IMPACTS OF INOCULATION STRATEGY ON SURVIVAL OF *SALMONELLA ENTERICA* AND SURROGATE *ENTEROCOCCUS FAECIUM* AT LOW WATER ACTIVITY ON DRY PEPPERCORN AND CUMIN SEEDS

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IMPACTS OF INOCULATION STRATEGY ON SURVIVAL OF *SALMONELLA ENTERICA* AND *ENTEROCOCCUS FAECIUM* AT LOW WATER ACTIVITY ON DRY PEPPERCORN AND CUMIN SEEDS

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ABSTRACT

Salmonella contamination of spices and other low water activity foods is a growing concern for the food industry due to increased frequency of salmonellosis outbreaks and detection-based product recalls. The impact of inoculation preparation on the survival of a *Salmonella enterica* and its proposed surrogate, *Enterococcus faecium* NRRL B-2385, on the whole black peppercorns and cumin seeds was examined. Three liquid inoculation methods (biofilm-inclusion, agar-grown, broth-grown) for *Salmonella enterica* and surrogate *Enterococcus faecium* and one dry transfer method for *Salmonella enterica* were developed then applied to whole peppercorn and cumin seeds. Spices were returned to original water activity (a_w 0.3) and stored for 28 days with periodic sampling (0, 1, 7, 14, 21, 28 days) and surviving bacteria enumerated. Average log reductions (LR) over time were statistically analyzed to determine differences in stability during storage. Inoculation preparation was associated with significant differences in recovered *Salmonella* and *Enterococcus* from both peppercorn and cumin over the storage period. At 28 days, the most stable inoculations of *Salmonella* resulted from the biofilm-inclusion (-0.04 CFU/g LR) and agar grown (-0.75 CFU/g LR) methods on peppercorn and the biofilm inclusion method (-0.28 CFU/g LR) on cumin. Log reductions of *Enterococcus faecium* (-0.02 CFU/g LR biofilm-inclusion-peppercorn, -0.19 CFU/g LR agar-grown-peppercorn, -0.61 CFU/g LR biofilm-inclusion-cumin) were comparable to *Salmonella* after 28d desiccated storage. These results will guide the inoculation

strategies for validating inactivation processes for reducing *Salmonella* on whole spices, and for comparisons of inactivation of *Salmonella* and its proposed surrogate *Enterococcus faecium*.

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DEDICATION

This work is dedicated to my son, Connor Stewart Bowman, who brought new joy and meaning to my research. He was my companion for the journey and is a source of motivation for the work that I do.

ATTRIBUTION

Multiple contributions were made to this research and the contributions are as follows:

Monica A. Ponder, PhD, (Food Science & Technology Department at Virginia Tech) is currently an Associate professor and served as the principal investigator of this project. Dr. Ponder designed the experiments, provided guidance and funding for this research project, serves as corresponding author of the manuscript in Chapter 3, and contributed to the data analysis.

Robert C. Williams, PhD, (Food Science & Technology Department at Virginia Tech) is currently an Associate professor of food microbiology and food safety, focused on detection and control of pathogenic and spoilage microorganisms in food. Dr. Williams was a co-author on Chapter 3 and provided consultation on the design of experiments.

Kim M. Waterman, MS, (Food Science & Technology Department at Virginia Tech) is currently microbiology lab manager, supporting departmental and graduate research. Ms. Waterman was a co-author on Chapter 3 and provided technical assistance in the laboratory.

P. Kumar Mallikarjunan, PhD, (Biological & Systems Engineering at Virginia Tech) is currently a professor of food process engineering, focused on process development and non-destructive sensing. Dr. Mallikarjunan provided consultation on design of experiments and apparatus design for future research.

Stephen McCartney (Virginia Tech ICTAS Nanoscale Characterization and Fabrication Laboratory) performed sample preparation and transmission electron microscopy of biofilm inoculated peppercorns in Chapter 3.

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CHAPTER 1: INTRODUCTION AND JUSTIFICATION

Salmonella enterica, the commonly occurring food-borne bacteria responsible for human salmonellosis, has been repeatedly associated with dry spices and spice products. Detection of the pathogen in commercial spice products has prompted multiple nation-wide voluntary recalls of domestic products and rejections of imported spice goods entering into the United States *(3, 33)*. Since 2006, the FDA has recorded twelve major outbreaks of salmonellosis resulting from consumption of contaminated spices and other foods of low water activity similar to spices including nuts, nut butters, cereals, dry pet food, chia powder, and tahini paste drawing attention to the issue of persistence of the pathogen at low water activity *(4)*. *Salmonella* contamination of spice products has also been detected in samples collected from commercial markets around the world *(7, 15, 17, 18, 26)*. Investigators have confirmed that multiple serovars of *Salmonella,* including those isolated from spice outbreaks, have improved survival capabilities in lower water activity foods, attributed to increased tolerances to processing controls such as heat inactivation, acid and solute exposure, and desiccation *(5, 20, 25)*.

Improved survival of *Salmonella* under low water activity conditions is associated with multiple factors. *Salmonella* cells in a variety of physiological forms may contaminate spice products during the course of cultivation, harvesting, processing, and storage due to wildlife exposure and agricultural practices in their source regions *(30)*. Physiological state of the cell during growth and inoculation plays a significant role in cell survival under stress. *Salmonella* have the ability to produce and coexist in mixed biofilm structures, which impart protections against simulated gastric passage and

common sanitizers to the cell *(6, 23)*. Sessile cell growth prior to inoculation enhances *Salmonella* survival during drying and refrigerated storage conditions compared to traditional liquid culturing methods *(16, 31)*. Cells in each of these states have evolved different mechanisms for adaptation to their environments. As a result, the method in which low water activity products are inoculated with the pathogen has the potential to impact the survival and resistance of the pathogen in processing and storage and therefore must be investigated.

Dry inoculation methods for inoculating spice products would be advantageous compared to traditional wet methods. Laboratory inoculations of low moisture foods commonly involve suspension of the bacteria within liquid, followed by an additional drying process *(10)*. This procedure may be problematic for some dry ingredients (nuts, spices, powders) because the texture and water activity of the product after wetting and drying can be irrevocably altered compared to the original product *(29)*. Dry transfer from a contaminated carrier has been described for nuts *(8, 11)*, ready to eat meats *(14)* and poultry feed *(21)*. Implementation of dry inoculation procedures for spices would also prevent release of water-soluble antimicrobials *(34)*, which may artificially reduce microbial numbers in the absence of processing.

Controlling for the broad array of external pressures that contribute to the risk of *Salmonella* contamination is unrealistic, therefore inactivation processes must be able to reliably and reproducibly neutralize the risk of pathogens. Process validation consists of executing a representative process with specific parameters and conducting post-process analysis sufficient to confirm that lethality of the pathogen has been achieved *(2, 27)*. Current practices for microbial reduction in spices involve minimal processing

techniques in order to preserve sensory attributes and product quality. Common methods include chemical fumigation, irradiation, and steam-based heat treatments *(2)*. However, there are gaps in the literature regarding inactivation of *Salmonella* on spices using these methods *(32)*. New regulations resulting from the 2011 Food Safety Modernization Act legislation motivate commercial processors to utilize preventative controls in order to minimize risks of pathogens entering the food supply *(1)*. Industry needs process methods that have demonstrated efficacy backed up by data to show that inactivation is achieved.

Non-pathogenic surrogate organism selection is another important consideration in the validation of a process method. Surrogates must be vetted through a thorough selection process to ensure that they exhibit similar behaviors, characteristics, and most importantly that the surrogate provides a conservative measure of the inactivation on the product in question compared to the target pathogen *(19)*. In commercial processing, surrogate bacteria are often used for process validation to prevent unintended contamination of the process facility with human pathogens, including *E. coli, L. monocytogenes,* and *S. enterica (12, 13, 24, 28)*. *Enterococcus faecium* NRRL B-2354 has been investigated as an appropriate surrogate for *Salmonella* in multiple inactivation applications including moist air and infrared heating of almonds, thermal processing of beef, and extrusion of protein meal *(9, 22, 24, 35)*. Successful applications in these and other low water activity food processes suggest that *Enterococcus faecium* may be appropriate surrogate for use in thermal inactivation of *Salmonella* on spices.

Further research is needed to evaluate inoculation strategies including biofilm-inclusion, sessile growth and liquid culture for *Salmonella enterica* and *Enterococcus faecium* on peppercorn and cumin seed surfaces. The testable hypotheses include:

1. Application of one of the above physiological states at inoculation will produce a more stable *Salmonella* inoculation on peppercorn and cumin than the other states.
2. *Enterococcus faecium* can be adapted for shelf stable inoculation on peppercorn and cumin in a similar manner to *Salmonella enterica*.

The null hypotheses include:

1. Inoculum preparation will have no effect on *Salmonella* stability on peppercorn and cumin.
2. *Enterococcus faecium* cannot be adapted for shelf stable inoculation on peppercorn and cumin in a similar manner to *Salmonella enterica.*

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CHAPTER 2: LITERATURE REVIEW

Salmonellosis

The CDC estimates that salmonellosis accounts for approximately 1.2 million cases of food-borne illness contracted in the United States annually and was the most frequently reported bacteria-induced foodborne infection identified by FoodNet in 2012 *(7, 8)*. This estimate adjusts for significant underreporting, based on evidence that reported cases of salmonellosis represent as little as 10% of actual cases *(73)*. Most cases are resolved without treatment with symptoms clearing up in 4-7 days, however, some individuals including young children (under 5 years), elderly adults (over 65 years), and pregnant women can be at higher risk for infection with more severe symptoms and even death *(8)*. The FDA reports that depending on host age and state of health, the infective dose to cause illness could be as little as 1 cell *(51)*. Salmonellosis symptoms include nausea, abdominal cramping, fever, headaches, diarrhea, and vomiting with the latter two symptoms creating complications of dehydration. Although commonly associated with chicken and egg products, salmonellosis cases have been linked to a wide variety of foods, including fruits, vegetables, nuts, cereals, and confectionary products *(17, 88)*.

Microbiological Quality and Safety of Spices

Throughout history, spices have been valued for not only for their unique flavor properties, but also for their inherent preservative attributes and antimicrobial characteristics. However in recent years, concerns have emerged about the microbiological safety of spice products, due to associations with cases of food-borne

illness. *Salmonella enterica,* the bacterial agent of human salmonellosis, has emerged as the most commonly isolated illness-inducing pathogen in contaminated spices *(98)*.

The presence of aerobic microbiota and bacterial pathogens in spices is expected. Spice products are commonly dried to reduce available water and mitigate microbial growth of bacteria, yeasts, and molds *(89)*. Dried spice products typically have a low to moderate water activity (a_w) between 0.20 and 0.60, making them unlikely substrates for bacterial growth which requires a minimum water activity of 0.87 *(39, 108)*. Although growth is limited, a variety of aerobic microbiota is readily detected on spice products, including common foodborne pathogens *such as Bacillus cereus, Clostridium perfringens,* and *Escherichia coli (86, 92)*. While serotypes of *Salmonella enterica* do not grow below a_w 0.93, the pathogen is not prevented from persisting once it has been introduced to the product *(32)*. Isolation of *Salmonella* has been confirmed in commercial spice markets in various countries around the world, including Austria, Brazil, India, Turkey, and the U.K. *(16, 52, 65, 79, 92)*.

Outbreaks of salmonellosis associated with consumption of contaminated spice products specifically have been well documented *(100, 110)*. Between 1973 and 2010, fourteen illness outbreaks were traced back to contaminated spices impacting nine different countries, with a significant portion (71%) of those reports implicating serovars of *Salmonella* as the responsible pathogen *(100)*. In Germany in 1993, more than 1000 people contracted salmonellosis after consumption of potato chips seasoned with *Salmonella*-contaminated paprika *(69)*. A 2009-2010 outbreak in the United States involving 272 cases of salmonellosis was traced to salami containing black and red pepper contaminated with *Salmonella* ser. Montevideo *(45)*. Several of the reported

outbreaks identified untreated spice ingredients, which were added after the point of the inactivation step as the root cause of illness.

In addition to significant outbreaks, notable increases in the number of recalls of spices and spiced products have been observed. Recalls of contaminated spices overseen by the FDA jumped from 2 in the 1990s to 16 recalls in the years 2000-2004. A total of 21 domestic recalls occurred from 1970 to 2003, nearly all of them citing the presence of *Salmonella* spp *(102)*. FDA sampling records for external spice shipments sent to the U.S. for importation in the years 2007-2009 indicated that 6.5% of samples (187 of 2844) tested positive for *Salmonella* contamination over the three-year window *(99)*. From 2013 to 2015, FDA oversaw fifteen recalls of dry products due to detection of *Salmonella* in products ranging from garlic, paprika, and chili powders, sunflower seeds, ground black pepper, and seed powders (carob, chia and flax) *(10)*. The rise and spread of *Salmonella* detection on spice products, together with the numerous outbreaks detailed above challenge the belief that low water activity sufficiently limits the possibility of finding food-borne pathogens in these foods.

Spices are raw agricultural commodities that may be contaminated with human pathogens during growth, harvest, and processing practices. Most spices are cultivated in tropical climates, largely in developing countries where public health infrastructure is underdeveloped. The risk of transmission of *Salmonella* and other enteric pathogens associated with human and animal fecal waste is increased due to disparities in access to clean water sources and inadequate public sanitation. Common local agricultural practices that could promote contamination with *Salmonella* include exposure to animal waste from native birds, rodents, and pests; contaminated irrigation water; soil contact

when spice berries are spread on the ground for sun-drying; and poor personal hygiene of farm workers conducting the harvest *(81)*. Growers in these regions are often unfamiliar with risk-minimizing good agriculture practices (GAPs) and good manufacturing practices (GMPs) and as a result the measures may not be practiced in these areas. While many pre-harvest contamination sources exist, the possibility of contamination of the dried product also exists through aerosols and cross contamination during storage, post-harvest transportation, and downstream processing. Prevention of these known risks in the cultivation, harvesting, and distribution pipeline outlined above is not economically feasible. Government and industry groups have identified these issues and offer limited guidance to processors *(6, 28)*. The 2013 risk profile on spices prepared by FDA details significant gaps that remain in validation data for effective post-harvesting process methods for spice applications *(98)*.

Salmonella survival at low water activity

Salmonella has the capacity to tolerate extremely low water availability, which enables adaptation for survival in a wide range of low water activity environments. *Salmonella* have been detected in a variety of low water activity food products, such as contaminated peanut butter (a_w 0.35), chocolate (a_w 0.4-0.5), nuts (0.4-0.5), and infant cereal (a_w 0.35-0.41) *(21, 40, 91, 93, 100)*. *Salmonella* also have the ability to persist on contact surfaces in food processing environments, including stainless steel, plastics (polyvinyl chloride, polystyrene, HDPE), paper, and cement *(15, 50, 55, 59, 75, 107)*. These traits become a challenge in the food processing industry, in which manipulation of product water activity is often utilized as a control measure for limiting microbial growth.

Limiting water activity in a food product provides a means to influence microbial activity independent of processing or kill steps such as applied heat, UV, or radiation technologies. Water activity as it relates to foods is defined as the measurement of the intensity of the association of available water molecules with non-aqueous components of the food product, on a scale from 0 to 1.0. This parameter is commonly used as an indicator to quantify the risk of microbial growth associated with a given food. Most bacteria require a minimum water activity of 0.87 for growth, although survival can occur at lower water activity. Water activity can also change in response to environmental conditions, such as temperature, humidity, and presence of solutes (salt or sugar) *(39)*.

Environmental persistence strategies: Biofilms

The ability to form biofilms is a common response of *Salmonella* to stress conditions. Biofilms are complex structures composed of layers of extra-cellular metabolites that result in the protective encasement of cells and attachment to environmental surfaces. *Salmonella* and some other Gram-negative bacteria excrete cellulose exopolysaccharides promoting adherence to a physical structure and to other bacteria *(104, 109)*. Characteristics of biofilm formation, chiefly presence of curli fimbriae used for initial adherence and cellulose production, have been described in the majority of *Salmonella* strains exhibiting high survivability to low water activity *(41)*. Desiccation tolerance has been directly linked to cellulose and O-antigen production *(44, 104),* both of which are primary components in biofilm structures. Biofilms provide many protective traits and are likely to be the most commonly occurring form that cells take in the surrounding environment.

Biofilm composition is dominated by exopolysaccharide compounds, primarily cellulose, which contribute to cell-cell interactions that help create the sticky texture needed for surface adhesion *(104, 109)*. Although biofilm matrix composition varies in response to the location of the biofilm attachment, cellulose is found to be crucial in a majority of biofilms regardless of surrounding environment *(94)*. Previous studies have investigated common traits found among biofilm-forming food-borne *Salmonella* strains, focusing on desiccation and low-water activity adapted strains. The presence of cellulose production, along with curli fimbrae production, was confirmed in a majority of strains tested *(41)*. Based on prior work in this lab, it is expected that inclusion of the enzyme cellulase in peptone recovery buffers during post-inoculation sample preparation will aid in breakage of the cellulose bonds and encourage release of cells for more effective biofilm enumeration *(12)*.

Biofilms also have a significant proteinaceous component, the most dominant protein being identified as Bap A *(68)*. Bap A protein is a secondary component of biofilm matrices that plays a significant role in aggregation of cells to form the biofilm complex *(31)*. It has been suggested that BapA works with the fimbrae structures to create long distance interactions between bacterial cells or clumps to allow clustering and overall film buildup to occur *(68)*.

A third crucial component to the biofilm matrix is O-antigen (O-Ag) capsule. O-Ag is believed to contribute to desiccation resistance specifically, due to links to desiccation-triggered expression of stress response genes *(44)*. The presence of these unique compounds in biofilm matrices serves to provide additional protections to biofilm cells that the planktonic and sessile counterparts have not adapted.

Biofilm structural composition can vary depending on the microbial community that produces the biofilm. The biofilm-associated compounds linked with desiccation tolerance in *Salmonella* (curli fimbriae, cellulose and O-Ag) also promote adherence to surfaces and to other bacteria *(41, 44, 104, 109)*. It is expected that a single biofilm would include a broad variety of microbial species in symbiotic relationships. It is likely that the biofilms in this study were complex bacterial communities, consisting not only of the strains used for inoculation, but also members of the native microbiota.

Encasement within biofilms increases bacterial resistance to various environmental stresses and can impart multiple protective benefits to the cell. Improved survival has been reported for biofilm-encapsulated cells under starvation conditions, which induce increased expression of multiple genes associated with biofilm production in *Salmonella enterica* ser. Enteritidis and Typhimurium *(34, 54, 104)* suggesting that these surroundings in fact trigger biofilm formation as a stress response for the bacterial cell. Evidence also suggests that biofilm formation protects the cells from the effects of desiccation *(12, 44, 47, 54, 104)*. Furthermore, changes induced by desiccation exposure appear to provide additional resistance, known as cross-resistance, to other stressors, such as heat and ethanol *(11, 48)*. Physiological state at time of inoculation, specifically biofilms, has been reported to influence the *Salmonella* Tennessee recovery after desiccation and storage in dry milk powder *(12)*.

Biofilms also provide a level of protection against heat and moderate acid exposures *(14, 67, 105)*. Biofilm cells are more tolerant to acid exposure than planktonic equivalents, both in vitro and when attached to plant tissues *(67, 105)*. Cells in a biofilm are more resistant to the effects of high temperature steam exposure, compared to liquid

grown equivalents which are killed by steam contact with shorter treatment durations and lower temperatures *(14, 77, 87)*. Biocide resistance is associated with the biofilm state, which creates significant challenges in eradication *(29, 40, 59)*. There is evidence of lesser efficacy for antimicrobials and standard cleaning methods against *Salmonella enterica* in biofilm in presence of antimicrobials *(29, 59, 83)*. Persistence within a biofilm likely provides a mechanism for dispersal to new environments *(25)*. All these traits in biofilm cells collectively produce physiologically resilient cells, which supports the needs for further focused testing of inactivation methods which target these types of cells specifically.

Considerations for strain selection and inoculation procedures for process validation

Process validation is necessary to ensure the successful, reliable inactivation of any pathogenic organism within a specific environment or food matrix. The Codex Alimentarius defines validation as "obtaining evidence that a control measure, if properly implemented, is capable of controlling the hazard to a specified outcome." *(5)*. In order for a process to be truly reliable in inactivation of a specific hazardous organism, initial validations must be conducted with representative organisms. Careful consideration should be given to strain selection due to variances in behavior and response to stress conditions. Inoculation method should also be considered due to impacts of physiological differences in cells resulting from variations in culture methods and growing media.

Serovar behavior is an important factor to consider in strain selection. *Salmonella* serovars exhibit varied responses to environmental and process conditions and no two serovars are guaranteed to behave in the same manner *(3, 72, 88, 101)*. Farakos et al. developed predictive models to quantify which factors have the most impact on

Salmonella survival in low water activity foods ($a_w < 0.7$). Using a Weibull model, analysis was conducted based on 1,064 data points selected from major reviews of *Salmonella* survival literature. The model showed that *Salmonella* serotype, along with temperature, water activity, and growth medium, has a significant impact on *Salmonella* survival in various product types and inactivation methods *(38)*.

Some types of *Salmonella* are more resistant to heat inactivation than others when heated under similar conditions. *Salmonella* serovar Tennessee required longer heating at 90 °C (120 minutes) for 7-log reduction compared to a cocktail of serovars Enteritidis, Heidelberg, and Typhimurium (86 minutes) in peanut butter *(72)*. *Salmonella* Alachua showed greater resistance to heat (larger mean D_{56} values) than *Salmonella* Typhimurium when challenged in milk with varying levels of milk solids *(33)*. In spray-inoculated corn flour, *Salmonella* Tennessee and Thompson were more resistant to inactivation by dry heating at 49 °C than six other serovars including Newington, Typhimurium, Anatum, Kentucky, Cubana, and Senftenberg *(101)*. However, heat resistance is not always predictable based on serotype alone. Ma et al. showed that *Salmonella* serovar Tennessee associated with an outbreak in contaminated peanut butter was more heat resistant than clinical isolates of the same strain. The outbreak-associated strain required 120 minutes of heating at 90 °C for 7-log reduction compared to 55 minutes *(72)*.

Serovars also exhibit variations in response to desiccation exposure. Cells of *Salmonella* Enteritidis, Senftenberg, Mbandaka, and Infantis were recoverable within 1 log of initial inoculation levels at 4 hours after inoculation and drying onto dry poultry feed (a_w 0.6), whereas, serovars Kentucky and Typhimurium 14028 exhibited a 3 log decrease in the same time frame *(2)*. Gruzdev et al compared the effects of desiccation on

Salmonella tolerance to multiple chemical and physical stresses, including exposures to bile, salt, ethanol, hydrogen peroxide, sodium hypochlorite, UV and citric acid *(48)*. Five serovars were examined (Enteritidis, Hadar, Typhimurium, Newportm and Infantis) and tolerance to each stressor was statistically compared based on log reductions after exposure. Significant differences in log reductions among serovars after exposure were found in response to each stressor. These differences in behavior may be explained by evidence that desiccation causes variations in regulation and expression of stress response genes among serovars *(70)*.

Due to the variation in *Salmonella* survival, heat resistance and desiccation tolerance among individual strains within the species, strains which have been isolated from dry spices and low-moisture foods and/or prior outbreaks in these food models are the most appropriate model organisms for study. The *Salmonella* serovar Tennessee strain in use during this research was isolated from a major outbreak traced to contaminated peanut butter (a_w 0.7) lasting from August 2006 to July 2007 in which 715 salmonellosis cases were in 48 states *(93)*. *Salmonella* serovar Ball samples provided by FDA were isolated from black pepper and have been associated with the spice in the EU *(9, 99)*. *Salmonella* Johannesburg cultures were provided by FDA after isolation from ginger *(98)*. It is expected that these strains would closely mimic those which might adapt for survival on low water activity products and would provide representative inactivation data that will be relevant to real-world manufacturing processes.

Inoculation strategies

Inoculation strategies need careful consideration in order to design appropriate inoculation methods that mimic actual contamination vectors and conditions. It is

hypothesized that the physiological state of the bacterial cell at the time of inoculation onto the product is related to resilience of the pathogen. Low water activity adaptable strains of *Salmonella* have been detected in major outbreaks arising from low water activity foods, such as peanut butter, chocolate, cereal, and infant formulas *(88)*. The ability of *Salmonella* to form biofilms helps to protect the organisms from the detrimental effects of desiccation and likely enhances survival at lower water activity levels than previously expected by the food industry *(12)*. Persistence may also be enhanced when cells are cultured in a reduced moisture environment, such as an agar plate as compared to in a liquid environment such as a broth culture *(49)*.

Dry Inoculations

Storage, transport and processing of spice products typically occurs under very dry conditions with the intent to minimize moisture in the spices. Potential sources of cross contamination occurring in these situations are likely to take non-liquid form. Dry transfers are a probable source of cross-contamination of spices in industry, which suggests that wet inoculation practices are impractical for spice validation work. *Salmonella* cross-contamination of foods and equipment during processing can be attributed to dry transfer from particles in the air, other contaminated equipment, and pests *(17, 36)*. Development of dry transfer inoculation methods may allow replication of true vectors of contamination and minimize the effects created by introducing moisture. Understanding how dry transfer inoculation influences recoverability could reveal a more realistic representation of *Salmonella* survival on spices under conditions in the processing and distribution pipeline.

A dry transfer inoculation method could also provide a beneficial alternative to negative impacts that liquid introduction may have on spice flavor and microbiological quality. Introduction of liquid to the spice surface can create unintentional changes to the spice product, such as manipulation of surface properties and water activity *(103)*. Several studies note that increasing substrate water activity by introducing liquid allows for increased aerobic bacterial and fungal growth *(46, 74, 103)*. Palipane and Driscoll suggested that water activity manipulation cannot always be reversed, citing dry products that had been introduced to water and re-dried did not exhibit the same water activity as the original unwetted product *(85)*. Liquid exposure also creates the potential for changing or removal of functional compounds. Volatile flavor and aroma compounds are more concentrated in dry spices than their fresh counterparts, lending to the pungency of dried spice profiles *(4)*. These compounds are susceptible to adulteration or leaching by liquid exposure, which contributes to dulling of organoleptic properties. Antimicrobial compounds could also be leached out, creating an artificially high presence on spice surface and unintentionally impacting surface microbiota *(81)*.

Inoculation via transfer from a dry carrier has been achieved in a variety of applications, including inoculated chalk on pecans, inoculated talc in peanut paste, and inoculated sand on walnuts and almonds *(19, 22, 37)*. A *Salmonella* cocktail of serovars Anatum, Enteritidis, Oranienburg, Sundsvall, and Tennessee inoculated onto chalk was successfully transferred onto pecans at an initial recovery of 8.28 log CFU/g *(18)*. Dry inoculated cells transferred by chalk were more resistant to subsequent inactivation by hot oil roasting than wet inoculated cells. *Salmonella* Typhimurium inoculated into chalk can be readily recovered 6 months after inoculation *(56)*. However, chalk may not be

suitable for use with spices due to difficulties with separation and collection of chalk residues after inoculation *(19)*. *Salmonella* ser. Tennessee inoculated onto talc ($a_w < 0.55$) at a rate ≥ 8.0 log CFU/g survived with little change in recovery after 30 days *(37)*. Blessington et al demonstrated that *Salmonella* ser. Enteritidis that was previously dried on sand particles could be successfully transferred to nut surfaces at levels between 4.2-5.1 log CFU/g for almonds and 4.7-5.2 log CFU/g for walnuts. Inoculum transferred by this method survived and was recoverable after 98 days in ambient storage. *Salmonella* survival of dry inoculated cells was comparable to that of wet inoculated cells during the storage period *(22)*.

Wet Inoculations

Laboratory-scale inoculation methods traditionally involve wet application techniques for transferring inoculum to food systems, in part because liquid inocula are high yielding and easy to work with. However, research shows that the mode of growth (liquid vs. biofilm inclusion vs. agar) impacts the resilience and recoverability of inoculum cells *(12, 37, 49, 62, 97)*. *Salmonella* Enteritidis phage type 30 cells inoculated onto almonds were more easily recovered after drying when grown on tryptic soy agar media than in trypic soy broth prior to inoculation suggesting superior adaptation to drying *(97)*. Similarly, *Salmonella enterica* (cocktail) that was dried on polystyrene 96-well plates had higher survival rates over 12 weeks when inoculum was grown on LB agar as compared to broth-grown cultures *(49)*. *Salmonella* sers. Tennessee and Oranienburg cultured on agar media prior to inoculation showed improved survival rates and increased thermal tolerance compared to broth-grown equivalents when inoculated into peanut butter *(62)*. Biofilm inclusion before inoculation can also improve survival of

Salmonella in some conditions as compared to growing cells in liquid media. *Salmonella* Tennessee grown in a biofilm state had higher recoveries after desiccation in dried milk powder and gastric passage as compared to cells grown in broth *(12)*. It is vital to verify any differences in survival and stability on spices imparted to *Salmonella* by growth medium and to consider how these techniques might impact inactivation resistance in subsequent processing methods. The following research is intended to provide a unique and valuable direct comparison of all the above methods within uniform environmental conditions and substrates.

Surrogate microorganisms

Surrogate organisms provide a practical alternative when working with the organism of interest is unpractical or unsafe *(80)*. Non-pathogenic surrogates are often preferred by processers when conducting inactivation processes in a commercial setting. An ideal surrogate organism should be non-pathogenic and mimic the behavior of the organism of interest in the specific process scenario, responding in a similar manner to temperature, chemical, and environmental controls *(53, 80)*. Common applications of surrogate organisms include heat-stable organisms serving as biological indicators for routine inactivation. One example is in the form of a convenient device for inclusion within laboratory autoclaves to confirm proper conditions were met to cause inactivation. Multiple U.S. patents exist surrounding commercially available biological indicators utilizing multiple heat-stable microorganisms in this capacity, including *Geobacillus stearothermophilus* and *Bacillus subtilis (30)*. Organisms within the same genus can make for advantageous surrogates for food borne pathogens by possessing similar genetic make up and physical structure to target organisms, such as in the case of non-pathogenic

Listeria innocua or *Clostridium sporogenes* strains substituting for *Listeria monocytogenes* or *Clostridium botulinum* respectively *(26, 60)*.

In some instances, it may be too difficult to replicate necessary time and temperature profiles outside the manufacturing setting, i.e., in a laboratory. Processors in industry must also be sure to avoid exposure of the processing facility, equipment, and operating personnel to pathogens that could inadvertently contaminate commercial products *(53)*. Substitution of a surrogate allows processors to validate actual processes on specific equipment and under the true process conditions with no risk of pathogen introduction.

Industry has many examples of surrogate organisms validated to adequately predict pathogen kill in thermal and non-thermal inactivation processes. Non-pathogenic *E. coli* strains are common alternative organisms in both the food and pharmaceutical industries, with applications including electron beam radiation, fermentation, and UV treatments *(82, 84, 90)*. Non-pathogenic *E. coli* K-12 MG1665 is suggested as a surrogate organism for multiple pathogens in electron beam radiation processes. Strain survival was compared against pathogens *E. coli* O157:H7, *L. monocytogenes*, and *Salmonella* Poona in a model food system (10% w/w gelatin) after irradiation in a 2 MeV Van der Graaf linear accelerator with radiation exposure up to 1.2 kGy. K-12 MG1665 was more resistant to radiation in the model food than *E. coli* O157:H7 and *Salmonella* (D_{10} = 0.36 kGy, 0.38 kGy, respectively) and statistically similar to *L. monocytogenes* (D_{10} = 1.09 kGy) with a D_{10} value = 0.88 kGy *(90)*. Various non-pathogenic *E. coli* strains can predict *Salmonella* survival through beef carcass cleaning and downstream fermentation of the tissue in mixed sausage *(82)*. Five non-pathogenic *E. coli* isolates and five *S. enterica*

strains (four Typhimurium and one Heidelberg) were inoculated onto beef and prepared in a summer sausage fermentation by incubation at 37.8 °C until pH ≤ 4.8 (~12 hrs). *E. coli* log reductions were statistically lower after fermentation (3 to 3.5 log CFU/g) compared to the *Salmonella* strains (5 log CFU/g reductions). The non-pathogenic *E. coli* strains had statistically similar survival to *Salmonella* through initial cleaning (repeated water, acid, and chlorine washes) of beef carcasses as well. During UV light processing of apple juice, non-pathogenic *E. coli* approximates inactivation of O157:H7 and non-O157:H7 STEC pathogenic clinical isolates *(84)*. Apple juice (pH = 3.5) was inoculated with five pathogenic STEC isolates, including an O157:H7 variant, and seven non-pathogenic *E. coli* before exposure to three wavelengths of UV (wavelength = 222, 254, and 282 nm) for varying durations corresponding to wavelength. Although inactivation of specific strains varied among wavelengths, at least one non-pathogenic *E. coli* strain was more resistant to UV than the most resistant STEC strain at each of the three wavelengths tested.

Other examples of non-*E. coli* organisms used to predict inactivation of *Salmonella enterica* include lactic acid bacteria and other native microbiota isolated from a target food product. *Pediococcus acidilactici*, a lactic acid bacteria, exhibited higher D values than a seven strain cocktail of *Salmonella enterica* in dry pet food thermal processing *(27)*. Freeze-dried cultures were ground to a fine powder and inoculated into dry pet food. Moisture levels were adjusted to 10%, 20%, or 30% and samples heated to 65.6 °C, 71.7 °C, 76.7 °C, and 82.2 °C (dependent on target moisture level) in a circulating water bath. By comparing D values after processing, *P. acidilactici* was more resistant to heating than the *Salmonella* cocktail at all three moisture levels (up to 1.75x,

5.1x and 6.5x respectively). In the application of thermal processing on beef jerky, lactic acid bacteria successfully predict adequate 5 log reduction of *Salmonella enterica* strains in varied thermal treatments *(23)*. Survival of two commercially available cultures of *Pediococcus* (one cocktail and one single strain, *acidilactici*) was compared against five strain cocktails of both *Salmonella* and *E. coli* during six different process schedules for beef jerky preparation, one in a small scale commercial dehydrator and five in a commercial grade smokehouse. Schedules varied in step time (15-420 min), cumulative time (30-420 min), presence of a smoking step, and set dry bulb (125-185°F) and wet bulb (125-161°F or not controlled) temperatures. Both *Pediococcus* cultures exhibited fewer log reductions than *Salmonella* in each of the six process schedules, supporting use as a surrogate in a variety of commercial jerky processes. *Hafnia alvei*, a native microorganism isolated from mushrooms, can predict inactivation of *S. enterica, E. coli, and L. monocytogenes* in chlorine dioxide (ClO_2) gas fumigation of fresh produce *(63)*. A study screened for resistance to ClO_2 treatment among a large group of pathogenic strains including, seven *E. coli*, three *L. monocytogenes*, and nine *S. enterica* strains. The most resistant strains were inoculated onto polystyrene plates and exposed to 0.3 mg/l gaseous ClO2 for between 0, 0.5, 1, 3, 5, and 10 minutes. *H. alvei* was significantly more resistant (D = 3.53 ± 0.95 min) than the next most resistant strain, *E. coli* O157:H7 C7927 (D = 1.95 ± 0.13 min) allowing inactivation of that strain to sufficiently predict kill of the pathogens investigated.

Enterococcus faecium as *Salmonella* surrogate

Enterococcus faecium has been suggested as an appropriate heat-stable surrogate for *Salmonella* in multiple food processes, specifically for low water activity foods such as

peanut paste, almonds, beef jerky, and extruded protein meal products *(20, 24, 58, 61, 95)*. *Enterococcus faecium* is an attractive surrogate candidate due to its ease of cultivation and quantification, simplicity of application, and confirmed non-pathogenicity. It is easily isolated from a variety of dairy-based foods and found within mammalian digestive tracts. Like *Salmonella*, *Enterococci* are able to grow within a wide temperature (5-50 °C) and pH (4.6-9.9) range and are tolerant of high salt and desiccated environments *(42, 64)*. Past concerns over an increase in hospital acquired enterococcal infections led to a 2010 study comparing the genome of *E. faecium* strain NRRL B-2354 and 125 other strains of *E. faecium* isolated from a mixture of innocuous and clinical sources. The study showed that B-2354 was more closely related to non-pathogenic strains than those that were known to cause clinical infections, stating that the strain lacked important virulence factors and could be controlled by common antibiotics. This strain, which was chosen for the following study, is considered commensal and non-pathogenic as compared to emerging nosocomial variants of the species *(53, 66)*.

Members of the *Enterococcus* family have shown the ability to incorporate into a biofilm *(13, 76)*. This characteristic is supported by the presence of physiological components that support biofilm formation, such as *Esp*, enterococcal surface protein, and the formation of pili which aid in attachment *(43)*. Integration of *Entercoccus faecium* into oral biofilms upon introduction is known to occur within 3-5 days of inoculation *(1)*. Inclusion into biofilms aids *Enterococcus* species survival in a variety of environments, such as oral cavities, gastrointestinal tissues, and polystyrene *(13, 35, 96)*. There is also evidence to suggest that biofilm inclusion helps sustain colonization by *Enterococcus* on the surfaces of implantable medical devices *(78)*, which contribute to

human nosocomial infections. Studies suggest that some enterococcal strains may be able to carry out biofilm production. Biofilm forming capacity of thirteen *Enterococcus faecium* strains on polystyrene plates confirmed biofilm formation in eleven of strains investigated *(78)*. Temperature and concentration of solutes such as glucose also impact enterococcal biofilm formation *(76)*. While it is not clear whether NRRL B-2354 has the ability to produce biofilms, it is likely that the strain is able to incorporate within them and gain protection as a means of survival.

Enterococcus faecium is likely adaptable for application to spices based on multiple examples of conservative surrogacy for *Salmonella* in other low water activity food processes and products. Suggested adaptations of use as a surrogate span multiple food types, including lean ground beef, peanut paste, almonds, extruded protein meal, and dry pet food *(20, 27, 37, 71, 106)*. Validated studies also touch on varied forms of heat inactivation, specifically dry heating, moist air heating, circulating water bath heating, and extrusion. In inoculated model dry pet food, NRRL 2354 exhibited larger D-values than a seven serovar cocktail of *Salmonella enterica* strains during heating to 76 and 87 C in a circulating water bath *(27)*. Similar results were observed when NRRL B-2354 was substituted for *Salmonella* ser. Enteritidis phage type 30 during moist air heating of almonds. Log reductions in recoverable NRRL-B2354 were lower than *Salmonella* recoveries at a majority of temperature (121, 149, 177, 204 °C) and humidity (0, 30, 50, 70, 90 %) combinations tested *(58)*. After inoculation onto a dry carrier (talc) and incorporation into a model peanut paste, *Enteroccocus faecium* demonstrated greater heat resistance compared to *Salmonella* ser. Tennessee (in the form of higher D_{85} °C values) for up to 30 days after inoculation *(37)*. The body of research suggests that *Enterococcus*

behaves similarly to *Salmonella* in a range of substrates and conditions and thus has the potential to be a conservative surrogate for multiple heat treatment techniques of interest for pathogen inactivation in spices.

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CHAPTER 3: INOCULATION PREPARATION EFFECTS SURVIVAL OF *SALMONELLA ENTERICA* ON WHOLE BLACK PEPPERCORN AND CUMIN SEEDS STORED AT LOW WATER ACTIVITY

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Abstract

Salmonellosis is increasingly attributed to contaminated spices. Identifying inoculation and stabilization methods for *Salmonella* on whole spices is important for future research on the development of validated inactivation processes. The study objective was to examine effects of inoculation preparation on the recoverability of *Salmonella enterica* from dried whole peppercorns and cumin seed. Whole black peppercorns and cumin seeds were inoculated with *Salmonella enterica* using different wet inoculation methods (24h growth in tryptic soy broth with the seeds likely leading to inclusion within native microbiota biofilms formed around the spice, application of TSB-grown cells and/or cells scraped from tryptic soy agar), that were followed by drying, and one dry transfer method. Post inoculation spices were dried to a_w 0.3 within 24h and held for 28 days. Spices were sampled after drying (time 0), and periodically during 28 days of storage. Cells were enumerated by serial dilution and plated onto XLT-4 and TSA. Recovery of *Salmonella* was high after 28 days storage but was dependent on inoculation method, with ranges of 4.05 to 6.22 log CFU/g and 3.75 to 8.38 log CFU/g recovered from peppercorns and cumin, respectively on XLT4. The changes in survival from initial inoculation levels (LR) after 28 days were significantly smaller for the biofilm-inclusion ($+0.142_{pepper}$, $+0.186_{cumin}$ LR) compared to other inoculation methods (-0.425_{pepper}, -2.029_{cumin} LR for TSA-grown; -0.641_{pepper}, -0.718_{cumin} LR for dry transfer; -1.998_{pepper} LR for TSB grown). In most cases, trends in total aerobic LR were similar to those of *Salmonella*. The inoculation method influenced the recoverability of *Salmonella* from whole peppercorns and cumin after drying. The most stable inoculum strategies were dry transfer, 24h incubation of *Salmonella* and spices in TSB (potential inclusion within

native microbiota biofilms) or using TSA- plate grown cells subsequent to drying, however it was difficult to achieve the large inoculum needed for inactivation studies using the dry transfer method.

Introduction

Spices have long been known to harbor bacteria, yeasts, and molds whose presence were considered to be a product quality issue rather than a safety issue *(34)*. Dried spice products are very low in moisture and water activity (a_w 0.20 to 0.60) *(13)* and well below the threshold water activity that supports microbial growth *(10)*. While serotypes of *Salmonella enterica* do not grow below a_w 0.93, salmonellosis outbreaks have been attributed to contaminated peanut butter (a_w 0.35), chocolate (a_w 0.4-0.5), infant cereal (a_w 0.35-0.41), and a variety of spice products *(14, 35, 38, 40)*. These outbreaks and an increasing frequency of recalls associated with *Salmonella* detection *(41)* indicate that the intrinsic low water activity of these products is not sufficient to prevent transmission of food-borne pathogens.

Understanding the survival of *Salmonella* over time on spice surfaces is crucial due to the vulnerability of spice products, which are raw agricultural commodities, to contamination during cultivation, harvest, and storage. Spices often originate from tropical climates, many of which are in developing countries where a lack of clean water sources and inadequate public sanitation contribute to an increased risk for transmission of pathogens. Typical cultivation and harvest practices that could lead to contamination with *Salmonella* include contact with animal waste from pests and birds, soil contact during sun-drying of spice berries on the ground, and poor personal hygiene of laborers involved in hand-picking and separation of berries and stalks by foot pressing *(28)*. Preventing all sources of contamination is impractical, therefore it is important to improve detection and validate methods for inactivation of pathogens on spices.

An important consideration for inactivation studies should be the methods used to inoculate the product before processing. Laboratory inoculations of low moisture foods

commonly involve suspension of the pathogen within liquid, followed by a drying process *(6)*. This procedure may be problematic for some dry ingredients (nuts, spices, powders) because the texture and water activity of the product after wetting and drying is not equivalent to the original product *(30)*. Dry transfer from a contaminated carrier has been described for nuts *(4, 7)*, ready to eat meats *(11)* and poultry feed *(21)*. Implementation of dry inoculation procedures for spices would also prevent release of water-soluble antimicrobials *(43)*, which may artificially reduce microbial numbers in the absence of processing. In addition to the method of inoculation, the media used and physiological state of the cells during preparation prior to inoculation should also be considered.

Improved *Salmonella* survival in a variety of environmental conditions, including desiccation, starvation, and acidity, has been reported for agar-grown cells and cells within biofilms compared to planktonic cells *(19, 20)*. Thermal destruction curves of agar-grown cells of *Salmonella* serotypes Tennessee and Oranienburg exhibited greater linearity compared to planktonic, liquid-cultured cells, however overall thermal resistance in product was similar *(23)*. Biofilms improve the resistance of *Salmonella* to a variety of conditions including heat *(2)*, presence of antimicrobials *(9, 29)*, moderate acid exposures *(45)*, and desiccation *(1)*. Physiological state at time of inoculation has been reported to influence the *Salmonella* Tennessee recovery after desiccation and storage in dry milk powder *(1)*.

The objective of this study was to compare the effects of inoculation preparation on the recoverability and stability of serotypes *Salmonella enterica* on whole black peppercorn and cumin seeds stored for 28 days of dry storage (a_w 0.3). Recoverability

was compared for *Salmonella* inoculated using 3 different wet inoculation methods (tryptic soy broth grown cells, tryptic soy agar grown cells, or 24h incubation of *Salmonella* in TSB with whole spices, likely resulting in inclusion within native microbiota biofilms formed around the spice), which were followed by drying, and one dry inoculation method.

Materials and Methods

Bacterial strains and growth conditions. Three *Salmonella enterica* serovars isolated from low water activity foods (Tennessee K4643, 2010, ConAgra peanut butter; Ball ARL-SE-085, 2011, Black pepper; and Johannesburg aRL-SE-013, 2010, dried ginger) were used to inoculate whole black peppercorns and whole cumin seed. Cultures from -80 °C freezer stocks were streaked onto Tryptic Soy Agar (Becton Dickinson, Franklin Lakes, NJ) and incubated for 24 h at 37°C to obtain isolated colonies. An isolated colony was transferred to XLT4 agar (Becton Dickinson, Franklin Lakes, NJ) and incubated for 24 h at 37°C. Single colonies from XLT4 were transferred to Tryptic Soy Broth (TSB, Becton Dickson) and incubated with shaking (180 rpm) for 24 h at 37°C. Cells were washed three times in 10 ml 0.1% (wt/vol) peptone (Sigma-Aldrich, Co., MO) with 0.1% Tween 80 (PW-Tween, Fisher Scientific, Kansas City, MO) to remove excess nutrients and spent media. Peppercorns were inoculated by using pure cultures of *Salmonella* Tennessee, and cumin seeds were inoculated with a cocktail of all three strains combined.

Spice varieties and sources. Whole peppercorns and whole cumin seeds were obtained from a major national spice processor. No additional treatments were applied after receipt of spices to remove background microbiota before inoculation.

Inoculation methods. The effects of inoculation method on survival of *Salmonella* over a 28 day period were compared for whole peppercorns and cumin seeds individually. Spice samples (10g, duplicate samples per time point) prepared using each of the inoculation methods were destructively sampled at 1, 7, 14, 21 and 28d post-inoculation. The inoculation strategies varied as described below. For each inoculation method enough inoculated spice was prepared to allow for enumeration of two separate 10g subsamples for each of the time points. In the case of all wet methods, the inoculated spices were spread out in a single layer on sanitized aluminum trays (46 cm. x 66 cm.) and dried for 24 h at room temperature (23-25 °C) in a biological safety cabinet (final a_w = 0.3). After drying the spices were held in a desiccator (RH 43-45%) at room temperature (23-25 °C) until sampling.

TSB-grown *Salmonella* wet inoculation. Planktonic (TSB-grown) cells were cultured as described above and applied directly to the peppercorn or cumin seed surface. Washed cells suspended in 20 ml sterile PT buffer were applied to 50 g of dry whole seeds within 27 oz Whirl-pak bags and massaged by hand for 1 minute to evenly coat the seed kernels. To prepare enough inoculated spices per experiment two 50g bags per spice were prepared and all spices combined into one batch before enumeration. This was performed for two biological/experimental replicates per spice.

TSA-grown cell wet inoculation. Overnight cultures of *Salmonella* were plated onto 150 x 15 mm (BD Falcon, Franklin Lakes, NJ) TSA plates and incubated for 24 h at 37 °C to cultivate a lawn of bacteria. Cells were scraped from the agar surface using a sterile cotton-tipped swab and suspended in 9 ml of PT buffer. Scraped cells were washed twice in sterile PT buffer, re-suspended in 20 ml of PT buffer and mixed by pipetting to

break up cell clumps. The washed cell suspension (20 ml) was applied to 50 g of dry whole seeds in 27 oz Whirl-pak bags and massaged by hand for 1 minute to evenly coat the seeds. To prepare enough inoculated spices per experiment two 50g bags per spice were prepared and all spices combined into one batch before enumeration. This was performed for two biological/experimental replicates per spice

24h incubation of *Salmonella* and seeds in TSB (Biofilm inoculation). This methodology was adapted from the method used by Aviles et al. 2013 *(1)* to form biofilms around silica beads, substituting whole seed spices. Briefly, *Salmonella* was incubated in TSB (1 cm depth) containing dry whole peppercorns (62 g) or cumin seeds (25 g) arranged in a single layer on the bottom of a 2L Erylenmeyer flask. The *Salmonella*-seed mixture was incubated statically for 24 h at 37 °C. After 24 h, liquid media was decanted and seeds were washed by vigorously swirling for 30 s in PT buffer to remove non-adherent cells and nutrients from the seed surface. To prepare enough inoculated spices for the run, two flasks were used for peppercorns and four flasks for cumin seeds 50g bags per spice were prepared and all spices combined into one batch before enumeration. This was performed for two biological/experimental replicates per spice. Wet mode environmental scanning electron microscopy was performed on inoculated and non-inoculated peppercorns to visualize biofilms. Peppercorns were stained according to the ruthenium red method described by Priester et al., 2007 *(31)* and visualized at 68% humidity, the pressure set to 4Torr, 5°C and an accelerating voltage of 10KV using a FEI Quanta 600 FEG environmental scanning electron microscope at the Virginia Tech ICTAS Nanoscale Characterization and Fabrication Laboratory.

Dry transfer inoculation. Dry transfer inoculation of *Salmonella* to seeds via contact transfer from inoculated silica sand carrier was adapted from the method of Blessington et al. 2013 *(7)*. Briefly, a concentrated inoculum (10 log CFU/ml) was prepared as described for the TSA-grown wet inoculation method. After collection, 3 ml of the resuspended inoculum was applied to dry silica sand (40-100 mesh, Acros Organics, New Jersey, USA) in 20 g batches achieving an average inoculum of 7.8 log CFU/g on the sand. The *Salmonella* inoculum was incorporated evenly into the sand by stirring and mashing with a sanitized mortar and pestle for 1 minute. Inoculated sand was dried within a biological safety cabinet for 48 h, achieving a water activity of 0.30. After drying, all batches were combined and stored at 4 °C in a desiccator for up to 7 days until use. The average loss of *Salmonella* on sand after drying was 0.72 ± 0.18 log CFU/g.

Dry transfer to the seeds was performed by combining 25 g inoculated sand with 50 g spices in a whirl-pak bag and mixing by hand for one minute. Contents of the bag were then transferred to a sanitized 8 in. x 8 in. container, covered with aluminum foil, and shaken for 24 h on a horizontal-rotating platform (Barnstead International, Dubuque IA) to maximize surface contact between seed and sand. After 24 h, the sand and seed mixture was transferred to a sanitized sieve (U.S.A. standard No. 7 sieve, mesh size 2.8 mm, Fisherbrand, Pittsburgh, PA) and shaken by hand in a horizontal circular motion for 30 s to separate the sand particulates. To prepare enough inoculated spices per experiment two 50g bags per spice and of 25g inoculated sand were prepared and all spices combined into one batch before enumeration. This was performed for two biological/experimental replicates per spice.

Microbiological detection. Enumeration of total aerobic bacteria and *Salmonella* were performed according to the following method. Seed samples (10 g) were homogenized in 90 ml of sterile peptone tween buffer (PT, both 0.10% w/v) for 60 s within a sterile filtered bag using a lab blender (Interscience BagMixer®, Guelph, Ontario). For biofilm-inoculated spices PT with 0.2% cellulase (Sigma-Aldrich, St. Louis, MO) was used. The homogenized liquid was vacuum filtered through #4 qualitative filter paper (Whatman, GE Healthcare, Pittsburgh, PA) to remove small seed particulates that were otherwise transferred by serial dilution. The filtered homogenate was serial diluted 1:10 in sterile PT buffer. From appropriate dilutions, 100 µl was spread plated onto duplicate XLT4 and TSA plates (BD, Franklin Lakes, NJ) and were incubated for 18-24 h at 37 °C.

Water activity and measurement. Water activity of whole peppercorns (3 g) and cumin seeds (5 g) was determined using an AquaLab 4TE water activity meter (AquaLab, Pullman, WA). Peppercorns and cumin seeds were determined to have an a_w of approximately 0.3 and 0.4, respectively, prior to inoculation. The water activity of non-inoculated seeds prepared using methods as described above with strains omitted was measured periodically over 48 h to identify the length of drying time needed to return the product to its original water activity. Drying for 24 h was determined to be a sufficient period to return both non-inoculated whole spice seed and dry carrier (sand) samples to water activities comparable to the original substrates. Inoculated spices and sand were stored in glass jar desiccators (RH 39-45%) after inoculation and drying to minimize fluctuations in a_w due to changes in relative humidity during storage.

Statistical analysis. Bacterial counts were log transformed to approximate normal distribution. Two biological replicates were performed for each inoculation, and duplicate replication was used for enumeration of survival. Statistical analyses were performed using JMP statistical software (version 10, SAS, Cary, NC). The effect of inoculation method over 28 days was compared within each spice at each time point using a 1-way ANOVA to test for differences in the average log reduction of recovered CFU/g on both peppercorn and cumin. P-values < 0.05 were considered significant.

Results

Total aerobic plate count (APC). The mean total aerobic bacteria of non-inoculated spices was 8.3 log CFU/g and 6.45 log CFU/g for whole peppercorns and cumin seeds, respectively when plated on TSA. No colonies with characteristic black centers indicative of *Salmonella* appeared on XLT4 plates from non-inoculated spices.

On inoculated peppercorns, declines in APC were minimal over 28 days, with less than 1.5 log reductions for most methods (Figure 3.1). The TSB-grown method was the exception, with -2.81± 0.06 log reductions on day 14. On cumin seeds, the APC for seeds inoculated by biofilms were significantly increased compared to the other methods at each time point (Figure 3.3). Overall small reductions of less than 1.5 log CFU/g were detected.

Comparison of *Salmonella* survival by method and time. *Salmonella* recovery after 28 days of dry storage was affected by method of inoculation (Figures 3.2, 3.4). Differences in log reductions associated with each inoculation method were compared within each time point on both selective and non-selective media (XLT4 and TSA). Statistical differences in overall log reductions of recoverable *Salmonella* for at least one

method of inoculation were observed at each time point from both peppercorn and cumin seeds (Figures 3.2, 3.4).

Recovery of *Salmonella* from peppercorns inoculated using the 24h incubation of *Salmonella* and peppercorns (hereafter referred to as biofilm inoculated samples) showed nearly zero net change (-0.04 ±0.07 log CFU/g) on XLT4 after 28 days, while larger reductions were observed for samples inoculated using the dry transfer and TSA-grown methods at day 28 (Figure 3.2). There were no statistical differences in recovery between the two methods when seeds were inoculated using the TSA-grown (-0.75 ± 0.04 log CFU/g) and dry transfer (-0.74 ± 0.06 log CFU/g) methods. The greatest reductions in *Salmonella*, on average -3.56 ± 0.20 log CFU/g, were determined for the TSB-grown method, and this method was therefore omitted from inoculation testing on cumin seeds.

Similar trends between inoculation methods were observed in *Salmonella* recovered during storage of inoculated cumin seeds. *Salmonella* cells inoculated onto cumin seeds likely within biofilms had the least change (-0.28 ± 0.12 log CFU/g on XLT4) over 28 days, with slight increases at 1, 7, and 21 days rather than reductions (Figure 3.4). The greatest log reductions of *Salmonella* were observed when cumin seeds were inoculated with the TSA-grown method, with overall reductions of -2.76 ± 0.04 log CFU/g on XLT4 at 28 days. Overall, these results indicated that inoculation method influenced the long-term recoverability of *Salmonella* from whole peppercorns and cumin seeds after drying compared to the initial recovery 24h after inoculation.

Discussion

Salmonella is able to survive and has been implicated in illness after extended periods of dry storage in a variety of low water activity foods including spices *(32)*. Dried milk powder, walnuts, and ground pepper can all support the persistence of the pathogen for more than 30 days, and in some cases viability has been confirmed after one year of storage *(1, 6, 24)*. Survival of *Salmonella* in very low water activity tahini (a_w = 0.17) was documented for up to 16 weeks *(39)*. In the current study, *Salmonella* persisted in high numbers on the inoculated spices for the 28 day period of storage at a_w 0.3. Log reductions in recoverable *Salmonella* ser. Tennessee from whole peppercorns at 14 days (-2.97 ± 0.09 log CFU/g) were comparable for TSB-grown cells to that previously reported for *Salmonella* ser. Rubislaw (-2.57 log CFU/g) in ground black pepper stored at 25 °C and a_w 0.66 for 15 days *(33)*. Results were comparable only for the TSB-grown *Salmonella* inoculation method, as much smaller log reductions of *S*. Tennessee were seen for TSA-grown, biofilm and dry transfer inoculation methods.

Inoculation method, amongst other factors including growth phase, temperature, presence of glucose, trehalose, NaCl, and speed of dehydration have been reported to affect subsequent desiccation persistence of *Salmonella (19)*. Increased desiccation tolerance of agar-grown (LB agar) *Salmonella* compared to planktonic cells has been reported on polystyrene plates stored at 4 °C, 40-45% RH for over 100 weeks *(19)*. Agar-grown *Salmonella* survived dry storage better than planktonic cells, with approximately 2 logs more LB agar-grown cells recovered at 4 weeks, a comparable period to the current study *(19)*. In the current study, the TSA-grown cells were quickly washed to remove residual nutrients. It is unknown if the cells changed physiology during these washing steps. However there were survival differences between TSA-grown and TSB-grown

cells in the current study, with a 2.81 log increase in recovery of TSA-grown cells compared to TSB-grown cells on peppercorns after 28 days. Since the main purpose of this experiment was to identify stable inoculation methods for spices to be used for inactivation studies, we did not continue the evaluation of the wet broth inoculation method for cumin seeds. In contrast to the small LR for TSA-grown cells on peppercorns, 2 log CFU/g reductions were seen for cumin seeds. This may reflect differences in spice properties including smaller surface areas, chemical compositions and smaller populations of native microbiota for cumin seeds compared to whole black peppercorns.

In each of the wet inoculation methods the same media rich in glucose was used for growth, however some differences in physiological state may have been present. Other differences included the amount of liquid used for deposition of the inoculum. For the TSA and TSB grown methods small volumes of liquid were applied across a larger volume of spice, likely resulting in only surface deposition of the *Salmonella*. In contrast, immersion of the spice within TSB for 24h prior may have resulted in altered surface properties of the spice. It is unknown if *Salmonella* became internalized within the spice, or if additional nutrients beside those present in TSB were available for use. Keller *et al*. has shown that a 4-strain cocktail of *Salmonella enterica* including the same *S*. Tennessee strain used here, was able to grow when inoculated into ground pepper at water activity above 0.97 at 35°C *(24)*. In the current study, while it is possible that some metabolism of the spice surface may have occurred, it is probable that *Salmonella* were incorporated within or attached to the surface of a biofilm originating from the native microbiota of the spice during the 24h immersion in TSB. Environmental scanning microscopy of the wet peppercorns showed small coccoid and rod shape cells (~7-8 uM) encased within a

mucoid layer (Figure 3.5) after performing the 24h immersion inoculation method. The net-like structure visible is similar to that reported for other strains of *Salmonella* on inert surfaces, and the mucoid appearance of the spice is likely due to the production of exopolysaccharide and proteases produced by the bacterial biofilm communities *(46)*. While it was not possible at this time to visualize the location of *Salmonella* in the biofilm, large populations of *Salmonella* were recovered from the spices even after vigorous prior wash steps were performed to remove non-adherent cells. It is possible that some planktonic cells were present on the surface of the biofilm, which would be characteristic of typical biofilm dispersal however, previous studies have shown the majority of cells to be encased within a matrix *(47)*. One component of this matrix was cellulose, a critical component of *Salmonella* biofilms *(37),* which is evident based on the larger numbers of *Salmonella* recovered when the enzyme cellulase was added to the diluent buffer. The inclusion of cellulase may explain the small increases in *Salmonella* recovered from cumin seeds inoculated by 24h immersion method, as it would increase cell dissociation, resulting in more colonies. It is likely that the native microbiota within the biofilm secrete different polysaccharides or proteins that are potentially encasing the *Salmonella* cells as well, and that addition of other enzymes could further promote disassociation and enumeration. Alternatively, the inclusion of cellulase may aid in removal of *Salmonella* that may have been entrapped within the seed coat.

While the present study does not definitely identify *Salmonella* within the biofilm on spices, previous research identifies that encasement within biofilms increases bacterial resistance to various environmental and sanitation stresses including organic acid *(26, 45)*, desiccation *(16, 44),* and cleaning *(22)*. In this study, no reductions in log CFU/g of

Salmonella were detected after 28 days for either peppercorn and cumin seeds when the 24h immersion inoculation method was used, further supporting the role of biofilms in resistance to desiccation. Desiccation tolerance in *Salmonella* has been highly associated with production of curli fimbriae, cellulose and O-antigen production *(14, 15, 16, 44)*. These are also important components of *Salmonella* biofilms, promoting adherence to surfaces and to other bacteria *(44, 47)*.

Salmonella cross-contamination in dry environments has been traced to transfer from contaminated equipment, dust in the air, and rodents *(3, 12)*. The potential for dry vehicles transferring *Salmonella* to low water activity foods supports the need for development of dry transfer inoculation procedures to mimic real-world contamination. Wet inoculation practices are particularly problematic for spices as introduction of liquid to the spice surface may allow growth of other native aerobic bacteria and fungi *(27, 43)* requiring lengthy drying of spices after harvest *(36)*. Water activity of dry products that have been previously introduced to water and re-dried may not be the same as original *(30)*. Moisture introduction can also release antimicrobial compounds, which could interfere with the intended inoculation procedure *(17)*.

Prior examples of successful dry carrier inoculations include transfer of *Salmonella* from inoculated sand to walnuts and almonds, and from *Salmonella* inoculated chalk to pecans *(4, 7)*. Transfer from silica sand resulted in 4.2 – 5.2 log CFU/g of *Salmonella* on nut surfaces *(7)*, which is comparable to the transfer procedures to peppercorns (5.5 ±0.1 log CFU/g) but greater for cumin seeds (6.5 ± 0.06 log CFU/g). However, no further increase in transfer was achieved by altering the ratio of carrier to product and increasing contact time (results not shown). Log reductions in *Salmonella*

ser. Enteritidis after 30 days were approximately 0.5 – 1.5 log CFU/g on almond and 0.6 – 1.25 on walnuts *(7)*. Comparable reductions in recovery of *S*. Tennessee after 28 days are reported here for peppercorns and cumin seeds suggesting that dry transfer of *Salmonella* from a sand carrier could be a useful inoculation method for whole spice applications where the carrier particles could be removed.

Prior exposure to low water activity, including desiccation, improves the survival of *Salmonella* spp. when exposed to multiple stressors, including those routinely experienced in food processing environments. Desiccated *S. enterica* serotypes Enteritidis, Hadar, Infantis and Typhimurium exhibit enhanced tolerance to chemical disinfectants, dry heat and UV irradiation *(18)*. Tolerance of *S*. Enteritidis to heat and hypochlorite stress is increased for cells grown at reduced water activities (a_w 0.94) *(25)*. Increased process times and/or elevated temperatures are necessary to kill *Salmonella* in a variety of low water activity products, including almond kernels, raw hide, and alfalfa seeds *(5, 8, 42)*. Future studies should consider cross-protection, physiological state of cells and inoculation methods when designing validation studies to assure inactivation of the most resistant *Salmonella* spp.

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Figures

Figure 3.1

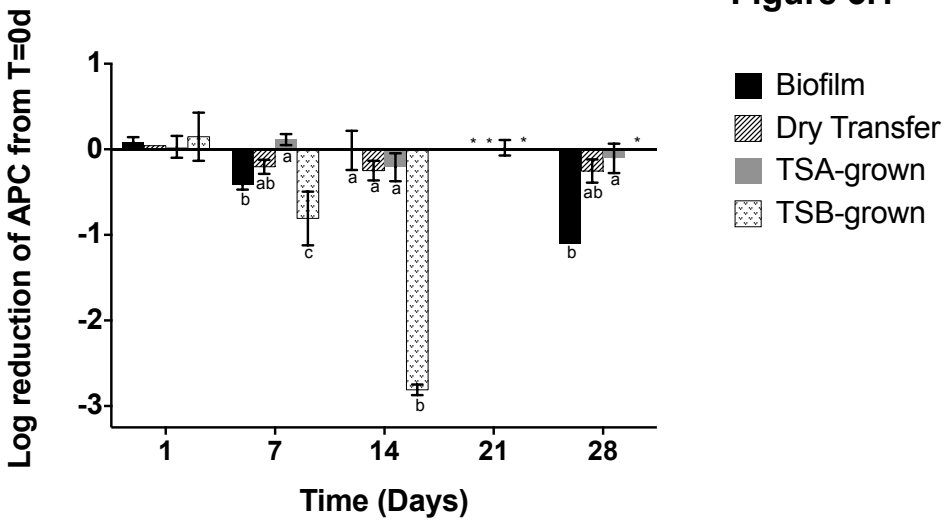


Figure 3.2

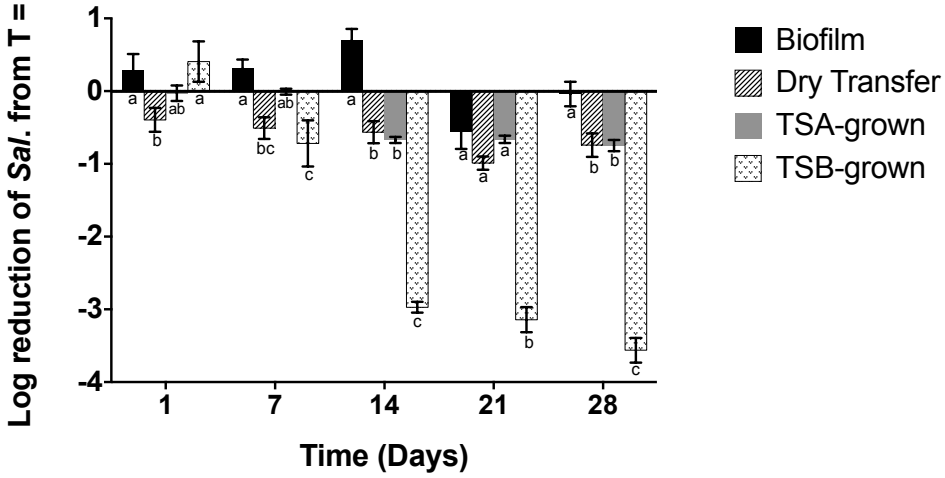
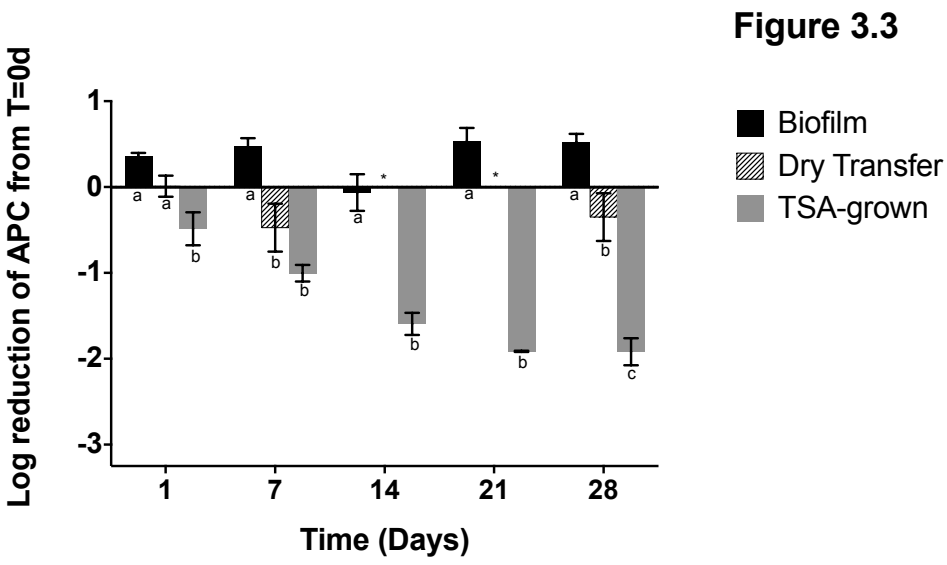


Figure 3.1, Figure 3.2: Comparisons of log reductions CFU/g of total bacteria and *Salmonella* recovered from peppercorn surfaces when inoculated by various methods over 28 days of storage at a_w 0.3. Bars not connected with the same letter are significantly different. Bars represent the average of two replicates. (*) denotes data not collected. (3.1) Total bacteria recovered from peppercorns on TSA. Gaps in data (21, 28 days) occur where data was not collected. Initial average inoculum per method after drying: 6.56 log CFU/g biofilm, 5.52 log CFU/g dry transfer, 7.56 log CFU/g TSA-grown, 8.15 log CFU/g TSB-grown. (3.2) *Salmonella* recovered from peppercorn on XLT4. Initial average inoculum per method after drying: 5.19 log CFU/g biofilm, 5.53 log CFU/g dry transfer, 6.79 log CFU/g TSA-grown, 7.89 log CFU/g TSB-grown.



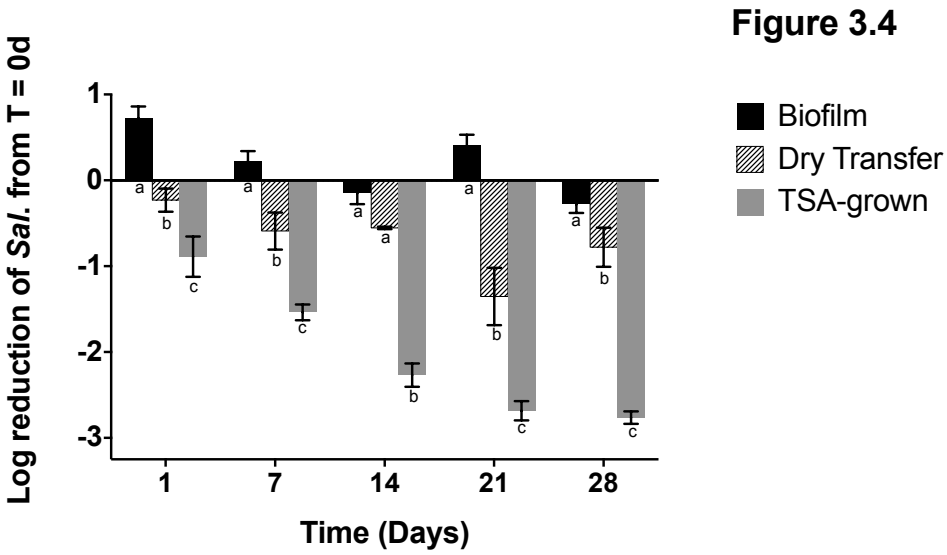


Figure 3.3, Figure 3.4: Comparisons of log reductions CFU/g of total bacteria and *Salmonella* recovered from cumin seed surfaces when inoculated by various methods over 28 days of storage at a_w 0.3. Bars not connected with the same letter are significantly different. Bars represent the average of two replicates. (*) denotes data not collected. (3.3) Total bacteria recovered from cumin on TSA. Initial average inoculum per method after drying: 9.08 log CFU/g biofilm, 6.79 log CFU/g dry transfer, 6.89 log CFU/g TSA-grown. (3.4) *Salmonella* recovered from cumin on XLT4. Initial average inoculum per method after drying: 8.46 log CFU/g biofilm, 6.47 log CFU/g dry transfer, 6.55 log CFU/g TSA-grown.

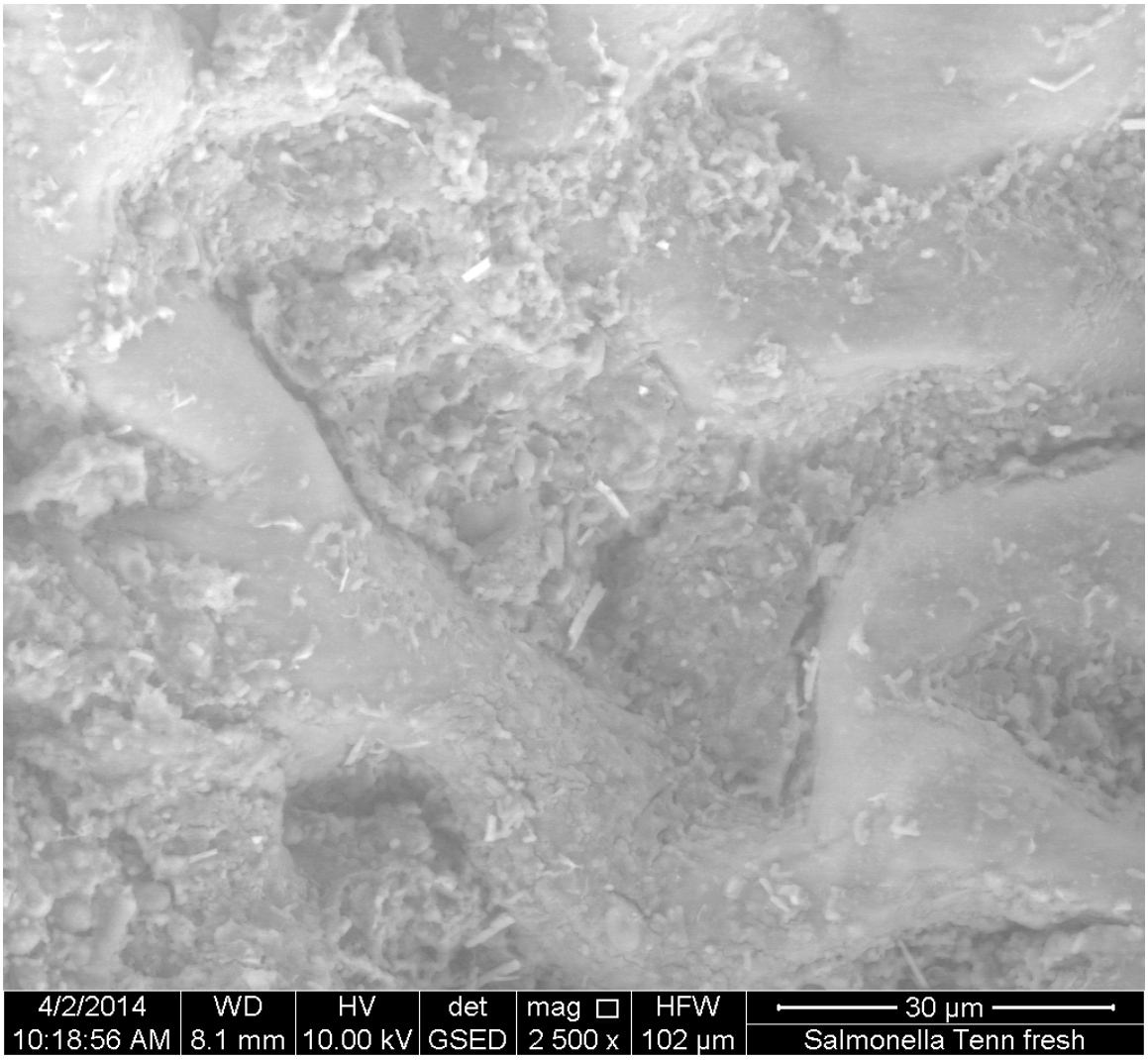


Figure 3.5: Environmental scanning electron microscopy image (2,500 magnification) of cells on peppercorn surface after biofilm inoculation method. Courtesy of M. Ponder

CHAPTER 4: SURVIVAL OF *ENTEROCOCCUS FAECIUM* ON WHOLE BLACK PEPPERCORN AND CUMIN SEEDS AT LOW WATER ACTIVITY IS INFLUENCED BY INOCULATION STRATEGY

Introduction

Foodborne *Salmonella enterica* is estimated to cause 1.2 million illnesses and 450 deaths annually in the United States *(3)*. Multiple extensive *Salmonella* outbreak events have been traced back to low water activity foods, including peanut butter, almonds, cereals, chocolate, and paprika *(1, 14, 20, 27, 32)*. While some of these foods have been the subject of *Salmonella* survival or inactivation validation research *(15, 22, 25)*, the literature is lacking information related to validation of *Salmonella* inactivation in inoculation or processing protocols for spice products. The industry urgently needs research in this area in anticipation of greater regulatory emphasis on process validation and efficacy of pathogen inactivation strategies once pending regulations of the Food Safety Modernization Act for preventative food safety controls are implemented *(2)*.

Establishing effective pathogen inactivation processes in a commercial environment often requires a non-pathogenic surrogate organism that responds to the inactivation process in a manner similar to the target pathogen. Processors do not wish to risk exposing raw materials, equipment, and facilities to contamination with the pathogen of interest. Use of a surrogate allows validation of specific processes on actual equipment under true process conditions while eliminating the risk of introducing the pathogen to the facility. An ideal surrogate organism should mimic the behavior of the pathogen in the process scenario, responding in a similar manner to temperature, chemical, and

environmental controls *(24)*. *Enterococcus faecium* has proved to be an appropriate surrogate organism for inactivation of *Salmonella* using thermal processing (pasteurization and steam treatment) in multiple food systems, including low water activity foods such as peanut paste, almonds, and extruded protein meal products *(6, 15, 16, 30)*. Based on similar properties of spices including moisture content and water activity we hypothesize that *Enterococcus faecium* NRRL B-2354 may serve as a conservative surrogate for *Salmonella* to examine the survival during dry storage.

Dried spices have low water availability, with an activity (a_w) ranging from 0.2-0.6 *(11)*. In general, *Enterococcus* cells require a minimum water activity of 0.93 for growth, but can survive in dry conditions for extended periods *(19)*. There is little evidence establishing the minimum water activity at which *Enterococcus* can survive and requires investigation. Research suggests that *Enterococcus* is adaptable to survival on dry carrier materials, such as talc (a_w 0.6), which could be helpful for dry inoculation of low water activity foods *(10)*.

Salmonella survival at low water activities and specifically on spice seed surfaces is affected by the method of cell growth prior to inoculation *(8, 12)*. The effect of inoculum preparation on the survival of *Enterococcus faecium* on spice surfaces must be understood to identify if the stability is comparable to *Salmonella* under the same target conditions. This study examined the survival of *Enterococcus* on peppercorn and cumin seed surfaces after inoculation, drying and 28 days storage. Three inoculation preparation strategies as described in Chapter 3 were adapted for use with *Enterococcus faecium* strain NRRL B-2354 *(8)*. The results of this research will enable comparisons between *Enterococcus* and *Salmonella* survival on dried spices held for 28d.

Materials and Methods

Bacterial strains and growth conditions. *Enterococcus faecium* (NRRL B-2354, ATCC strain #8459) isolated from cheese products was obtained from American Type Culture Collection (Manassas, VA). Cultures were prepared for each experiment from -80 °C freezer stocks plated onto Bile Esculin Agar (BEA) plates (Becton Dickinson, Franklin Lakes, NJ) and incubated 24h at 37 °C. A single isolated colony was transferred to Tryptic Soy Broth (TSB, Becton Dickinson) and incubated for 24h at 37 °C with shaking (180 rpm).

Spice varieties and sources. A major national spice processor provided whole peppercorns and whole cumin seeds in bulk for this research. No further treatments were applied after receipt of spices to reduce background microbiota prior to inoculation.

Microbiological analysis. Initial aerobic populations were determined by enumerating microorganisms from non-inoculated peppercorn and cumin seeds. Seed samples (10 g) were homogenized in 90 ml of sterile peptone tween buffer (PT, both 0.10% w/v) for 60 s within a sterile blender bag using a lab blender (Interscience BagMixer®, Guelph, Ontario). The homogenized liquid was vacuum filtered through #4 qualitative filter paper (Whatman, GE Healthcare, Pittsburgh, PA) to remove small seed particulates to avoid transfer in serial dilution. The filtered homogenate was serial diluted 1:10 in sterile PT buffer. From appropriate dilutions, 100 µl was spread plated onto duplicate BEA and TSA plates (BD, Franklin Lakes, NJ) and were incubated for 18-24 h at 37 °C.

Water activity and measurement. Water activity (a_w) of whole peppercorns (3 g) and cumin seeds (5 g) was determined using an AquaLab 4TE water activity meter (AquaLab, Pullman, WA). Peppercorns and cumin seeds were determined to have an a_w of approximately 0.3 and 0.4, respectively, prior to inoculation. The water activity of non-inoculated seeds prepared using comparable methods as described below with strains omitted was measured periodically over 48 h to identify the length of drying time needed to return the product to its original water activity. Drying for 24 h was determined to be a sufficient period to return non-inoculated whole spice seeds to water activity levels comparable to the original substrates. Inoculated spices were stored in glass jar desiccators after inoculation and drying to minimize fluctuations in a_w due to changes in relative humidity during storage.

Inoculation methods. The effects of inoculation method on survival of Enterococcus over a 28 day storage period were investigated. The inoculation strategies varied as detailed below and were adapted from methods previously described by Bowman et al *(8)*. In all methods, the inoculated spices were spread out in a single layer on sanitized aluminum trays (18 in. x 26 in.) and dried for 24 h at room temperature (25 °C) in a biological safety cabinet (final a_w = 0.3) (Figure 4.1). Dried inoculated spices were collected into sterile 27 oz Whirl-pak bags (Nasco, Ft. Atkinson, WI) and stored in a desiccator at 25 °C for 28 days.

TSB-grown *Enterococcus* wet inoculation. Planktonic (TSB-grown) cells were cultured from freezer stocks as described above and applied directly to the peppercorn or cumin seed surface. Cells were washed in 0.10% (wt/vol) peptone (Sigma-Aldrich, Co., MO) with 0.1% Tween 80 (PW-Tween, Fisher Scientific, Kansas City, MO) to eliminate

spent media and excess nutrients prior to inoculation. Washed cells suspended in 20 ml sterile PT buffer were applied to 50 g of dry whole seeds within 27 oz Whirl-pak bags and massaged by hand for 1 minute to evenly coat the seed kernels before drying.

TSA-grown cell wet inoculation. The inoculation procedure was adapted from a method described by the Almond Board of California for *Enterococcus* inoculation of almonds *(30)*. Liquid cultures of *Enterococcus* were spread plated onto large 150 x 15 mm (BD Falcon, Franklin Lakes, NJ) TSA plates and incubated for 24 h at 37 °C to cultivate a lawn of bacteria. Cells were scraped from the agar surface using a sterile cotton-tipped swab and suspended in 9 ml of PT buffer. Scraped cells were washed twice in sterile PT buffer, re-suspended in 20 ml of PT buffer and mixed by pipetting to break up cell clumps. The washed cell suspension (20 ml) was applied to 50 g of dry whole seeds in 27 oz Whirl-pak bags and massaged by hand for 1 minute to evenly coat the seeds before drying.

Biofilm-inclusion inoculation. Biofilm formation with *Enterococcus* inclusion on whole spices was adapted from the method used by Aviles et al. 2013 for *Salmonella* inoculation *(4)*. Briefly, *Enterococcus* was incubated in TSB (1 cm depth) containing dry whole peppercorns (62 g) or cumin seeds (25 g) arranged in a single layer on the bottom of a flask (Figure 4.2). The *Enterococcus*-seed mixture was incubated statically for 24 h at 37 °C. After 24 h, liquid media was decanted and seeds were washed by vigorously swirling for 30 s in PT buffer to remove non-adherent cells and nutrients from the seed surface before drying. To facilitate more accurate microbiological enumeration and replicate the procedure used for *Salmonella* recoveries in chapter 3, cellulase enzyme was

included in the initial PT buffer homogenation diluent to encourage release of *Enterococcus* cells entrapped in the biofilm matrix.

Statistical analysis. Bacterial counts were log transformed to approximate normal distribution. Three biological replicates were performed for each inoculation, and triplicate replication was used for enumeration of survival. Statistical analyses were performed using JMP statistical software (version 11, SAS, Cary, NC). The effect of inoculation method over 28 days was compared within each spice at each time point using a one-way ANOVA to test for differences in the average log reduction of recovered CFU/g on both peppercorn and cumin. P-values < 0.05 were considered significant. Regression analysis was conducted to evaluate the relationships between inoculation method and survival over time (Figures 4.7-4.11). The following general first order model is used to describe the microbial curve:

$$\frac{dN.}{dt} = -kN^n$$

where $n = 1$ as the order of the model and k is a rate constant. This equation describes the change in microbial population (N) as it relates to time (t) *(29)*. Analysis was conducted by plotting the mean log reductions of *Enterococcus* and *Salmonella* (data from Chapter 3) over 28 days within each inoculation method and fitting the data for each organism with both linear and logarithmic trend lines. Correlation values (r^2) were determined to approximate goodness of fit for each organism. Performance (survival) of *Enterococcus* was directly compared to *Salmonella* survival by plotting the corresponding mean log reductions at a given time point during storage against each other. The data were fit with a linear trend line and correlation values (r^2) were determined to approximate goodness of fit.

Results

Total aerobic plate count (APC). The mean total aerobic microbiota for non-inoculated spices was 8.3 log CFU/g and 6.45 log CFU/g for whole peppercorns and cumin seeds respectively, when cultured on TSA. Non-inoculated samples plated on BEA did not yield any colonies with characteristic black coloration to suggest *Enterococcus* presence *(33)*. After peppercorn inoculation, APC (log CFU/g) remained relatively stable over time, with statistically significant decreases in recovery limited to d1 for BF-inclusion and d28 for TSB-grown inoculations. Total log reductions were less than 0.40 log CFU/g for all methods over the 28d period (Figure 4.3). The BF-inclusion preparation method showed the largest total reductions in total APC (mean LR 0.38 ± 0.18 log CFU/g) and was the only method with statistically significant reductions in APC compared to other peppercorn inoculations. Similarly on cumin seeds, declines in APC were small in all inoculation methods, with total reductions less than 0.9 log CFU/g (Figure 4.5). Significant decreases in APC during the 28d storage period occurred for the BF-inclusion inoculated cumin seeds, but while some APC reduction occurred at different time points there was no overall trend in reduction for cumin seeds inoculated using the TSB-grown and TSA-grown methods. Changes observed may be due to expected natural variability in recovery of cells. Preparation using the BF-inclusion method for cumin yielded the largest and only statistically significant decrease from other methods, with an average reduction of 0.86 ± 0.47 log CFU/g.

Evaluation of *Enterococcus* survival by method and time. Statistical analysis indicated that inoculation method impacted *Enterococcus* recovery from both cumin and

peppercorn over the 28d dry storage period (Figures 4.4, 4.6). Respective log reductions were compared between inoculation methods at each time point (1, 7, 14, 21, 28d) on selective and non-selective media (BEA and TSA). Significant decreases in *Enterococcus* recovered from both spices were observed for most inoculation methods by d 28, however the trend associated with the timing of this decrease varied between methods. Significant differences in overall log reductions between methods were noted at 7 and 28d for peppercorn and 1, 14, and 28d for cumin seeds. In contrast, no significant reduction in *Enterococcus* inoculated on whole peppercorns using the biofilm-inclusion methods occurred with time. From peppercorns, BF-inclusion inoculated samples yielded minimal net change in *Enterococcus* recovery (mean LR 0.02 ± 0.37 log CFU/g) on BEA after 28d, while samples inoculated by the TSB-grown method were associated with a small yet statistically significant decrease in *Enterococcus* (mean LR 0.54 ± 0.15 log CFU/g, Figure 4.4). Recovery of *Enterococcus* inoculated by the TSA-grown method was statistically similar to other methods (mean LR 0.19 ± 0.01 log CFU/g).

In general, decreases in *Enterococcus* recovery were larger on inoculated cumin seeds as compared to recovery from peppercorns. There were significant decreases in *Enterococcus* recovered with time in all three methods, however the time points at which those decreases occurred were different (Figure 4.6). Cumin seeds inoculated with BF-inclusion and TSA-grown cells yielded similar declines at 7, 21, and 28d, with no statistical differences between methods at these time points. Total average reductions for these methods were 0.61 ± 0.2 log CFU/g and 0.56 ± 0.15 log CFU/g respectively on BEA (Figure 4.6). The largest decreases in *Enterococcus* recovery were observed when cumin seeds were inoculated via the TSB-grown method, in which total reductions

averaged 1.25 ± 0.29 log CFU/g after 28d storage. These were significantly larger than reductions observed with other methods.

Comparison of *Enterococcus* survival to *Salmonella* survival. Survival (log CFU/g recovered) of *Enterococcus* and *Salmonella* on peppercorn and cumin seed over 28 days of dry storage was statistically compared using one-way ANOVA ($\alpha = 0.05$, Tables 4.1, 4.2). *Enterococcus* was more stable, with significantly fewer decreases in recovered cells than *Salmonella* after 14, 21, and 28 days storage when organisms were inoculated onto peppercorn using the TSA and TSB-grown methods. Conversely, biofilm-included *Salmonella* had fewer initial decreases in recovered population, however there were no differences in recovery from peppercorn by 21 and 28 days of storage (Table 4.1). From cumin seed surfaces, *Enterococcus* was more stable, with significantly fewer log reductions in recovery at all time points during storage compared to *Salmonella* when both were inoculated onto cumin using the TSA-grown method. *Salmonella* had fewer log reductions at 1, 7, and 21d when cumin was inoculated using the biofilm method. The TSB-grown method was not applied to cumin (Table 4.2).

Regression analysis showed the relationships between change in survival of *Salmonella* and *Enterococcus* on peppercorn (Figures 4.7-4.9) and cumin (Figures 4.10-4.11) and the storage time within each inoculation method. From peppercorn, linear fitted regressions indicated very poor correlations between survival and storage time for both organisms when inoculated using the biofilm-inclusion method (Figure 4.7, Table 4.3). When inoculated via TSA- and TSB-grown methods, linear models had similarly weak correlations with time for *Enterococcus,* however decreases in *Salmonella* survival were more linear in correlation to storage time (Figures 4.8-4.9, Table 4.3). From cumin,

survival of both organisms showed stronger linear relationships with storage time when inoculated via the TSA-grown method than with the biofilm-inclusion method (Figures 4.10-4.11, Table 4.3). For cases in which the linear model was not a good fit, a logarithmic model was applied and often produced higher correlation coefficients (Table 4.3, graphs not shown).

Performance comparisons revealed whether any linear relationships exist between *Salmonella* and *Enterococcus* survival when inoculated onto seed by the same methods (Figures 4.12-4.16). From peppercorn, change in recovery of the two organisms displayed weak linear relationships when seeds were inoculated via TSA-grown and TSB-grown methods (r^2 = 0.40 and 0.31 respectively). Minimal correlation (r^2 = 0.0016) resulted when the biofilm-inclusion method was applied. From cumin, strong linearity was observed in the change in recovery of both organisms when seeds were inoculated via the TSA-grown method (r^2 = 0.98) and minimal correlation in recovery from biofilm-inclusion seed (r^2 = 0.11).

Discussion

Enterococcus faecium has been discussed as a surrogate for *Salmonella* in a variety of low water activity and low moisture-food processing applications, however there is little information describing the survival and use of the organism on spices. Food systems in which *Enterococcus* surrogacy for *Salmonella* has been explored include peanut paste, low moisture extruded foods, and almonds in multiple thermal process methods *(6, 15, 16)*. The suitability of *Enterococcus faecium* for surrogacy in validating *Salmonella* inactivation in dry food processes is further substantiated by recommendations from various industry groups *(13, 30)*. To expand *Enterococcus*

surrogacy into spice process applications, this study explored the stability and recovery of *Enterococcus faecium* NRRL-B2354 on whole peppercorn and cumin through multiple inoculation methods. This approach enabled comparison to prior survival studies performed using *Salmonella enterica*.

Salmonella enterica is known to survive for extended periods at low water activity on a variety of food products, including spices *(8, 26)*. The results suggest that *Enterococcus* is similarly able to persist for extended periods of time at low water activity when inoculated onto peppercorn and cumin seed surfaces. Throughout the 28 day storage, *Enterococcus* recovery showed only small log reductions (< 0.68 logs) in all inoculation methods on both spices, with the exception of the TSB-grown inoculations on cumin at the 28 day time point (Figures 4.4, 4.6). This trend is similar to *Enterococcus* survival observed in other low water activity environments, including peanut paste and on various food contact surfaces. Kataoka et al. observed smaller decreases in *Enterococcus faecium* NRRL B-2354 survival in peanut paste (a_w 0.3) after 12 months post-inoculation, heat treatment (75°C for 30 min) and storage (12 mo. at 20°C) compared to *Salmonella* serovars Tennessee and Typhimurium DT104 *(16)*. Decreased recovery may have also been influenced by changes in water activity, as they noted greater *Enterococcus* survival at a_w 0.3 compared to a_w 0.6. A closely related strain, *Enterococcus faecalis* is known to persist up to 16 weeks on various dry food contact surface materials (a_w approaching zero), such as stainless steel, glass, and polystyrene *(7)*.

Collectively, the results of this study suggest that long-term recoverability of *Enterococcus* from peppercorn and cumin seeds is influenced by inoculation method as compared to initial recovery 24h after application and drying. *Enterococcus* cells

incorporated into a biofilm matrix have higher survival rates at low water activity on peppercorn and cumin than agar-grown and liquid-grown equivalents after 28d dry storage (Figures 4.4, 4.6). Smaller total reductions in *Enterococcus* recovery were observed after inoculation and storage with BF-inclusion method than either of the other methods applied; this was true for both spices. While it unclear whether the *Enterococcus* is responsible for the biofilm production here, it is likely that the stable recovery of cells is a result of inclusion within the biofilm structure. These observations mirror the stability and recovery results of *Salmonella* inoculations on peppercorn and cumin described in Chapter 3, in which *Salmonella* inoculations with the BF-inclusion method showed minimal change in recovery from initial inoculation levels (Figures 3.2, 3.4). The ability to produce biofilms is attributed to *Enterococcus* species, allowing survival in a variety of environments, such as oral cavities, gastrointestinal tissues, and polystyrene *(5, 9, 31)*. There is evidence to suggest that growth in a biofilm helps sustain colonization by *Enterococcus* on the surfaces of implantable medical devices *(23)*, which contribute to human nosocomial infections. Overall, the data presented here suggest that stability and recovery of *Enterococcus* from whole peppercorn and cumin seed surfaces is comparable to that of *Salmonella* when inoculated using the same methods and stored dry for 28d.

The superior stability and recovery of BF-inclusion cells after storage can be attributed to multiple survival-enhancing mechanisms. Biofilm development is a complex and multi-layered process, often carried out by diverse microbial populations in which constituent organisms lend symbiotic support to one another. One possible mechanism is the acquisition of stress resistance genes from other *Enterococcus* or native bacteria due to proximity in the biofilm. *Enterococcus* is known to transfer resistance genes from

neighboring members of the microbial community at a high rate *(18)*, a rate which increases when cells are incorporated into a multi-species biofilm. Although this type of transfer would be less likely to have occurred in the TSA- and TSB-grown methods, the incubation period included in the BF-inclusion inoculation method would provide some opportunity for these transfers to occur. Additionally, biofilms allow significant resistance to antibiotic compounds, allowing tolerance to antibiotic concentrations from 10-1000 times greater than planktonic equivalents *(21)*. Peppercorn and cumin both contain compounds that have been shown to be antibacterial at high concentrations, however it is unlikely that these concentrations are present on the surface *(17, 28)*. Encasement within the biofilm likely allows some *Enterococcus* cells to be shielded from the effects of these antimicrobial compounds on the peppercorn and cumin seed surfaces.

To serve as a surrogate for *Salmonella enterica* in future process validation, an organism must behave in a similar manner to the pathogen itself in the specified food matrix, process parameters and conditions. In the case of spices, surrogate stability at low water activity is a critical factor due to the low availability of free water on the spice surface. When the data collected in this study is compared with *Salmonella* recovery data discussed in Chapter 3, the stability on the spice surface during long-term storage is comparable (Tables 4.1, 4.2). Data was first analyzed using one-way ANOVA statistical analysis in JMP, however wide variations in the *Enterococcus* data set affected the statistical significance of the outcomes. Experimental error may have been introduced as a result of multiple lab personnel participating in various aspects of dilution pipetting, spread plating and colony counting. Additional experimental sources of error may include

fluctuations in atmospheric conditions within the laboratory that were not precisely measured or held constant (i.e., room temperature (~5°C)).

From peppercorn, there were significantly fewer decreases in *Enterococcus* recovery as compared to *Salmonella* recovery in two of three inoculation methods (TSA-grown, TSB-grown) (Table 4.1). This suggests that *Enterococcus* could similarly or conservatively predict *Salmonella* survival when using these methods. When comparing the recoveries within the biofilm-inclusion method, there were two instances (7, 14 days) in which *Salmonella* log reductions were smaller than *Enterococcus*. However this was somewhat negated by the lack of statistical differences found at other time points (1, 21, 28 days) within the same method, meaning that *Enterococcus* surrogacy may also be considered appropriate when using the biofilm-inclusion method. In the case of cumin, *Enterococcus* showed significantly fewer decreases in recovery after 28d storage than *Salmonella* when both organisms were inoculated using the TSA-grown method (Table 4.2). *Salmonella* had fewer log reductions in recovered cells at 1, 7, and 21 days when cumin was inoculated using the biofilm method, suggesting that *Enterococcus* may not appropriately predict *Salmonella* recovery when using this inoculation method. Overall, these trends suggest that *Enterococcus* inoculations may be similar to or more stable than *Salmonella* inoculations during long-term dry storage of peppercorn and cumin seeds.

Overall, the regression analyses showed mixed results for the appropriateness of the fitted models (Table 4.3). The *Salmonella* data tended to be characterized by linear relationships, showing strong linear correlations between inoculation method and storage time in two inoculation methods on peppercorn (TSA- and TSB-grown), and one on cumin (TSA-grown). The linear models fitted to the *Enterococcus* data produced

relatively poor correlation coefficients (r^2 approaching zero) for peppercorn (Figures 4.7-4.9) and for BF-inclusion on cumin (Figures 4.10-4.11). Potential tailing effects present in the data could be distorting the fitted lines and therefore compromising the ability of the linear model to accurately predict the true population change. When a logarithmic model was applied, correlation coefficients improved marginally for two methods on peppercorn (TSA- and TSB-grown) and the TSA-grown method on cumin suggesting a more appropriate fit to the data. These factors must be taken into consideration when evaluating the appropriateness of the model for use in these applications. Though it is demonstrated that survival behavior of the two organisms is similar and rate of death is comparable, correlations suggest that these models alone may not appropriately predict organism survival during storage of these spice seeds, and should not be used to predict survival after the 28d time period.

Performance comparisons, which directly compare the change in survival of both organisms, are helpful to visualize any linearity in behavior during storage (Figures 4.12-4.16). *Enterococcus* reductions tended to be lower than *Salmonella* reductions in a majority of methods, including TSA- and TSB-grown on peppercorn and TSA-grown on cumin. This suggests a conservative underestimate of *Salmonella* survival within these methods, as indicated by points falling below the 1:1 line (Figures 4.13, 4.14, and 4.16). There was no overall trend when comparing organism performance in the BF-inclusion method on peppercorn (Figure 4.12). Conversely, *Enterococcus* reductions were larger than *Salmonella* decreases when BF-inclusion inoculations were applied to cumin, but with poor correlation ($r^2 = 0.1063$) the trend is weak. These poor correlations within the BF-inclusion method are due to the very small reductions that were detected, which make

it difficult to discern a true pattern. Based on these criteria, *Enterococcus* may be useful as a conservative surrogate under TSA-grown and TSB-grown inoculations of peppercorn and TSA-inoculation of cumin for spices stored 28d desiccated. However, it is important to note that these trends apply only to these applications and should not be extrapolated beyond 28d of storage.

Generally, the results contribute to the use of *Enterococcus faecium* as a conservative surrogate for *Salmonella enterica* recovery in spice stored for a period of 28d because the organism appears to be less affected by long term storage at low water activity than *Salmonella*. However, the poor model fit indicates that further research must be performed to compare survival of *Salmonella* and *Enterococcus* for longer periods of time. However, based on this data it seems promising that *Enterococcus faecium* may be a conservative indicator for persistence of *Salmonella*. The suitability of *Enterococcus* as a surrogate during process interventions beyond shelf life storage can not be inferred from this data. Comparison of inactivation under the target process parameters must be performed within a lab setting before the suitability of *Enterococcus* can be implemented in a commercial setting. Understanding strain persistence as it relates to the inoculation method onto the spice seed surface is the critical first step to confirming the surrogacy potential of *Enterococcus* on peppercorn and cumin seeds and in future process validations.

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Figures

Figure 4.1: Drying set up for inoculated seeds. Inoculated seeds were spread in a thin, single layer on foil-lined metal baking sheets suspended on cooling racks and allowed to dry for 24 hours in a BSL2 continuous air-flow hood to achieve 0.30 water activity.

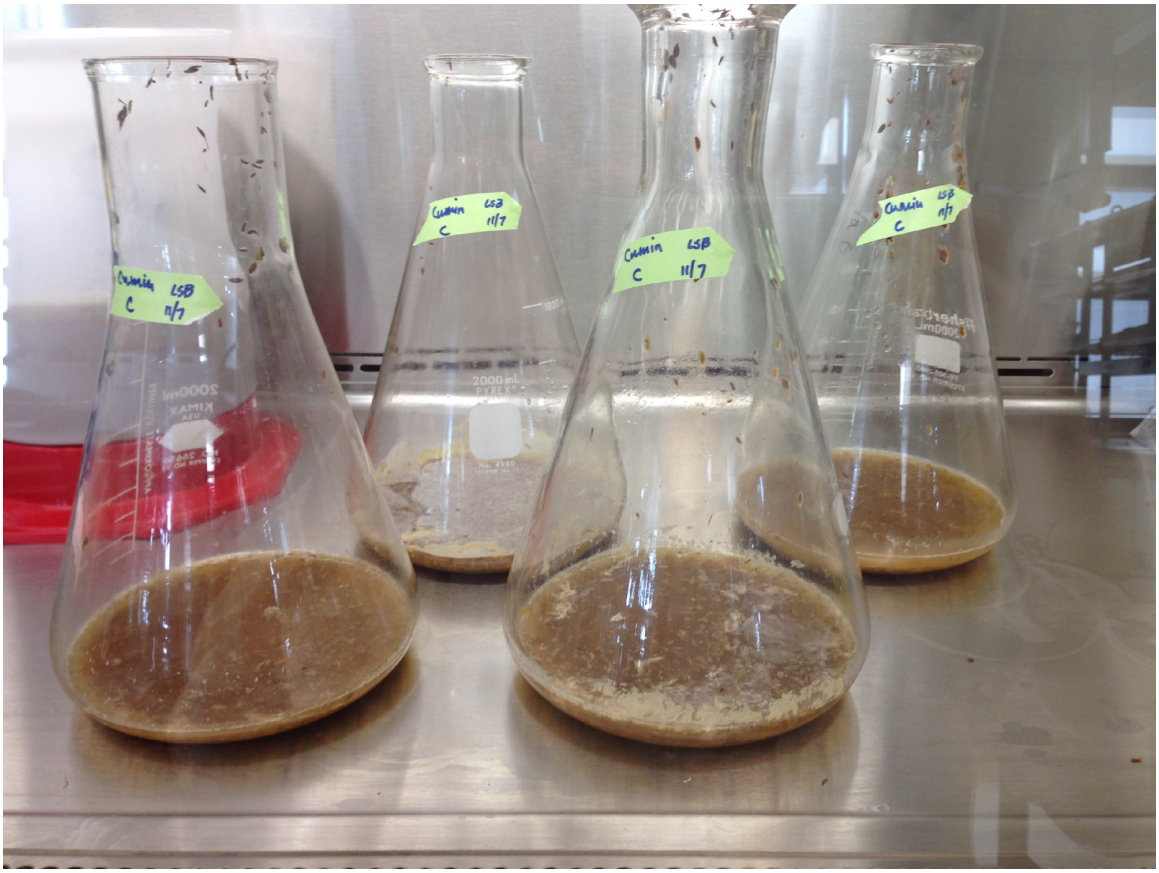

Figure 4.2: Biofilm inclusion method on cumin. Single layer of spice seeds under shallow volume of TSB. Static incubation for first 24h with 5 ml of 8 log broth culture added. Broth decanted at 24h and replaced over inoculated cumin seeds with new sterile TSB to encourage growth of the biofilm matrix previously adhered to seed surface.

Figure 4.3

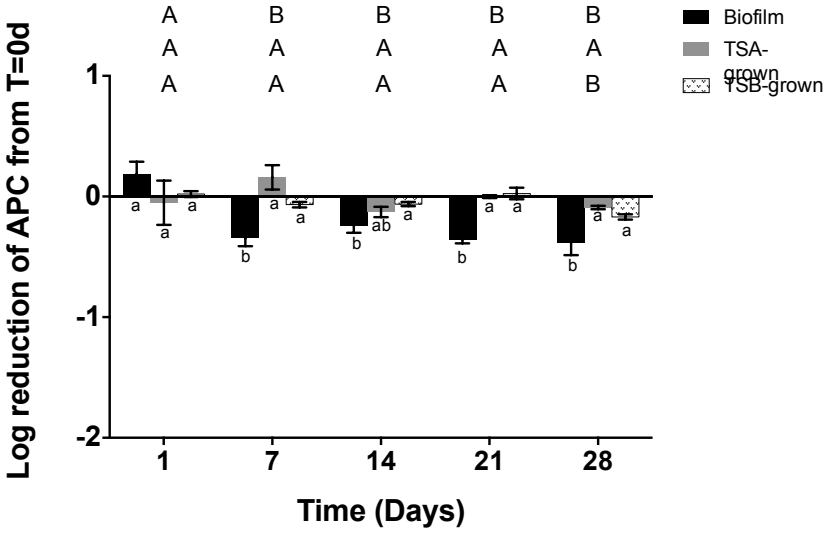

Figure 4.4

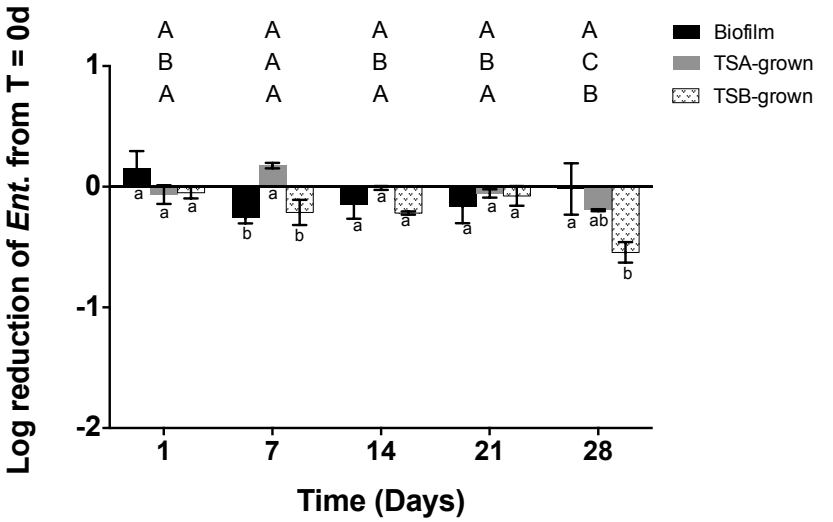

Figure 4.3, Figure 4.4: Comparisons of log reductions (CFU/g) of total bacteria and *Enterococcus* recovered from peppercorn surfaces when inoculated by various methods over 28 days of storage at water activity a_w 0.3. Bars not connected with the same letter are significantly different. Bars represent the average of three replicates. Lowercase letters show significance between methods at a time point. Uppercase letters show significance over time within a method. (4.3) Total bacteria recovered from peppercorns on TSA. Initial average inoculum per method after drying: 7.53 log CFU/g BF-inclusion, 8.67 log CFU/g TSA-grown, 7.28 log CFU/g TSB-grown. (4.4) *Enterococcus* recovered from peppercorn on BEA. Initial average inoculum per method after drying: 6.65 log CFU/g BF-inclusion, 8.56 log CFU/g TSA-grown, 6.31 log CFU/g TSB-grown.

Figure 4.5

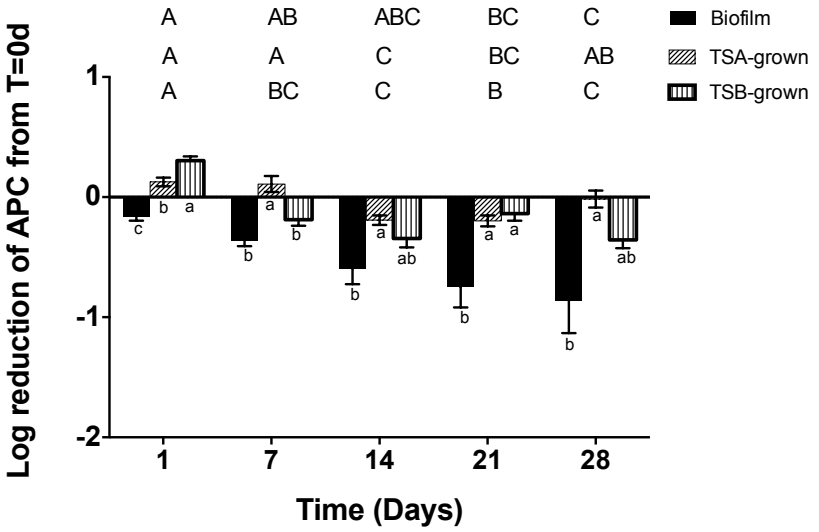

Figure 4.6

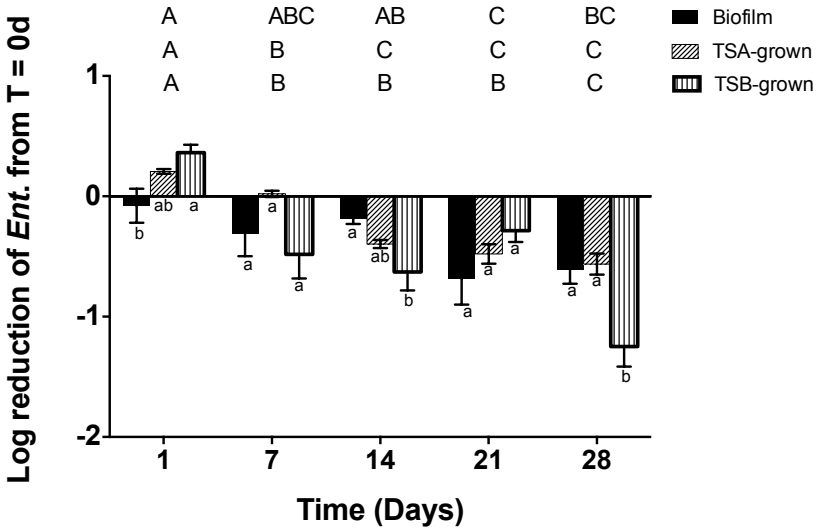

Figure 4.5, Figure 4.6: Comparisons of log reductions (CFU/g) of total bacteria and *Enterococcus* recovered from cumin seed surfaces when inoculated by various methods over 28 days of storage at water activity a_w 0.3. Bars not connected with the same letter are significantly different. Bars represent the average of three replicates. Lowercase letters show significance between methods at a time point. Uppercase letters show significance over time within a method. (4.5) Total bacteria recovered from cumin on TSA. Initial average inoculum per method after drying: 9.61 log CFU/g BF-inclusion, 8.32 log CFU/g TSA-grown, 6.28 log CFU/g TSB-grown. (4.6) *Enterococcus* recovered from cumin on BEA. Initial average inoculum per method after drying: 7.76 log CFU/g BF-inclusion, 7.95 log CFU/g TSA-grown, 4.97 log CFU/g TSB-grown.

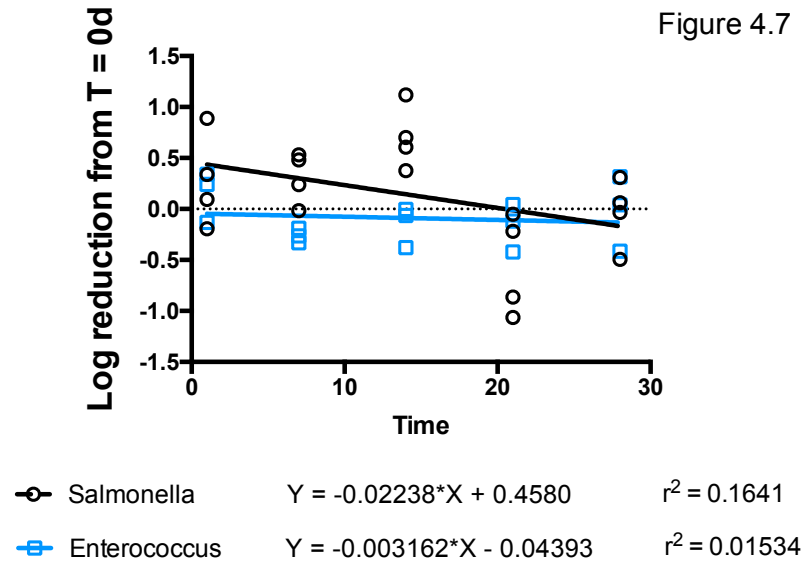

Figure 4.7: Linear regression of log reductions of *Salmonella* and *Enterococcus* recovered from biofilm-inclusion inoculated peppercorn. Lines are not statistically different (p_{slope} = 0.2128, $p_{intercept}$ = 0.1284). *Enterococcus* conservatively underestimates log reductions of *Salmonella* when using the biofilm-inclusion method on peppercorn.

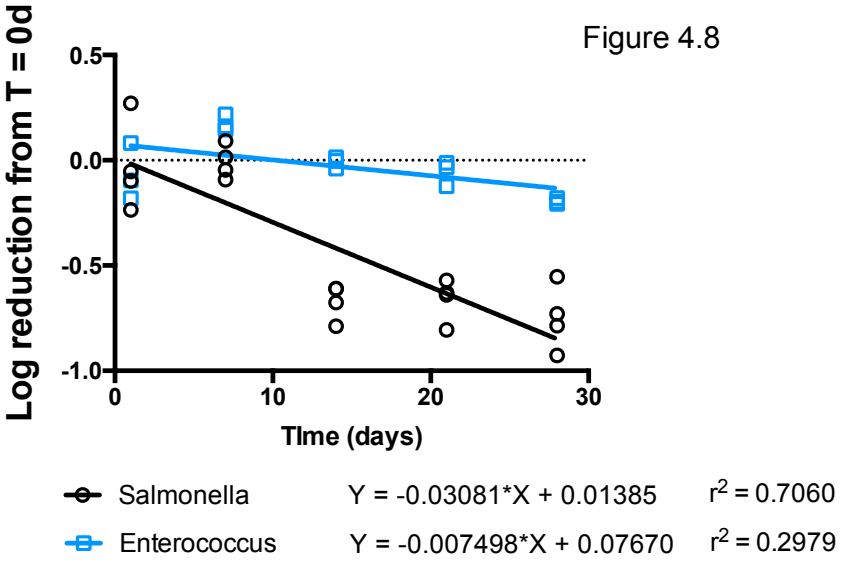

Figure 4.8: Linear regression of log reductions of *Salmonella* and *Enterococcus* recovered from TSA-grown inoculated peppercorn. Lines are statistically different (p_{slope} = 0.0006). *Enterococcus* conservatively underestimates log reductions of *Salmonella* when using the TSA-grown method on peppercorn, although estimates may be less accurate during later storage.

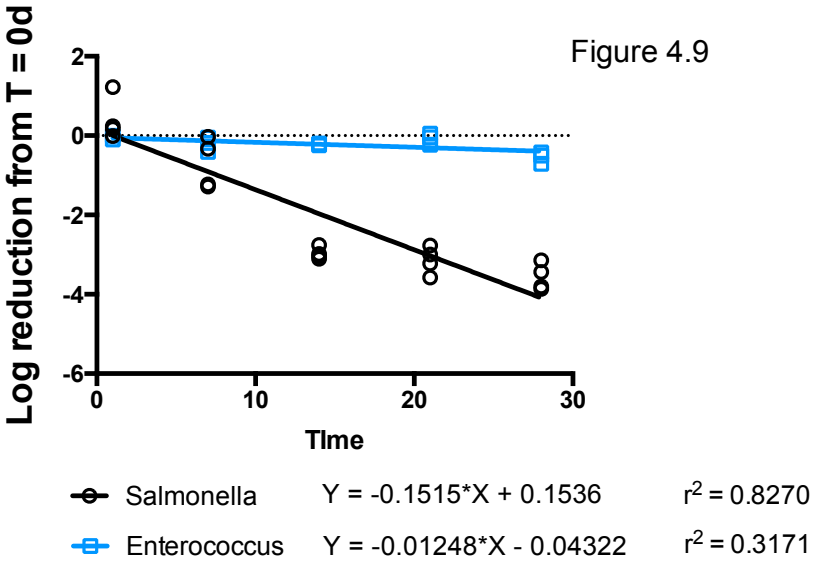

Figure 4.9: Linear regression of log reductions of *Salmonella* and *Enterococcus* recovered from TSB-grown inoculated peppercorn. Lines are statistically different (p_{slope} = <0.0001). *Enterococcus* conservatively underestimates log reductions of *Salmonella* when using the TSB-grown method on peppercorn, although estimates may be less accurate during later storage.

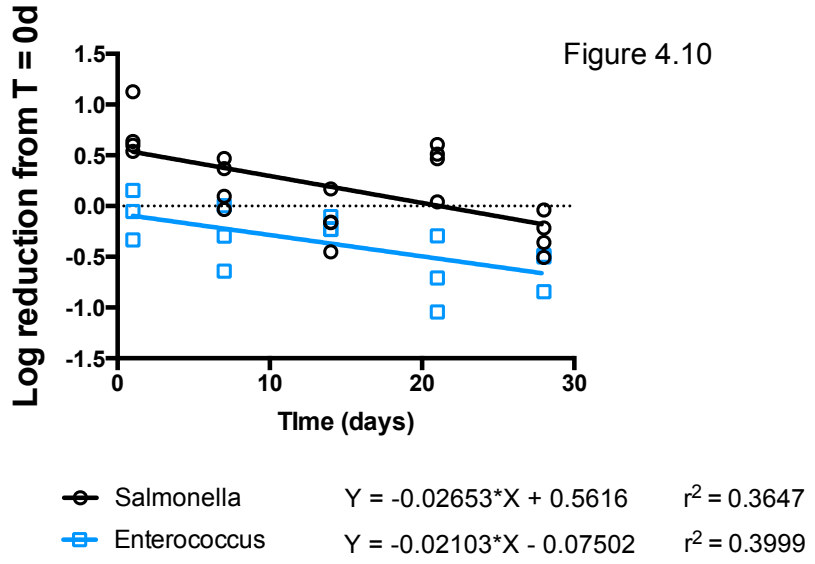

Figure 4.10: Linear regression of log reductions of *Salmonella* and *Enterococcus* recovered from biofilm-inclusion inoculated cumin. Lines are different (p_{slope} = 0.6329, $p_{intercept}$ = <0.0001). *Enterococcus* overestimates log reductions of *Salmonella* when using the biofilm-inclusion method on cumin, therefore may not be an appropriate surrogate for *Salmonella* shelf life using the biofilm-inclusion inoculation method.

Figure 4.11

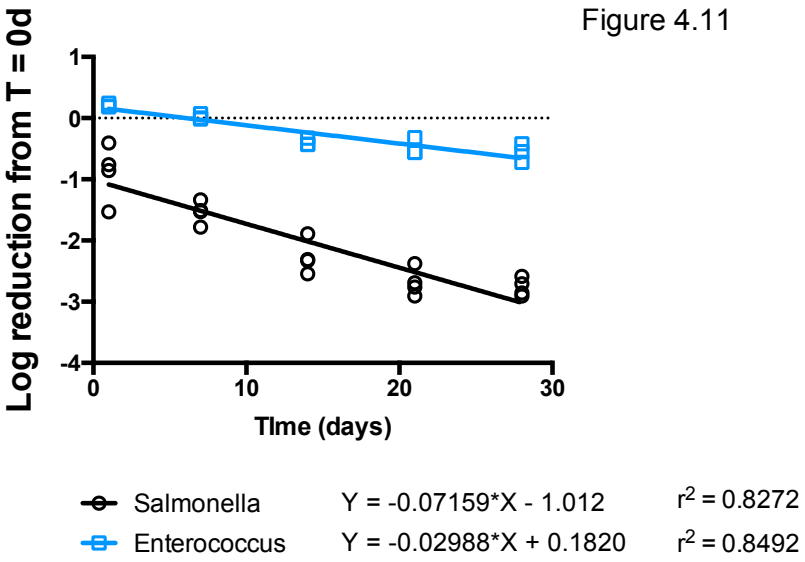

Figure 4.11: Linear regression of log reductions of *Salmonella* and *Enterococcus* recovered from TSA-grown inoculated cumin. Lines are different (p_{slope} = 0.0001). *Enterococcus* conservatively underestimates log reductions of *Salmonella* when using the TSA-grown method on cumin, although estimates may be less accurate during later storage.

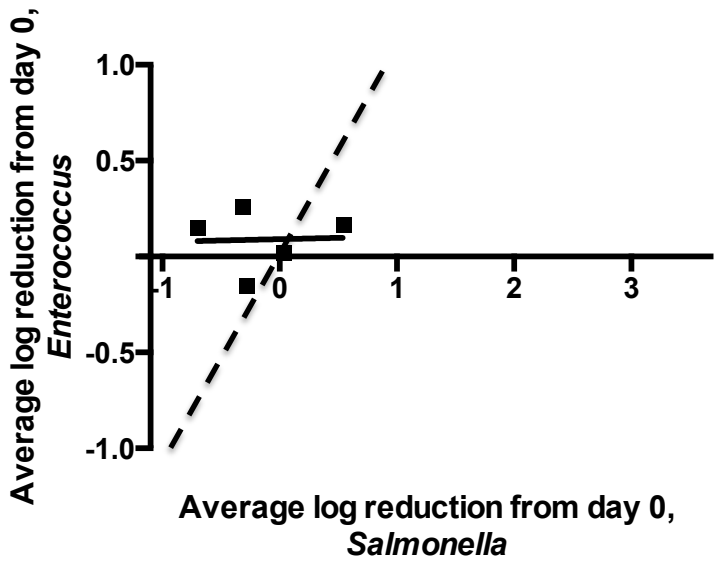

Figure 4.12: Performance comparison of average log reductions of *Salmonella* and *Enterococcus* recovered from biofilm-inclusion inoculated peppercorn over 28 days storage using linear regression. Dashed line represents 1:1 relationship. Equation of the fitted regression line is Y = 0.01374*X + 0.09075, with a correlation of r^2 = 0.001616.

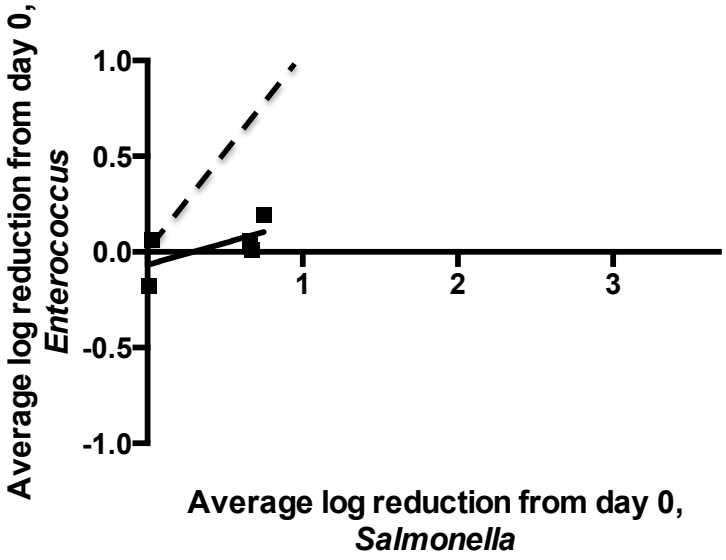

Figure 4.13: Performance comparison of average log reductions of *Salmonella* and *Enterococcus* recovered from TSA-grown inoculated peppercorn over 28 days storage using linear regression. Dashed line represents 1:1 relationship. Equation of the fitted regression line is Y = 0.2285*X - 0.06704, with a correlation of r^2 = 0.4036.

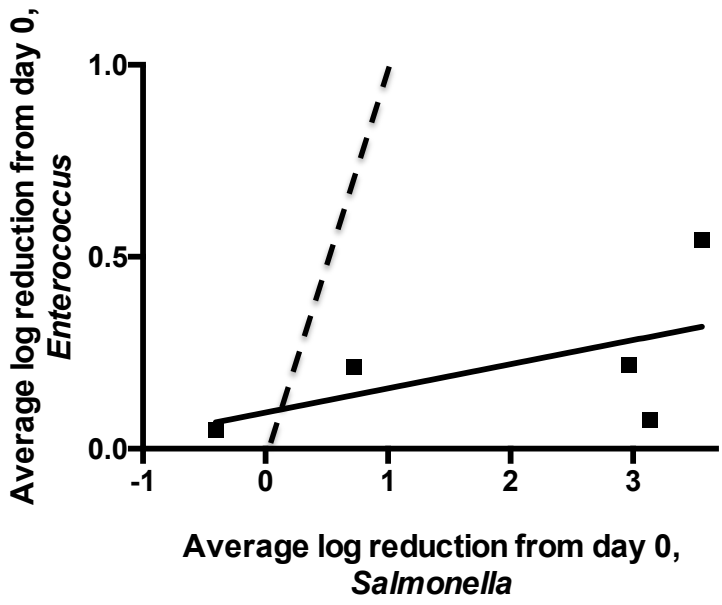

Figure 4.14: Performance comparison of average log reductions of *Salmonella* and *Enterococcus* recovered from TSB-grown inoculated peppercorn over 28 days storage using linear regression. Dashed line represents 1:1 relationship. Equation of the fitted regression line is Y = 0.06280*X + 0.09454, with a correlation of $r^2 = 0.3076$.

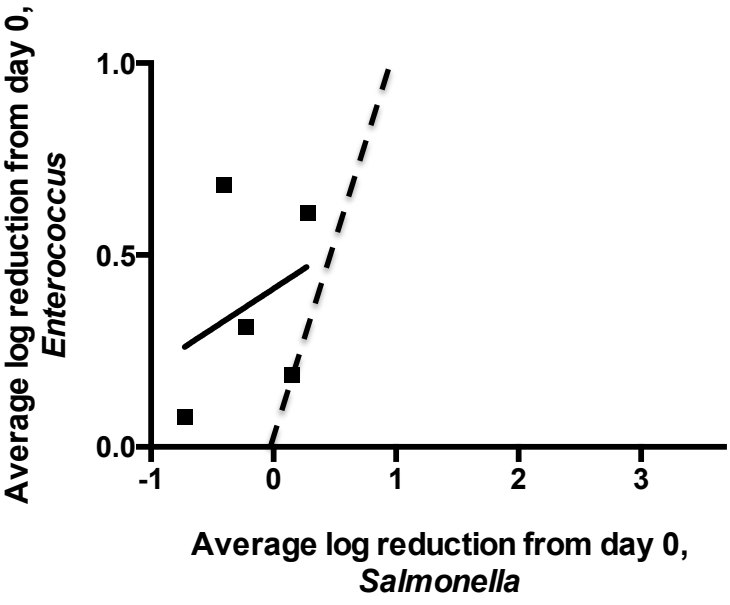

Figure 4.15: Performance comparison of average log reductions of *Salmonella* and *Enterococcus* recovered from biofilm-inclusion inoculated cumin over 28 days storage using linear regression. Dashed line represents 1:1 relationship. Equation of the fitted regression line is Y = 0.2089*X + 0.4122, with a correlation of $r^2 = 0.1063$.

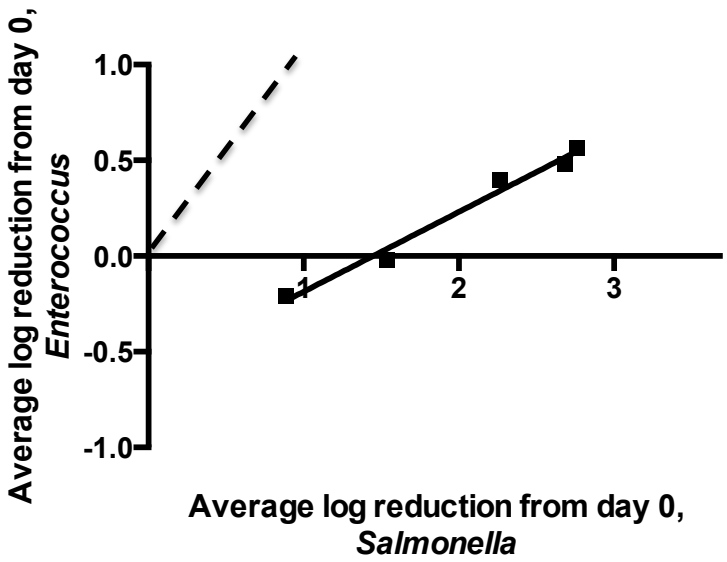

Figure 4.16: Performance comparison of average log reductions of *Salmonella* and *Enterococcus* recovered from TSA-grown inoculated cumin over 28 days storage using linear regression. Dashed line represents 1:1 relationship. Equation of the fitted regression line is Y = 0.4166*X - 0.6029, with a correlation of r^2 = 0. 9814.

Tables

Peppercorn inoculation method	Time after inoculation and drying (days)	*Enterococcus faecium (EF)*		*Salmonella enterica (SE)*		ANOVA comparison of EF and SE LRs ($\alpha < 0.05$)
		Average recovery (Log CFU/g ± SD)	Average LR ± SD	Average recovery (Log CFU/g ± SD)	Average LR ± SD	
BF-inclusion	0	6.65 ± 0.13	N/A	5.19 ± 0.15	N/A	N/A
BF-inclusion	1	6.80 ± 0.13	0.15 ± 0.25	5.48 ± 0.50	0.28 ± 0.46	0.679
BF-inclusion	7	6.39 ± 0.18	-0.26 ± 0.07	5.50 ± 0.15	0.31 ± 0.25	0.014^
BF-inclusion	14	6.50 ± 0.09	-0.15 ± 0.20	5.90 ± 0.16	0.70 ± 0.31	0.009^
BF-inclusion	21	6.48 ± 0.17	-0.17 ± 0.24	4.65 ± 0.37	-0.55 ± 0.49	0.273
BF-inclusion	28	6.63 ± 0.24	-0.02 ± 0.37	5.16 ± 0.26	-0.04 ± 0.34	0.941
TSA-grown	0	8.56 ± 0.10	N/A	6.79 ± 0.03	N/A	N/A
TSA-grown	1	8.50 ± 0.04	-0.07 ± 0.13	6.76 ± 0.22	-0.03 ± 0.21	0.813
TSA-grown	7	8.74 ± 0.07	0.18 ± 0.04	6.78 ± 0.06	-0.01 ± 0.08	0.015^
TSA-grown	14	8.55 ± 0.10	-0.01 ± 0.03	6.12 ± 0.06	-0.67 ± 0.08	<0.0001*
TSA-grown	21	8.50 ± 0.07	-0.06 ± 0.06	6.13 ± 0.12	-0.66 ± 0.10	0.0003*
TSA-grown	28	8.37 ± 0.11	-0.19 ± 0.01	6.04 ± 0.13	-0.75 ± 0.15	0.002*
TSB-grown	0	6.31 ± 0.14	N/A	7.88 ± 0.06	N/A	N/A
TSB-grown	1	6.32 ± 0.06	-0.05 ± 0.07	8.28 ± 0.57	0.41 ± 0.56	0.338
TSB-grown	7	6.09 ± 0.05	-0.21 ± 0.18	7.16 ± 0.58	-0.72 ± 0.64	0.247
TSB-grown	14	6.09 ± 0.11	-0.22 ± 0.02	4.91 ± 0.10	-2.97 ± 0.15	<0.0001*
TSB-grown	21	6.23 ± 0.01	-0.08 ± 0.14	4.74 ± 0.29	-3.14 ± 0.34	<0.0001*
TSB-grown	28	5.76 ± 0.08	-0.54 ± 0.15	4.31 ± 0.34	-3.56 ± 0.34	<0.0001*

Table 4. 1 Comparison of *Enterococcus faecium* and *Salmonella enterica* recovery from inoculated peppercorn after 1, 7, 14, 21, and 28 days dry storage. Statistical analysis by oneway ANOVA compared *Salmonella* and *Enterococcus* log reductions based on log CFU/g recovery. *Enterococcus* showed significantly fewer log reductions in recovery after 14, 21, and 28d storage than *Salmonella* when both were inoculated onto peppercorn using the TSA and TSB-grown methods. Initially, biofilm-included *Salmonella* had fewer log reductions, however there were no differences in recovery by 21 and 28 days of storage. (^) denotes significant difference in which *Enterococcus* overestimates *Salmonella* log reductions. (*) denotes significance in which *Enteroccocus* underestimates *Salmonella* log reductions.

Cumin inoculation method	Time after inoculation and drying (days)	*Enterococcus faecium (EF)*		*Salmonella enterica (SE)*		ANOVA comparison of EF and SE LRs ($\alpha < 0.05$)
		Average recovery (Log CFU/g ± SD)	Average LR ± SD	Average recovery (Log CFU/g ± SD)	Average LR ± SD	
BF-inclusion	0	7.76 ± 0.10	N/D	8.46 ± 0.29	N/D	N/D
BF-inclusion	1	7.68 ± 0.17	-0.08 ± 0.24	9.19 ± 0.13	0.73 ± 0.27	0.001^
BF-inclusion	7	7.45 ± 0.36	-0.31 ± 0.32	8.69 ± 0.47	0.23 ± 0.23	0.050^
BF-inclusion	14	7.57 ± 0.10	-0.19 ± 0.07	8.31 ± 0.08	-0.15 ± 0.25	0.824
BF-inclusion	21	7.08 ± 0.31	-0.68 ± 0.38	8.87 ± 0.38	0.41 ± 0.25	0.006^
BF-inclusion	28	7.15 ± 0.11	-0.61 ± 0.20	8.18 ± 0.22	-0.28 ± 0.20	0.085
TSA-grown	0	7.95 ± 0.12	N/D	6.55 ± 0.16	N/D	N/D
TSA-grown	1	8.15 ± 0.14	0.21 ± 0.03	5.66 ± 0.40	-0.89 ± 0.47	0.011*
TSA-grown	7	7.97 ± 0.07	0.02 ± 0.05	5.01 ± 0.06	-1.54 ± 0.18	<0.0001*
TSA-grown	14	7.55 ± 0.07	-0.40 ± 0.06	4.28 ± 0.17	-2.27 ± 0.27	<0.0001*
TSA-grown	21	7.47 ± 0.24	-0.48 ± 0.14	3.87 ± 0.16	-2.68 ± 0.22	<0.0001*
TSA-grown	28	7.38 ± 0.21	-0.56 ± 0.15	3.79 ± 0.04	-2.76 ± 0.15	<0.0001*
TSB-grown	0	4.97 ± 0.21	N/D			
TSB-grown	1	5.33 ± 0.26	0.36 ± 0.11			
TSB-grown	7	4.49 ± 0.48	-0.48 ± 0.35		N/D	
TSB-grown	14	4.34 ± 0.48	-0.63 ± 0.27			
TSB-grown	21	4.68 ± 0.37	-0.29 ± 0.16			
TSB-grown	28	3.72 ± 0.50	-1.25 ± 0.29			

Table 4. 2 Comparison of *Enterococcus faecium* and *Salmonella enterica* recovery from inoculated cumin after 1, 7, 14, 21, and 28 days dry storage. Statistical analysis by oneway ANOVA compared *Salmonella* and *Enterococcus* log reductions based on log CFU/g recovery. *Enterococcus* showed significantly fewer log reductions in recovery at all time points during storage compared to *Salmonella* when both were inoculated onto cumin using the TSA-grown method. *Salmonella* had fewer log reductions at 1, 7, and 21d when cumin was inoculated using the biofilm method. The TSB-grown method was not applied to cumin. (^) denotes significant difference in which *Enterococcus* overestimates *Salmonella* log reductions. (*) denotes significance in which *Enteroccocus* underestimates *Salmonella* log reductions. N/D is not determined.

Method, Spice	Linear model coefficients (r^2)		Logarithmic model coefficients (r^2)	
	Enterococcus	*Salmonella*	*Enterococcus*	*Salmonella*
Biofilm, Peppercorn	0.02	0.16	-	0.24
TSA-grown, Peppercorn	0.29	0.71	0.73	-
TSB-grown, Peppercorn	0.32	0.83	0.90	-
Biofilm, Cumin	0.40	0.36	0.45	0.40
TSA-grown, Cumin	0.85	0.83	0.87	0.90

Table 4. 3 Correlation coefficients from fitted regression analysis of Enterococcus and Salmonella survival over 28 days storage. Regression analysis was conducted by plotting the mean log reductions of *Enterococcus* and *Salmonella* (data from Chapter 3) over 28 days within each inoculation method and fitting the data for each organism with both linear and logarithmic trend lines. Correlation values (r^2) were determined to approximate goodness of fit for each organism.

CHAPTER 5: CONCLUSIONS AND FUTURE RESEARCH

The purpose of this research was to determine the survival capabilities of *Salmonella enterica* and *Enterococcus faecium*, a potential surrogate for *Salmonella*, on whole dry spices after inoculation, drying, and a period of storage at low water activity. This research concluded that *Salmonella* is able to survive at or near initial inoculation levels for 28 days beyond the initial drying step as a function of the method of inoculation (liquid-grown, agar-grown, dry transfer, or biofilm inclusion). Cells included in a biofilm matrix or grown on agar medium during inoculation preparation were the most recoverable after storage, suggesting that these methods of inoculation yield cells more resistant to drying and storage at low water activity than other methods, specifically traditional liquid culture. These findings indicate that choosing inoculation methods other than traditional liquid culture methods may be beneficial when working with dry spices. Additionally, it should be noted that the dry transfer method showed positive preliminary recoveries (Appendix B), despite low inoculation performance during shelf life studies. The dry transfer concept remains an attractive alternative to liquid application on spices and its practicality could be improved if higher initial inoculations could be achieved. It is recommended that these methods be considered for adaptations in future work when validating thermal processes for *Salmonella* inactivation on spices.

Since the publishing of the manuscript in Chapter 3, there has been an interest in the consistency of *Salmonella* recovery between samples within a batch of inoculated seed. Repeatability of *Salmonella* recovery within a single batch was determined to be satisfactorily consistent, with 0.2 log CFU/g difference in recovery for peppercorns prior to drying, and 0.6 log CFU/g after drying (n=5) (Appendix C).

The research further concluded that *Enterococcus faecium* is able to survive at or near initial inoculation levels in a similar pattern to *Salmonella* after drying and storage at low water activity (a_w 0.3). This data gives confirmation of behavioral similarities in survival during dry storage to *Salmonella* that are desirable in order to consider *Enterococcus faecium* as an appropriate conservative surrogate organism for the pathogen in thermal processes. However, it must be emphasized that the results and application of the methods described here are applicable only to *Enterococcus* acceptability as a *Salmonella* surrogate as it relates to shelf life. Any application of *Enterococcus* as a surrogate within inactivation processes will require further experimentation to collect inactivation data before any validation of the surrogacy for the process can be confirmed.

Given that inoculation can impact survivability of cells, future research must look at whether survival of those cells through inactivation processes is effected by inoculation method. By using inoculation methods that produce cells with tolerance to low water activity, researchers can design protocols that more closely mimic the types of cells associated with the outbreak strains isolated from contaminated low water activity foods. It is important to collect processing data for inactivation validation using representative cells in order to produce an accurate estimation of parameters needed for pathogen inactivation. Implementing methods that yield longer surviving cells will also enable more flexibility in preparation of inoculated materials and a greater likelihood that the use of weak or unrepresentative cells will be avoided.

Future research should focus on determining process parameters that will reliably and repeatably inactivate *Salmonella enterica* on peppercorn and cumin substrates. Current and alternative industry practices should be evaluated, specifically ethylene oxide

fumigation and steam treatments. Ethylene oxide (EtO) fumigation is a widely used method for reduction of microbial load in spices in the United States. The ASTA estimates that between 40-85% of all spices imported into the U.S. are treated with EtO annually *(1)*. The EtO fumigation process has historically been favored by industry in part because it has little to no detectable impact on product quality and sensory attributes. In a similar application, it was demonstrated that a related gas, propylene oxide, was capable of inactivation *Salmonella* ser. Enteritidis on dry almonds *(4)*. However, little data has been published documenting the efficacy of inactivation of *Salmonella* species in spices specifically.

Steam is widely used in the food industry as a treatment method for microbial inactivation. It can be a highly beneficial processing alternative in applications in which the addition of liquid water or chemicals for microbial reduction would compromise the quality of the food product. Low moisture steam processes are becoming increasingly used in place of chemical fumigation processes such as ethylene oxide treatment. Ethylene oxide is recognized as a pesticide by the EPA and as such, alternative methods are desired as a means to circumvent concerns over potential health effects from residues left behind. Validated steam processing is a desirable approach based on industry support, favor by international regulatory agencies, and reduced health risks associated with the process.

Steam treatment is known to be effective at deactivating *Salmonella* spp. in a variety of low water activity applications, including food processing equipment surfaces and on almonds *(2, 3)*. There is also evidence in the literature to suggest steam is adaptable for spice processing, specifically in black peppercorn, based on successful multi-log

reduction of aerobic mesophile bacteria *(5)*. However, very little data has been published demonstrating efficacy and acceptability of steam treatment targeted specifically for *Salmonella* in the application of spices. Developing process parameters for ethylene oxide and steam treatments that have demonstrated efficacy in *Salmonella* inactivation on spices, as well as the confirmation of *Enterococcus faecium* as a *Salmonella* surrogate in these processes, will enable manufacturers to improve the uniformity and reliability of their pathogen inactivation strategies and minimize the risk of *Salmonella* exposure to the consumer.

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APPENDICES

Appendix A: JFP Copyright permission

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Customer: Lauren Bowman
Account Number: 3000925924
Organization: Lauren Bowman
Email: stewie16@vt.edu
Phone: +1 (757)6729550
Payment Method: Invoice

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Journal of food protection

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Title of the article or chapter the portion is from	INOCULATION PREPARATION EFFECTS SURVIVAL OF SALMONELLA ENTERICA ON WHOLE BLACK PEPPERCORN AND CUMIN SEEDS STORED AT LOW WATER ACTIVITY
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Appendix B: Preliminary data for *Salmonella* inoculation of dry carrier and transfer to peppercorn

Methods: A dry inoculation method similar to that described in Chapter 3 was performed to transfer *Salmonella* Tennessee from an inoculated, pharmaceutical-grade silica particle to whole peppercorns. *Salmonella* Tennessee was grown overnight at 37 °C on large petri dishes (150 x 15 mm). After 24 hr, cells were scraped from the plate using sterile cotton swabs and suspended within 15 ml of sterile 0.1% peptone. The inoculated peptone was transferred to 100g of silica sand. Several approaches to disperse the liquid within the sand were undertaken including: hand massaging, mixing by inverting and constant shaking for 1 min were performed. The silica particles were dried within a biosafety cabinet for 48h. The length of drying time to achieve a_w 0.3 was determined using non-inoculated sand inoculated with the same volume of liquid and a_w measured every 12h for 48h. Water activity was measured using a dew point water activity meter (Aqualab 4TE, Aqualab, Pullman, WA). The inoculated sand and peppercorns were combined in different ratios, and several approaches to disperse the carrier throughout the peppercorns were undertaken including: hand massaging, constant shaking for 1 min and constant shaking overnight were performed. A sterile sieve was used to remove the inoculated sand from the peppercorns prior to enumeration. To ensure adequate transfer from the silica particle to the spice, bacteria were enumerated from wet sand, dry sand, and peppercorns after transfer. Bacteria were enumerated as described in Chapters 3, 4.

Results: In preliminary investigations, the average transfer of *Salmonella* from dry sand to peppercorns was 66%, achieving on average 5.1 log CFU *Salmonella*/g peppercorn (Table B-1). There was no significant increase in final log CFU *Salmonella*/g when larger amounts of peppercorns (100g) were used compared to 50g samples (results not shown). *Salmonella* were recovered in high concentrations from dry sand for up to 7d after drying (9d after inoculation), indicating the sand carrier can be prepped in advance for inoculation of large amounts of peppercorns (Table B-2). An increase in the transfer of *Salmonella* from sand to peppercorns occurred when sand and peppercorns were mixed for 24h compared to an initial mixing for 1 min (Table B-3).

Table B.1. Concentrations of *Salmonella* in liquid inoculum, and recovered from wet sand, before and after drying, and after transfer to dry peppercorns. Samples represent 50g peppercorn, 25g inoculated sand mixed for 24h at 25 °C.

	Liquid culture	Wet sand	Dry sand	Peppercorns
Log CFU/g *Salmonella*	9.9	8.4	7.7	5.1

Values are means of n= 2 for liquid culture and wet sand, n=3 for dry sand and peppercorns.

Table B.2. Concentrations of *Salmonella* recovered after inoculation on sand.

	Wet Sand	Dry sand 48h post-inoculation	Dry sand 3d after drying	Dry sand 7d after drying
Log CFU/g *Salmonella*	9.9	7.7	7.6	7.9

Table B.3. Concentrations of *Salmonella* recovered from peppercorns after dry transfer from sand particles using two mixing methods.

	Hand mixing	Shaking 24h
Log CFU/g *Salmonella*	5.07	5.54

Appendix C: Consistency of *Salmonella* recovery within inoculated peppercorn batches

Methods: A wet inoculation method similar to the method described in Chapter 3 was performed to transfer *Salmonella* Tennessee directly to whole peppercorns. *Salmonella* Tennessee was grown overnight in Tryptic Soy Broth at 37 °C with shaking at 180 rpm. Cells were washed in 10 ml 0.1% peptone buffer, and suspended in 20 ml sterile PT buffer. The 20 ml suspension was applied to 50 g of dry whole seeds within 27 oz Whirl-pak bags and massaged by hand for 1 minute to evenly coat the seed kernels. Inoculated spices were spread out in a single layer on sanitized aluminum trays (46 cm. x 66 cm.) and dried for 24 h at room temperature (23-25 °C) in a biological safety cabinet (final a_w = 0.3). Samples (10 g each) were taken for enumeration upon inoculation (wet) and at 24h after inoculation (dry). Enumerations were performed as described in Chapter 3.

Results: Repeatability of *Salmonella* recovery within a single batch was 0.2 log CFU/g for peppercorns prior to drying, however the variation in recoverable *Salmonella* increased to 0.6 log CFU/g after drying (n=5).

Table C.1. Consistency of *Salmonella* recovery within inoculated batches

Sample	TSA-Recovered aerobic bacteria (log CFU/ml)	XLT4-Recovered *Salmonella* (log CFU/ml)
Wet inoculation sample 1 (wet)	8.25 ± 0.05	8.08 ± 0.02
Wet inoculation sample 2 (wet)	8.59 ± 0.16	8.28 ± 0.01
Wet inoculation sample 3 (24h dry)	9.06 ± 0.27	8.19 ± 0.39
Wet inoculation sample 4 (24h dry)	8.62 ± 0.07	7.54 ± 1.06
Wet inoculation sample 5 (24h dry)	8.60 ± 0.01	8.7

Each sample represents 10 g inoculated peppercorn all taken from within a single batch of inoculated peppercorn, homogenized in 90 ml peptone recovery buffer for enumeration