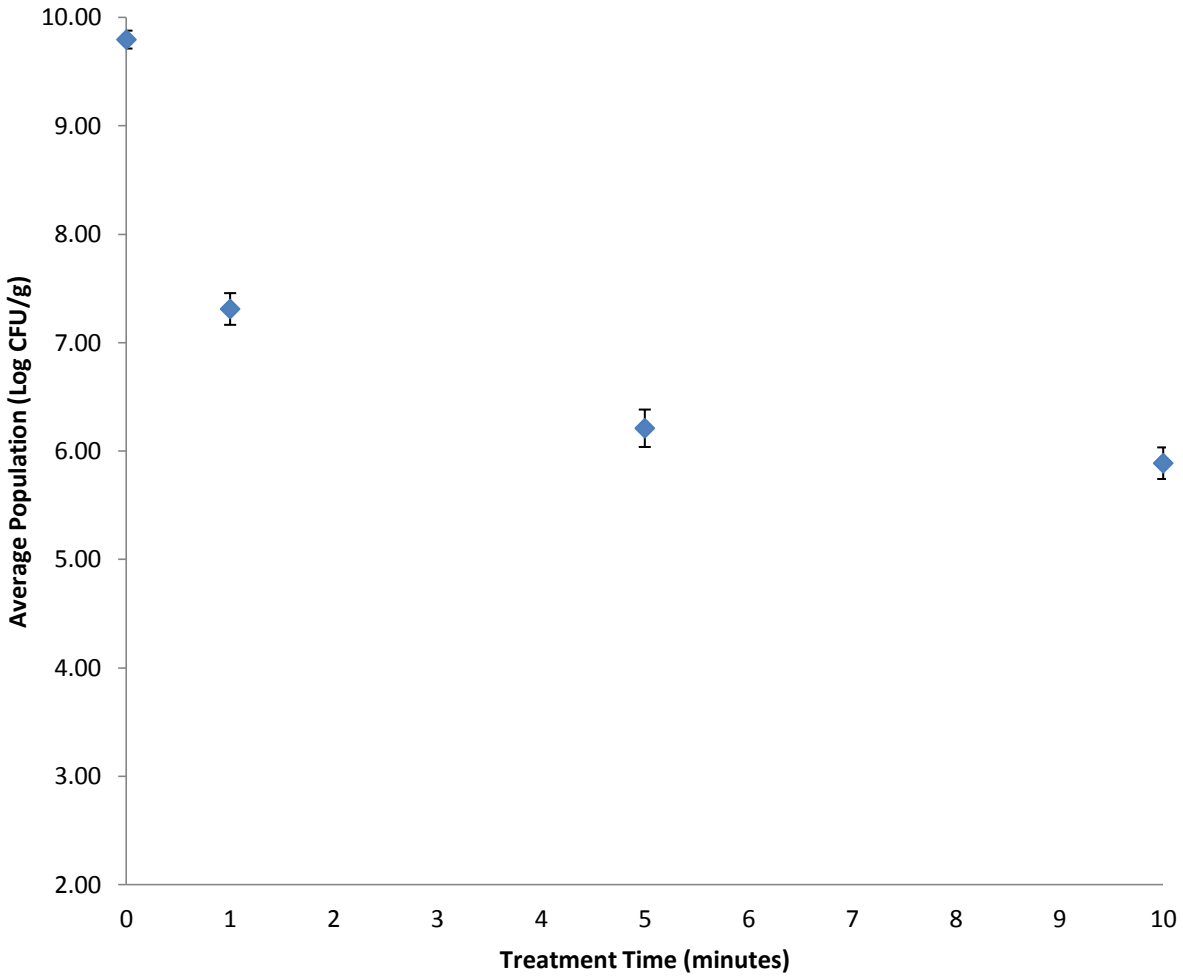

Figure 3.1 Mean populations (log CFU/g) of microbiota on *Salmonella embedded biofilm*-coated black peppercorns treated with ethanol vapor at atmospheric boiling temperature (sample chamber temperature 70±2°C) as determined on TSA using the Atmospheric Pressure Boiling Method with Standard Error bars shown. Three replicates.

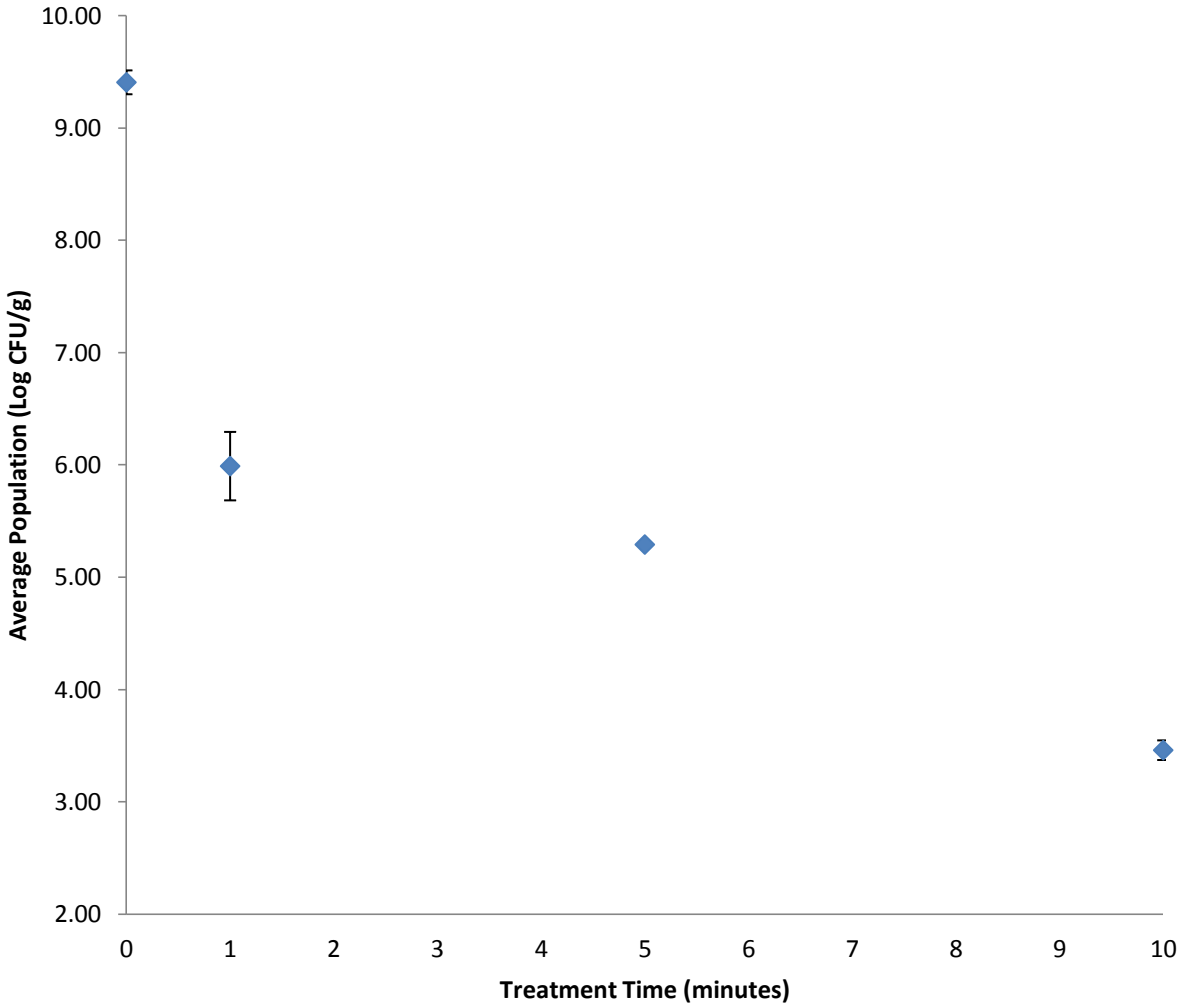

Figure 3.2 Mean populations (log CFU/g) of *Salmonella embedded biofilm*s on black peppercorns treated with ethanol vapor at atmospheric boiling temperature (sample chamber temperature 70±2°C) as determined on XLT-4 (Atmospheric Pressure Boiling Method) with Standard Error bars shown. Three replicates.

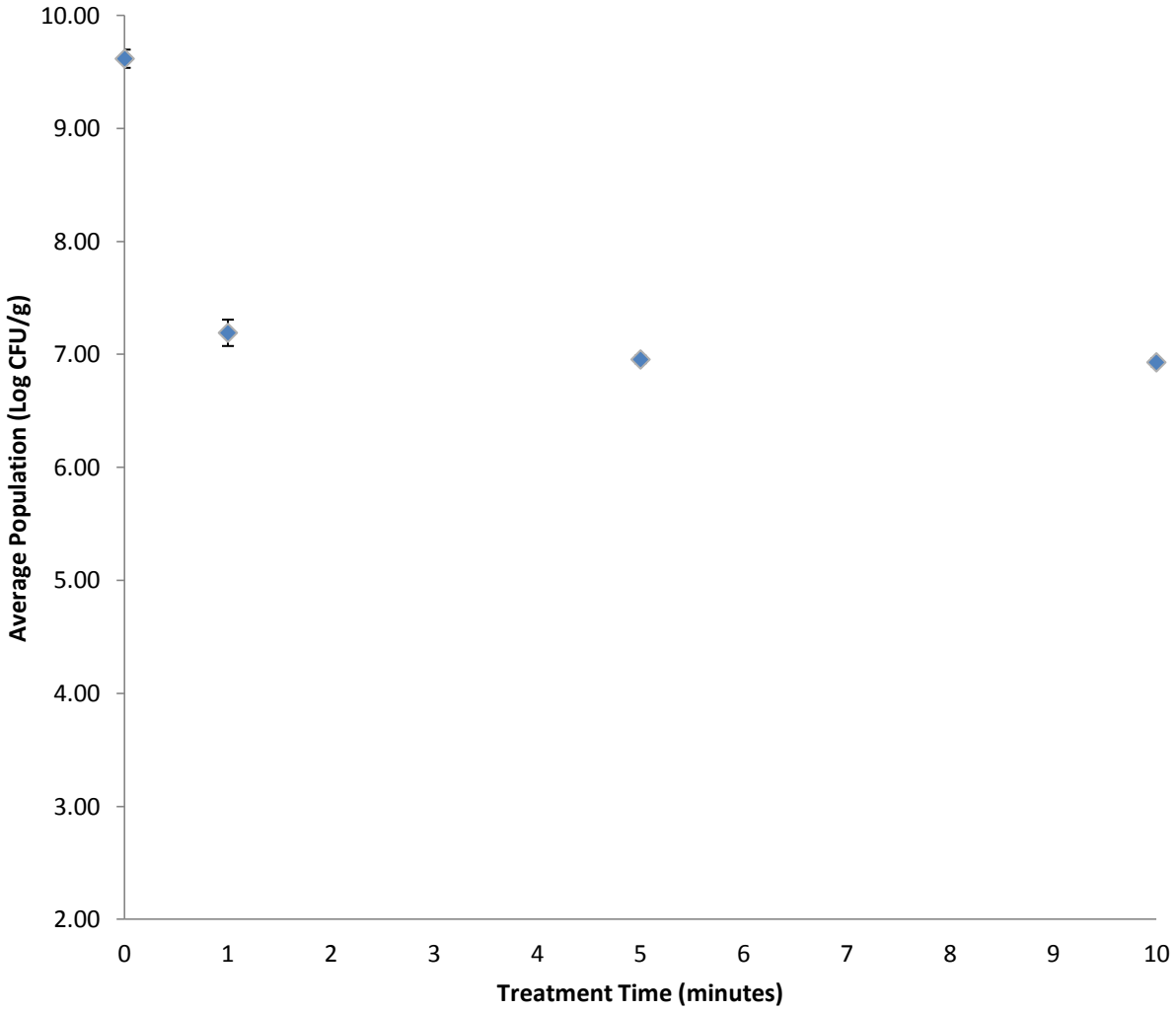

Figure 3.3 Mean populations (log CFU/g) of microbiota on *Salmonella* embedded biofilm-coated black peppercorns treated with ethanol vapor at partial vacuum (-20 inHg) boiling temperature (sample chamber temperature 50±2°C) as determined on TSA (Vacuum-Assisted Boiling Method) with Standard Error bars shown. Three replicates.

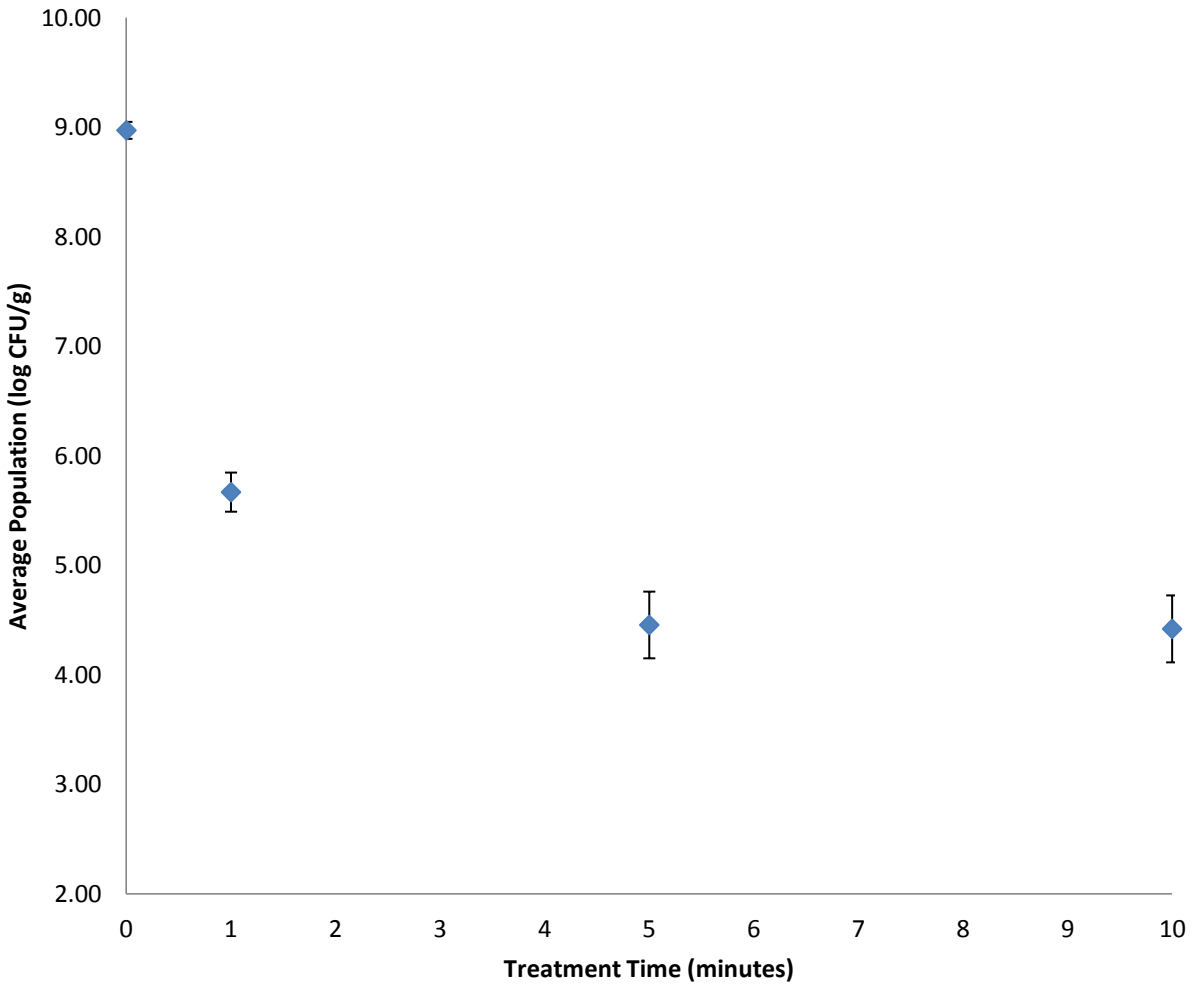

Figure 3.4 Mean populations (log CFU/g) of *Salmonella* embedded biofilms on black peppercorns treated with ethanol vapor at partial vacuum (-20 inHg) boiling temperature (sample chamber temperature 50±2°C) as determined on XLT-4 (Vacuum-Assisted Boiling Method) with Standard Error bars shown. Three replicates.

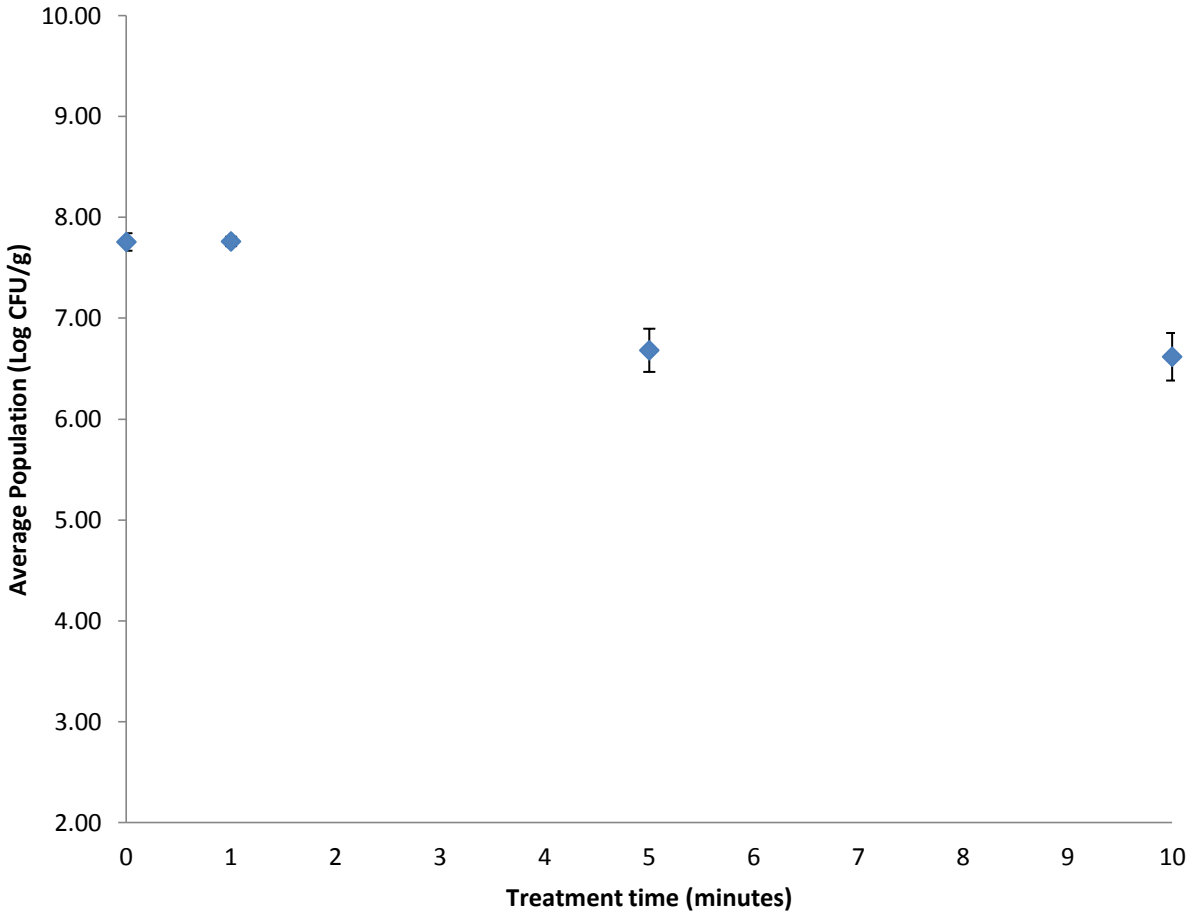

Figure 3.5 Mean populations (log CFU/g) of microbiota on non-inoculated (negative control) black peppercorns treated with ethanol vapor at atmospheric boiling temperature (sample chamber temperature 70±2°C) as determined on TSA (Atmospheric Pressure Boiling Method) with Standard Error bars shown. Three replicates.

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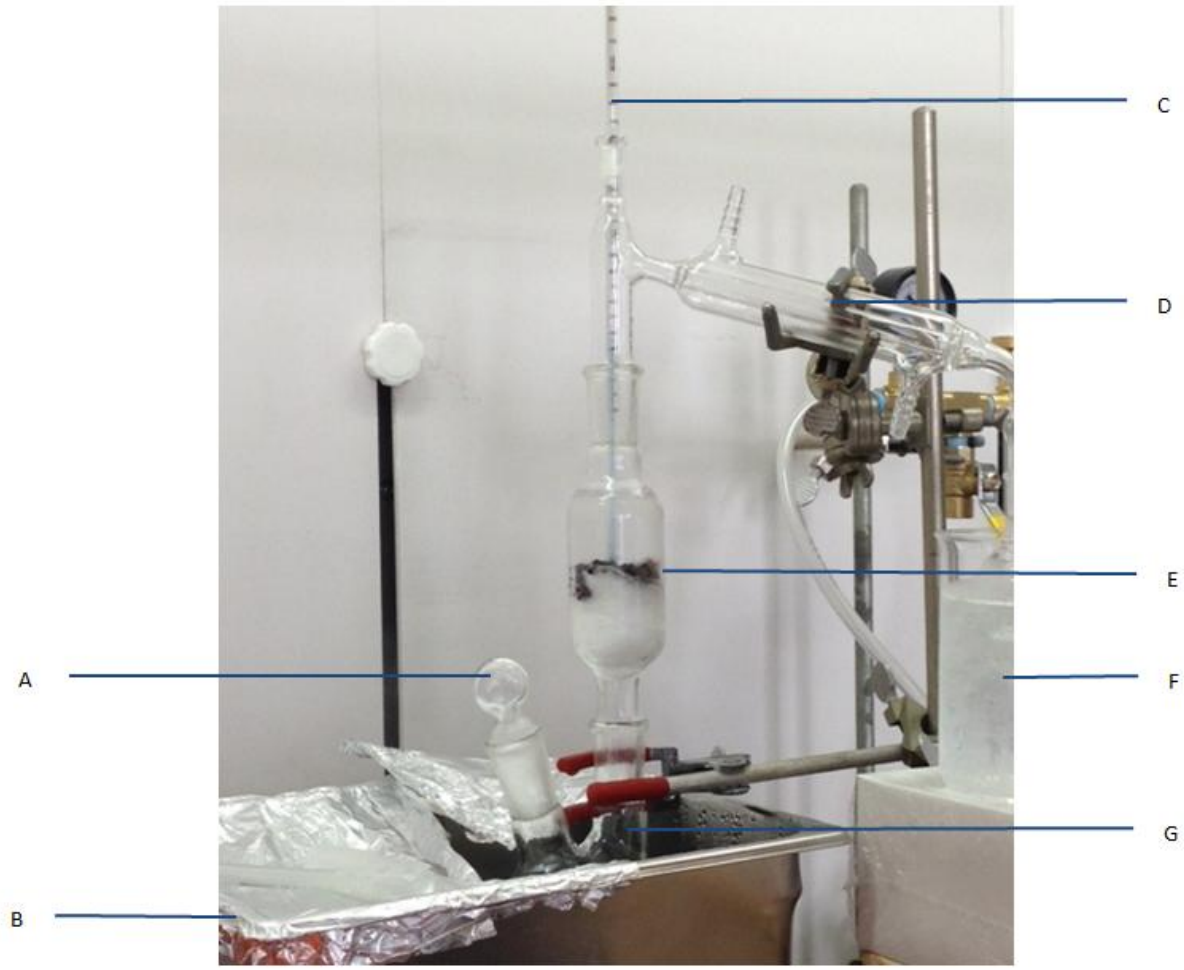
APPENDIX A

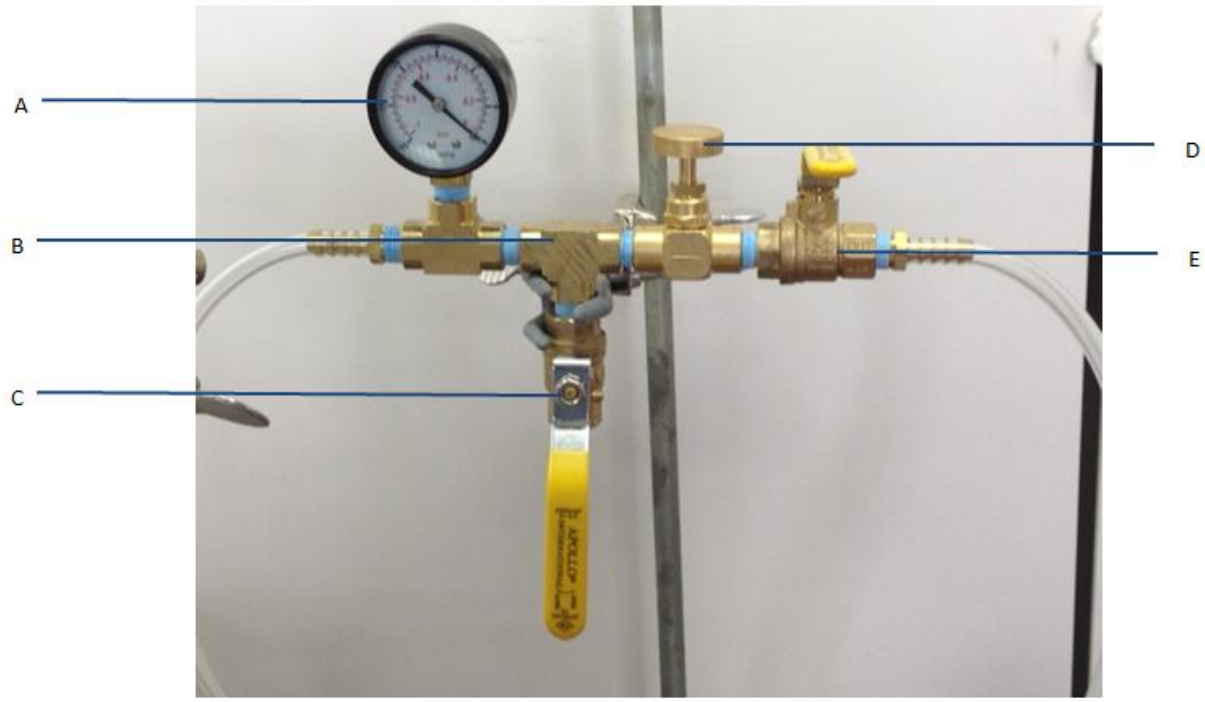
Nitrogen gas input for the Ethanol Vapor Apparatus. A) Nitrogen gas regulator; B) brass ball valve; C) brass needle valve; D) compressed nitrogen gas; E) water bath with thermometer, custom lid and quarter inch diameter copper tubing coiled connected to quarter inch interior diameter surgical tubing.

APPENDIX A



Central glassware components of the Ethanol Vapor Apparatus. A) Glass stopper which can be replaced with a gas inlet barb; B) stainless steel vessel with hot plate; C) thermometer; D) distillation arm with thermometer port and impinger attachment; E) sample chamber with silicon wool filter and peppercorns; F) pear shaped flask with 5 ml distilled water submerged in ice bath; G) two-neck flask containing ethanol and distillation beads.

APPENDIX A



The gas outlet set-up for the Ethanol Vapor Apparatus. A) Vacuum gauge with barb attachment; B) T joint; C) ball valve; D) needle valve; E) ball valve with barb attachment

APPENDIX B

+	X	003	X	014	X	085	X	021	X
+	-	003	013	014	039	085	087	021	Empty
+	-	003	013	018	039	085	087	021	Empty
+	001	003	013	018	039	085	088	021	Empty
+	001	005	013	018	039	085	088	029	Empty
+	001	005	013	018	056	085	088	029	Empty
-	001	005	013	018	056	087	088	029	Empty
-	001	005	014	018	056	087	088	029	Empty
-	001	005	014	039	056	087	088	029	Empty
-	003	005	014	039	056	087	021	029	Empty
X	003	X	014	X	056	X	021	X	Empty

An example Bioscreen C honeycomb. The positive control, as denoted by "+" was inoculated with *Salmonella* Tennessee. The negative control, "-" was non-inoculated tryptic soy broth. "X" on the diagram depicts a well that was sealed by the manufacturer to stagger the samples, and Empty denotes a well that was not filled. The numbers correspond to the serovar identification numbers listed in Table 2.1.

APPENDIX C

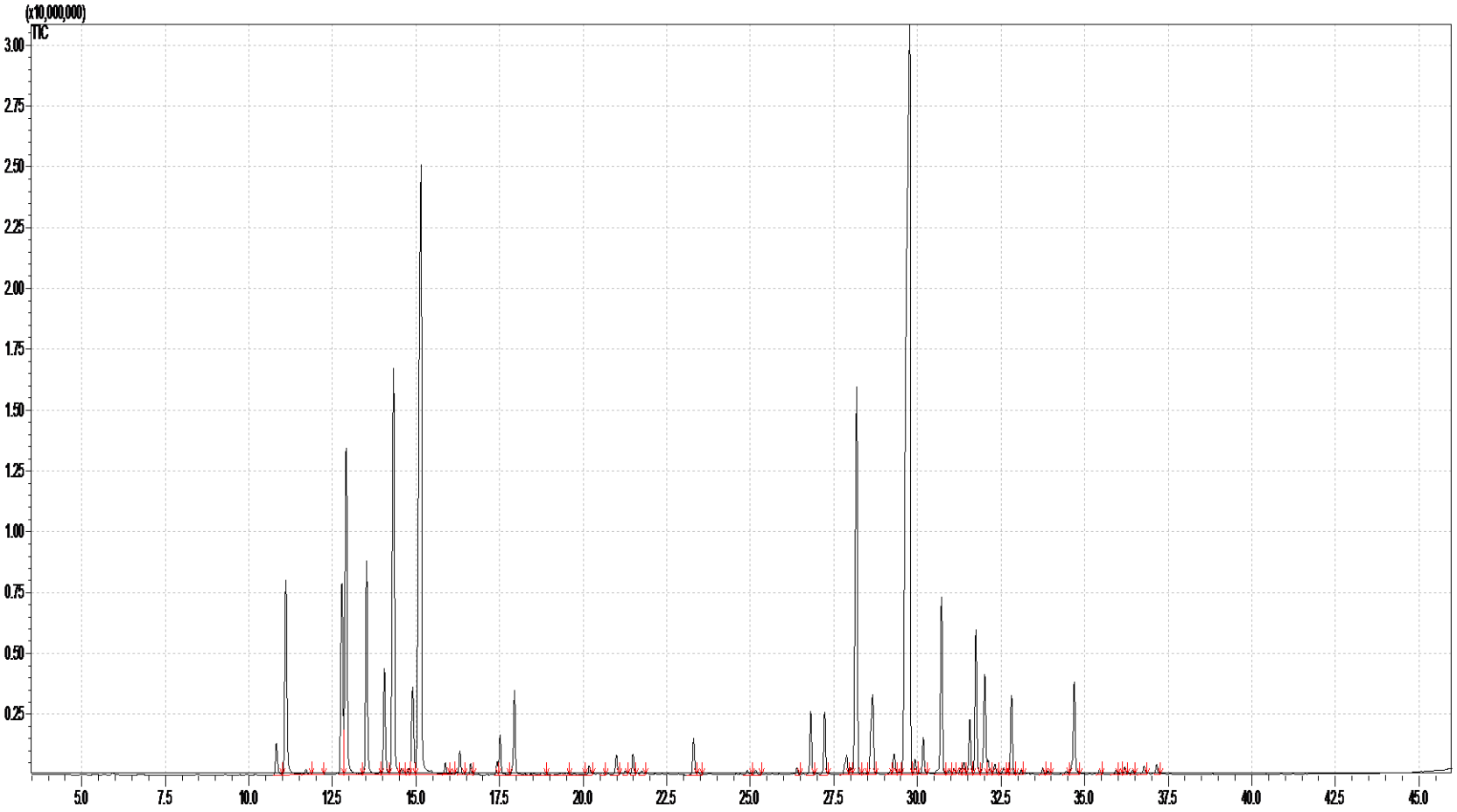


Figure C.1 Gas chromatogram image of whole black peppercorns that received no ethanol vapor treatment time.

APPENDIX C

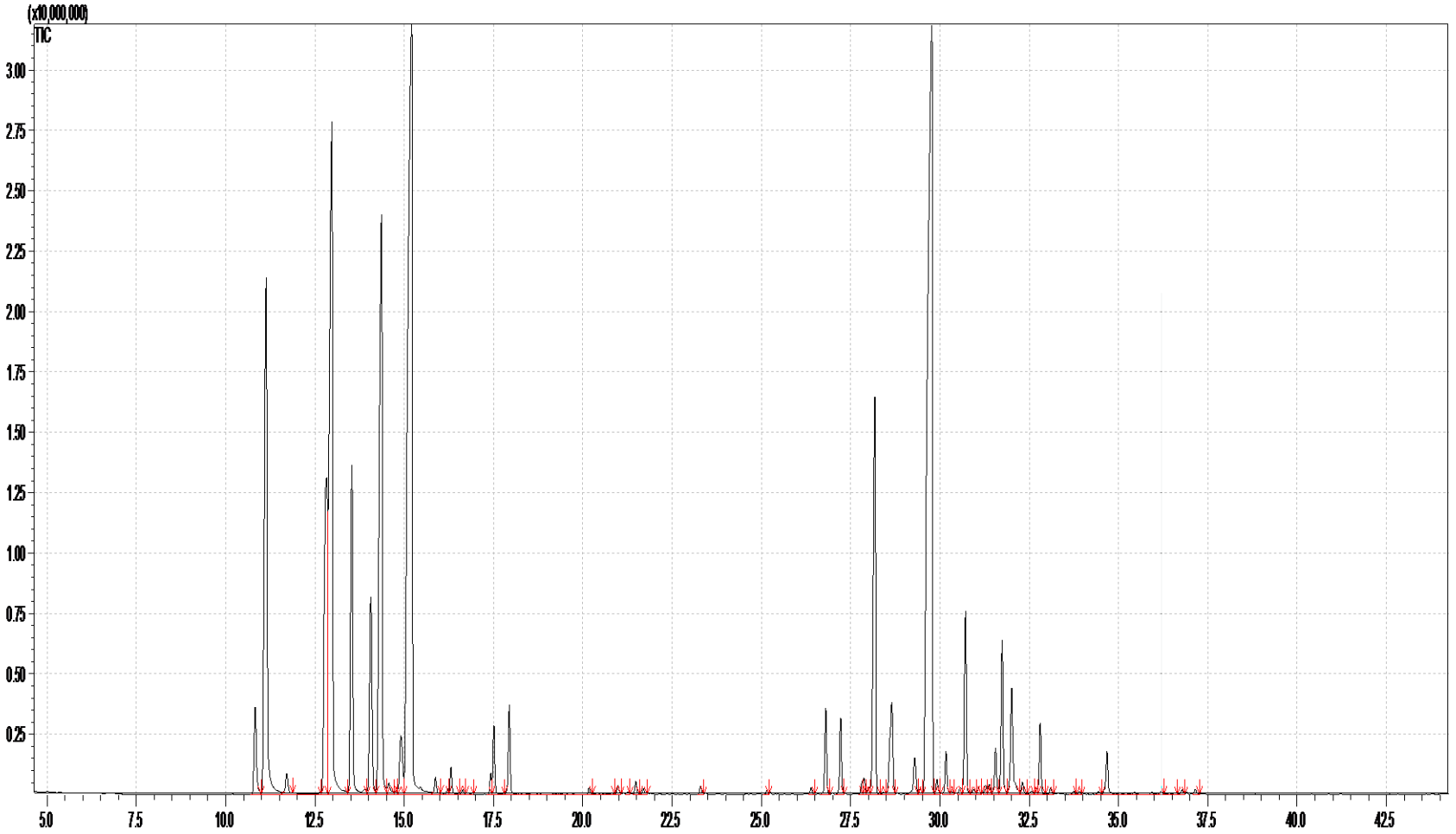


Figure C.2 Gas chromatogram image of whole black peppercorns that received ten minutes of ethanol vapor treatment time.