INACTIVATION OF *SALMONELLA ENTERICA* AND *ENTEROCOCCUS FAECIUM* ON WHOLE BLACK PEPPERCORNS AND CUMIN SEEDS USING STEAM AND ETHYLENE OXIDE FUMIGATION

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SCIENTIFIC ABSTRACT

Current methods to reduce the native microbiota and potential pathogens on spices include steam treatments and ethylene oxide (EtO) fumigation. The objectives of this research were to identify the effectiveness of a lab-scale steam apparatus and a commercial EtO process on the inactivation of *Salmonella enterica* or *Enterococcus faecium* NRRL B-2354 inoculated whole black peppercorns and cumin seeds.

Peppercorns and cumin seeds were inoculated with *Salmonella or Enterococcus* and processed in a lab-scale steam apparatus at 16.9 PSIA and two references temperatures (165°F and 180°F) and in a commercial ethylene oxide fumigation chamber using a standard commercial EtO fumigation process. Cells were enumerated by serial dilution and plating onto TSA with a thin overlay of selective media.

Inoculation preparation influenced inactivation of *Salmonella* on peppercorns with greater reductions reported for TSA-grown cells compared to within a biofilm. To achieve an assured 5-log reduction of TSA-inoculated *Salmonella* on peppercorns exposure for 125s and 100s at 165°F and 180°F, respectively is required. For cumin seeds temperatures of 165°F for 110s were needed or 65s at 180°F to assure 5 log reduction. EtO fumigation significantly reduced both microorganisms on both spices ($p<0.05$), however significant variation existed between bags in the same process run. Reductions of *Enterococcus* were comparable or less than that of *Salmonella* under the majority of conditions, however a direct linear relationship cannot

be used to compare the microbes. This study demonstrates that the effectiveness of *Enterococcus faecium* NRRL B-2354 as a surrogate for *Salmonella* can vary between spices and processes.

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PUBLIC ABSTRACT

Spices are used widely throughout the world to enhance the flavor of food. In the past couple of decades, however, outbreaks have been associated with the consumption of *Salmonella* contaminated spices. The majority of spices consumed in the United States are imported from countries with tropical climates. Most large spice producers follow guidelines to ensure that their product is safe. However, contamination by human pathogens may still occur prompting the need for interventions that improve the quality and safety of spices. Current methods to reduce microorganisms in spices include steam treatment, and ethylene oxide fumigation. The effectiveness of steam and ethylene oxide fumigation for reducing *Salmonella* on whole black peppercorns and cumin seeds was investigated. Furthermore, a surrogate, *Enterococcus faecium* NRRL B-2354 was compared to *Salmonella* to determine the potential of using this harmless microorganism to validate the effectiveness of steam and ethylene oxide processes within food plants where foodborne pathogens cannot be used.

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DEDICATION

This work is dedicated to my parents, Jamie and Michelle Newkirk, who made sure to continuously push me out of my comfort zone. As much as I disliked it at times, it has shaped me into the leader, student, athlete, and independent woman I am today. Their unconditional love and support is something I will forever cherish.

ATTRIBUTION

Multiple contributions were made to this research from the following:

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 provided guidance and funding for this research, assisted with data analysis as well as experimental design. She is a co-author on the manuscripts in Chapters 3 and 4.

Robert C. Williams, PhD, (Food Science & Technology Department at Virginia Tech) is currently an Associate professor of food microbiology and food safety. Dr. Williams was a co-author on Chapters 3 and 4 and provided guidance throughout the duration of the research.

Kim M. Waterman, MS, (Food Science & Technology Department at Virginia Tech) is currently the food microbiology lab manager, supporting departmental and graduate research. Ms. Waterman provided a great deal of technical assistance throughout the duration of the research.

Brian D. Wiersema, MS, (Food Science & Technology Department at Virginia Tech) is currently the pilot plant manager of the Human and Agricultural Biosciences Building 1. Mr. Wiersema helped develop and assemble the steam processing apparatus discussed in Chapter 3.

Brett Driver (Food Science & Technology Department at Virginia Tech) is currently a lab specialist. Mr. Driver provided assistance with the operation of the steam processing apparatus discussed in Chapter 3.

Jian Wu, PhD, (Food Science & Technology Department at Virginia Tech) is currently a project associate for the food safety and food microbiology lab. Dr. Wu provided assistance on the ethylene oxide research discussed in Chapter 4.

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 guidance on the design of the steam processing apparatus discussed in Chapter 3.

Sean F. O'Keefe, PhD, (Food Science & Technology Department at Virginia Tech) is currently a professor of flavor chemistry and food product development. Dr. Sean O'Keefe provided guidance on the heating element of the steam processing apparatus.

TABLE OF CONTENTS

CHAPTER 1: INTRODUCTION AND JUSTIFICATION

Culinary traditions, methods, and flavors vary widely between cultures across the world. One thing in common, however, is that they all utilize spices to enhance the flavor in food. In many cultures, spices are considered ready-to-eat and are added to foods after cooking.

Spices have been linked to many outbreaks of Salmonellosis, which can cause serious and sometimes fatal infections in young children, the elderly, or people with weakened immune systems. Several outbreaks linked to the consumption of *Salmonella* contaminated spices have occurred (40, 107, 160). A recent outbreak associated with contaminated black pepper occurred between July 1, 2009 and April 28, 2010, when 272 people from 44 states had been infected with *Salmonella* Montevideo after consuming salami containing the contaminated spice (40). Since then, at least 49 recalls of spices sold by wholesale clubs, grocery stores, and of other food products containing spices have occurred due to reasons such as *Salmonella* and undeclared allergen contamination (63). Such recalls pose a challenge for the food industry because it can be very difficult to trace back more than one or two levels in the spice chain (18).

The United States does not have the tropical climate necessary for most spice growth; therefore, the majority of spices consumed in the United States are imported from countries such as Indonesia, Mexico, China, and India. Most large spice producers base their food safety and hygiene practices based on specifications from third party auditors such as the Global Food Safety Initiative (GFSI), and International Standardization Organization (ISO) 22000, which encompasses food safety management, and guidance documents. The American Spice Trade Association (ASTA), a trade organization has also created benchmark guidelines for suppliers and manufacturers. However, small spice producers might not follow these recommendations and these spices may be unwittingly obtained in a global marketplace. Quality standards in place

by the U.S. Food and Drug Administration (FDA), called food defect action levels, indicate that black peppercorns are allowed to contain an average of 1% or more pieces by weight of each of the following: insect filth and/or insect mold, mammalian excreta, and foreign matter. Cumin seeds, on the other hand, are allowed to contain an average of 9.5% or more ash and/or 1.5% or more acid insoluble ash (60). If the product exceeds these levels, the FDA will then consider it adulterated and subject to enforcement action (60). An ongoing recall of ground cumin and cumin-containing foods due to undeclared peanuts or almonds is currently under investigation. It is hypothesized that the spice was intentionally adulterated with these nut products to increase the product volume for economic gain (8). Food agencies in the United States, Europe, and Canada have been trying track the source of the contamination, however, the more intermediates the spice passes through, the more difficult it is to find the offender.

Due to the potential for not only pathogen contamination but also food fraud, companies purchasing these spices are seeking additional verification of safety including having them processed using methods such as steam, ethylene oxide fumigation, or irradiation to further reduce the risk of pathogen contamination. There is published literature on these methods with other low water activity products but information on the effectiveness of steam and ethylene oxide on the inactivation of *Salmonella* on spices is scarce (44, 51, 106, 109, 155, 161). The goal of this research is to determine how these methods can further reduce the risk of pathogen contamination of spices.

Objectives of this research were the following:

1. Identify the effectiveness of a lab-scale steam apparatus at inactivating *Salmonella enterica* on whole black peppercorns and cumin seeds at one

pressure (16.9 PSIA) and two commonly used reference temperatures (165°F and 180°F).

2. Determine if a commercial ethylene oxide fumigation process is able to achieve a 5-log reduction of *Salmonella enterica* on whole black peppercorns and cumin seeds.
3. Determine if *Enterococcus faecium* is a compatible surrogate for the inactivation of *Salmonella* on spices using these two processes.

The null hypotheses include the following:

1. The custom-built steam apparatus will not be effective at inactivating *Salmonella enterica* on whole black peppercorns and cumin seeds at the specified pressure and temperatures.
2. A commercial ethylene oxide process is not able to achieve a 5-log reduction of *Salmonella* on whole black peppercorns and cumin seeds.
3. *Enterococcus faecium* is not a compatible surrogate for the inactivation of *Salmonella* using a lab-scale steam apparatus and ethylene oxide fumigation.

CHAPTER 2: LITERATURE REVIEW

Spices

Spices are defined by the United States Food and Drug Administration (FDA) as, "*Any aromatic vegetable substance in the whole, broken, or ground form, except for those substances which have been traditionally regarded as foods, such as onions, garlic, and celery; whose significant function in food is seasoning rather than nutritional; that is true to name; and from which no portion of any volatile oil or other flavoring principle has been removed"* (62). During ancient times, spices were used as medicines, perfumes, and cosmetics but were finally introduced in western cooking in the 1st century AD (162).

Spices are often considered to be the foundation of the modern world. They were introduced in North America toward the end of the nineteenth century and beginning of the twentieth when Europeans started migrating to the new world (59). Due to a large variety of cultures and customs, the demand for spices grew rapidly and United States quickly became one of the largest importers of spices. Spices are typically fumigated with methyl bromide to eradicate pests (131), shipped into the United States in the whole form in various containers including bulk shipping containers or burlap sacs, and are then inspected by the FDA for wholesomeness and cleanliness on the docks. The spices are then stored by the importer and eventually sold to spice companies throughout the country (59). The company is then in charge of processing and packaging the spices for retail distribution.

Most of the spices imported into the United States originate from the eastern hemisphere from nations such as India, China, and Indonesia. However, Central and South America as well as the West Indies are also contributing high quantities to the world market (59). The most

popular types of spices imported into the United States include black and white pepper followed by mustard seed, capsicum, cassia, paprika, coriander, ginger, and oregano (59).

Black Peppercorn

Piper nigrum L.*,* also known as black pepper, is a tropical vine that originated on the Malabar coast of south-western India (47). Throughout history, this spice has been considered one of the most precious of them all. In fact, it used to be so expensive that it was substituted for money. Pepper is a round, berry-like fruit that grows in tropical climates (4). The vines that the fruit grows on can grow as tall as 20 feet high (59). The leaves on the vines are almond-shaped with a long, dangling string of pendant berries called drupes. They change from green to red in color as they ripen. To produce black peppercorns, the fruit is harvested when fully grown but still green and shiny (4). Cultivation practices include picking the fruit and drying them in the sun until the berries turn black. Alternatively, the berries are picked and blanched using boiling water and then dried out in the sun for several days so that they turn black in color. Once dried, the fruit is known as a peppercorn. The flavor has been described as warm, sweetly spicy, woody, fruity, musty, with a pleasantly warm aftertaste (59). Pepper is easily incorporated into sweet and savory dishes, which is why the spice is so popular. The climate in the United States is not able to support the growth of *Piper nigrum* L. Therefore, all of the black (and white) pepper consumed in the U.S. is dependent upon imports exclusively (59).

Cumin

The spice, cumin, is made from the dried seed of the herb *Cuminum cyminum*, a plant in the Apiaceae family (59). It is mostly grown in warm climates but can also grow as far north as Norway (47). It is currently cultivated in Argentina, China, Cyprus, Denmark, India, Iran, Lebanon, Malta, Mexico, Morocco, Russia, Sicily, Syria, and Turkey (59). The herb grows to a

height of about 24 inches and has fine leaves with white or rose-colored flowers (59). The seed that is produced from this herb is a yellow-brown color, long, skinny, oval-like shape, with multiple vertical ridges and a short stem. Cumin seeds are known for their warm, spicy, aromatic, and pharmaceutical taste (59). The two largest importers of cumin seeds are the United States and EU countries (162).

Microbiological Quality and Safety of Spices

Spices have been used throughout history as a way to enhance the flavor in foods and for their antimicrobial properties and preservative characteristics. Since the beginning of the spice trade, this commodity has been traded as dry product for easy transportation and storage. However, spices grown in humid tropical regions are difficult to dry and have limited availability of equipment for mechanical drying (133). Drying and storage can reduce the number of vegetative cells and cause die-off that is enhanced by oxidation (58). The remaining microbiota consists mostly of spore-forming bacteria and molds (80). For this reason, the microbiological quality of spices is often dependent upon the hygienic situation of the region where they are produced and handled (58).

The original background microbiota, proliferation, and die-off all contribute to the number of viable cells on spices post harvest (58). Sources of contamination in spices include but are not limited to: dust and soil, and fecal matter from birds, rodents, and other animals. Likewise, microbial counts of spices can differ depending on storage conditions and moisture content. Spices and herbs have a wide range of microbial contamination (120). The majority of the microbial population that survives on spices are spore formers and yeasts or molds due to their ability to tolerate desiccation environments (58). Of the spore formers, the *Bacillus* species is the most commonly identified (58). Spore-forming bacteria such as *Bacillus cereus* and

Clostridium perfringens that can cause gastroenteritis when consumed in large numbers are frequently found in spices (52, 53, 97, 120, 126, 132). However, a large variety of non spore-forming bacteria can be present in spices (58, 120). Coliforms, a group of bacteria that may indicate fecal contamination, are the most common non-spore forming bacteria found on spices, although they are typically found in numbers less than 10 per gram. *Aspergillus* spp., *Penicillium* spp., and *Fusarium* spp., *Alternaria* spp., *Rhizopus* spp., and *Mucor* spp. are some of the most common mold species found on spices (58).

Some of the spices most commonly associated with high levels of native microbiota include black pepper, turmeric, the capsicum spices, coriander, and allspice (19, 53, 66, 96). Background microbiota of whole black peppercorns and cumin seeds are listed in Table 1. Out of about 130 pepper samples, over 90 percent contained aerobic plate counts at least 6-logs CFU/g or greater and mold counts between 3-5 logs CFU/g (80, 120). Although not as high as black pepper, cumin seeds also have a moderately high level of background microbiota. Out of 27 cumin samples, over 50 percent had aerobic plate counts of 5-6 logs CFU/g and mold counts between 2-4 logs CFU/g (80, 120). Black peppercorns and cumin seeds sampled from Mexican markets both contained high levels (5-7 logs CFU/g) of aerobic bacteria (67). Likewise, in India, 6 samples of both black pepper and cumin seeds were evaluated to determine their microbial status. For black peppercorns, 83 percent of the samples were in between 6.1 and 9.0 log CFU/g whereas 44 percent of cumin seeds samples were within the same range (17).

The bacteria *Salmonella* is not found as frequently in spices. However, in recent years, spices have been noted as a vehicle of human illness because of this bacterium. *Salmonella* is the most common bacterial pathogen linked to outbreaks and product recalls of spices (3). Black peppercorns (n=87) were sampled for *Salmonella* and resulted in a fairly high frequency of 8.2%.

These results are contrary to previous studies that reported a low frequency of *Salmonella* contaminated spices (85, 104). Contamination caused by this bacterium is a major concern because the product is normally consumed raw and added to foods after the food is cooked.

Table 1. *Distribution (%) of Aerobic Plate Counts (APC) and yeast and mold counts of black peppercorns and cumin seeds (80, 120).*

		<2	2-3	3-4	4-5	5-6	6-7	>7		<2	2-3	3-4	4-5	5-6	6-7
Spice	**No. Tested**	**Aerobic Plate Count (Log CFU/g)**							**No. Tested**	**Yeast & Mold Count (Log CFU/g)**					
Black Pepper	144				2	4	43	51	118	30	10	22	11	10	17
Cumin Seeds	27		4	0	37	44	15		23	35	13	35	8	9	

Salmonellosis

Salmonellosis is an infection caused by the pathogenic bacteria *Salmonella.* These bacteria are Gram-negative, facultatively anaerobic, straight, small rods that are usually motile with peritrichous flagella (20). In developed countries, Salmonellosis is recognized as one of the most prevalent foodborne illnesses associated with outbreaks and sporadic cases (20). The Centers for Disease Control and Prevention (CDC) estimates that *Salmonella* causes 1.2 million illnesses, as well as 19,000 hospitalizations and 380 deaths in the United States every year (42).

Salmonellosis arises because *Salmonella* causes an infection which means that the bacteria grows and multiplies in the host's body. As a gastrointestinal pathogen, it is able to penetrate the intestinal barrier and function as an intracellular pathogen in phagocytic cells (45). Symptoms of Salmonellosis include diarrhea, fever, and abdominal cramps within 12 to 72 hours after infection and can last for 4-7 days. Hospitalization of patients with Salmonellosis is typically due to dehydration from diarrhea. In developed countries, patients with Salmonellosis

typically recover with treatment methods such as fluid and electrolyte replacement. The use of antibiotics can actually increase the time that the organism is excreted so they are not recommended as a method for treatment (20). Immunocompromised individuals are particularly at risk because *Salmonella* can overcome the body's natural defense systems and reach the blood stream, resulting in severe illness.

Between the years 2007 and 2010, three large-scale Salmonellosis outbreaks in the United States were attributed to the consumption of *Salmonella* contaminated spices (41, 77, 143, 156). These outbreaks occurred within a 40 month period (January 2007-April 2010) (157). Between January 2007 and December 2007, there were 69 cases of *Salmonella* Wandsworth illness that spread across 23 states (157). It was confirmed that the illness was due to the consumption of seasoning mix containing broccoli powder, parsley powder, and other spices used to coat a puffed snack product (143). Between December 2008 and April 2009, 87 cases of *Salmonella* Rissen illness occurred across five states. Epidemiologic investigations, traceback investigations, and product sampling determined that the culprit was contaminated white pepper consumed in restaurants and hospitals (2, 77). Lastly, between the years of July 2009 and April 2010, there were 272 confirmed cases of *Salmonella* Montevideo illness from 44 states (41, 70). Epidemiological and traceback investigations determined that the outbreak was linked to the consumption of ready-to-eat salami products that contained *Salmonella* Montevideo contaminated black pepper and red pepper (41, 70).

Salmonella **survival at low water activity**

The drying of food is a traditional preservation method that inhibits the growth of microorganisms. Water activity (a_w) is a quantitative measurement used to determine the shelf life of a product (64) on a scale of 0 to 1.0. This term denotes the vapor pressure of a substance

compared to the vapor pressure of pure water at the same temperature. In other words, it is the amount of free or unbound water in a product that is available for microbial growth. There is no clear definition of low water activity foods. Some foods are considered to have a low water activity if they have an a_w below 0.7 (137) while others use <0.85 to define low water activity foods (22). The a_w of a food product is dependent upon the storage conditions and food composition (64). Common examples of low water activity foods include chocolate, peanut butter, preserved meat, powdered infant formula, soup mixes, and spices. It is sometimes inaccurately assumed that these foods are low risk because they are unable to support microbial growth. However, these food commodities remain very susceptible to contamination and have been implicated in many foodborne outbreaks (37, 38, 39, 46, 93, 134, 141).

In general, growth of pathogenic bacteria should not occur below a water activity of 0.85 (58). Although, growth of halophilic bacteria can occur at a_w as low as 0.75 (22). In a desiccated state, metabolism is greatly reduced which means that growth does not occur but vegetative cells and spores can be viable for months and sometimes years (22). *Salmonella* has proven that is able to grow at an a_w as low as 0.93 (21) and is also able to survive for months or even years in certain low moisture foods (123). In ground black pepper specifically, the a_w threshold for growth of *Salmonella* is 0.9793 ± 0.0027 at 35^{o}C (90).

The survival of *Salmonella* during storage conditions has been investigated (90). Ground black pepper was inoculated with approximately 8 logs CFU/g and stored at 25 and 35^{o}C at high (97% RH) and ambient (equal to or less than 40% RH). At high relative humidity, no *Salmonella* was detected after 100 days and 45 days at 25 and 35^{o}C, respectively. However, the ground pepper stored in ambient humidity conditions showed an initial decrease of *Salmonella* by 3-4 log CFU/g and then remained stable for over 8 months at 25 and 35^{o}C (90). Likewise, almond

kernels were inoculated with *Salmonella* Enteritidis (PT) 30 to 7.1 or 8.0 log CFU per almond and stored for 171 and 550 days, respectively, at various temperatures (154). On average, the calculated reduction of *Salmonella* was 0.25 ± 0.05 log CFU/month at 23^{o}C. Halva, a confectionary product with a a_w of 0.18, contains tahini, sugar, citric acid, and soapwort root extract. *Salmonella* cannot multiply in the product because of the very low water activity. However, *Salmonella* Enteritidis survived in vacuum-packed halva stored for 8 months under refrigeration (103). In another instance, the survival of *Salmonella* in peanut butter and peanut butter spread was investigated (35). Peanut butter and peanut butter spread was inoculated with 5.7 log CFU/g of *Salmonella* and stored at 21 and 5^{o}C. After 24 weeks, *Salmonella* was reduced by 4.1 to 4.5-log at 21 ^{o}C and 2.9 to 4.3-log at 5^{o}C.

Not only can *Salmonella* survive in low water activity foods but it has also demonstrated that it can survive on common contact surfaces in food processing environments such as stainless steel, plastic, paper, and cement (15, 75, 78, 84, 112, 167). The combination of these facts poses very large threats for the food industry.

Heat resistance of *Salmonella*

Processing methods such as mild heat treatment is very effective at inactivating *Salmonella* in high-a_w foods. However, low water activity foods pose a challenge because as the a_w of a food decreases, the heat resistance of *Salmonella* increases (35, 79, 92, 114). The extent of heat resistance of pathogens in low-a_w foods is difficult to predict because it varies greatly between pathogen and type of solute present (28, 54, 69, 138). The thermal resistance of *Salmonella* can be greatly influenced by both intrinsic and extrinsic properties of a food. For this reason, it is difficult to compare the heat resistance of *Salmonella* in different food commodities, especially if there are variations in processing parameters. However, understanding how

Salmonella responds to heat in various foods can help predict how it might respond in the target food.

Chocolate and chocolate candies have a water activity of about 0.4-0.5, so low that organisms subjected to heat are essentially experiencing dry heat (123). In one instance, the heat resistance of *Salmonella* in milk chocolate exhibited D-values of 4.5, 4.6, and 6.6 hours at 71^{o}C for *Salmonella* Eastbourne, *Salmonella* Senftenberg, and *Salmonella* Typhimurium, respectively (108). Likewise, in molten chocolate, *Salmonella* Typhimurium had a D-value of 6.6 hours and 13.6 hours at 71.1 and 65.6^{o}C, respectively (71). These two studies (71, 108) illustrated that *Salmonella* Typhimurium is more heat resistant than *Salmonella* Senftenberg 775W in milk chocolate and the increase in D value is associated with the low a_w of chocolate.

The heat resistance of *Salmonella* serovars Agona, Enteritidis, and Typhimurium was demonstrated when Shachar and Yaron (139) inoculated peanut butter at 4 and 8 logs CFU/g and incubated in water baths at 70, 80, and 90^{o}C for 5 to 50 minutes at each of the temperatures. Results indicated that there was no significant difference in heat resistance between the *Salmonella* serovars ($P > 0.05$). Furthermore, the serovars were so heat resistant that there was only a 3.2-log reduction after remaining in the 90^{o}C water bath for 50 minutes. Shachar and Yaron (139) were able to predict that more than 260 minutes (> 4 hours) were needed to reduce *Salmonella* by 7 logs at 70^{o}C and more than one hour was needed at 90^{o}C. Therefore, some treatments used in the industry for example, 70^{o}C for 20 minutes, do not provide a sufficient inactivation of *Salmonella* in peanut butter. The authors believe that the combination of high fat content and low a_w in peanut butter provide a protective element for *Salmonella* and therefore make it more heat resistant (139).

Almonds are traditionally roasted by using high temperature or long roasting time (123). Commercial oil roasting of almonds requires that almonds remain at temperatures higher than 260^{o}F (126.7^{o}C) for longer than 2 minutes to achieve a 5-log reduction of *Salmonella* in almonds (1). The effect of steam treatment on the reduction of *Salmonella* serovar Enteritidis on the surface of raw almonds was investigated (106). The surface of the raw almonds (cultivars 'Nonpareil' and 'Mission') were inoculated and then treated with steam (93^{o}C ± 1^{o}C) for 5, 15, 25, 35, 45, 55, and 65 s. The effect of steam treatment improved with increasing treatment time. After 65 seconds of treatment, reductions in populations of *Salmonella* Enteritidis were 5.7 log CFU/g and 5.8 log CFU/g for 'Nonpareil' and 'Mission' cultivars, respectively. Likewise, a higher D-value of 16.1 seconds was calculated for *Salmonella* Enteritidis inoculated Mission almonds versus 12.2 seconds for the Nonpareil cultivar; indicating that varietal differences in the almonds resulted in differences in the inactivation of *Salmonella*. These results suggest that varietal differences should also be considered when inactivating *Salmonella* on almonds using steam.

Considerations for strain selection and inoculation procedures for process validation

Process validation involves the collection and evaluation of data that establishes scientific evidence that a process is consistently and repeatedly able to deliver a safe, quality product. Steps required for process validation include: identifying the pathogen of concern, identifying preventative control(s), identifying the most resistant pathogen, determining the level of control necessary, assessing the impact of the food matrix on the inactivation of the pathogen, determining an approach and the efficacy of the preventative control(s), and defining the critical limits of the proposed process (124). Strain selection is a critical part of process validation as

there can be differences in behavior and response to stress. *Salmonella* serovars vary in responses to environmental and process conditions (9, 111, 123, 158).

Temperature, water activity, substrate, culture media, serotype, and strain are factors that can influence the survival of *Salmonella* in low water activity foods (137). Likewise, additional factors could include the addition of solutes to the matrix, acidity, growth medium, stage of growth of the cells, stress prior to heating, and species and strain (123). Assuming log-linear kinetics can be applied to inactivate *Salmonella,* survival numbers can be estimated (137). However, survival curves of *Salmonella* in low-a_w foods typically do not follow log-linear kinetics. Instead, they show significant asymptotic tails (7, 111). Santillana Farakos et al. (136) used the Weibull model to examine the survival of *Salmonella* in low water activity foods at temperatures ranging from 21 to 80^{o}C and a_w levels below 0.6. This model was useful at predicting the survival of *Salmonella* in other low-a_w foods. It was also determined that the food composition, a_w, and temperature influenced the survival of *Salmonella*. To validate previously developed models, 1,064 data points were collected from *Salmonella* survival literature and fit into a log-linear model and Weibull model (137). Results demonstrated that temperature, a_w, product and serotype all influenced time required for first log-decimal reduction.

The reduction of *Salmonella* can be influenced by the water activity of a food product. Sucrose solution at various a_w levels (0.98 to 0.83) was inoculated with *Salmonella* Typhimurium and heated at 65.6^{o}C for the same time intervals (149). The decimal reduction time of *Salmonella* Typhimurium at a_w of 0.98 and 0.83 was 0.29 min and 40.2 min, respectively. *Salmonella* serovars also influence heat resistance under similar conditions. The rates of inactivation of three *Salmonella* strains of various serotypes (Enteritidis, Typhimurium, and Heidelberg) were compared with that of three strains of *Salmonella* Tennessee (111). A 5-log

reduction was achieved for the mixture of *Salmonella* Tennessee strains when the peanut butter remained at 90°C for 41 ± 3 min. On the other hand, only 26 ± 2 min was required for the other *Salmonella* isolates (111). Likewise, in milk chocolate, *Salmonella* Eastbourne, *Salmonella* Senftenberg, and *Salmonella* Typhimurium had D-values of 4.5, 4.6, and 6.6 hours at 71°C, respectively (105).

Inoculation strategies

Inoculation methods in a process validation study are important because they provide a high initial cell concentration to demonstrate the potential to achieve the desired reduction. The goal when determining inoculated strategies is to imitate potential contamination conditions of the product under investigation. Wet inoculation techniques are frequently used on a laboratory-scale because they are easy to control and produce high yields. However, in spice validation studies, such strategies are impractical because dry transfers during storage, transport, and processing of spices are probable sources for the contamination of spices (34).

Wet Inoculations

Studies have shown that such preparation methods of inoculum (liquid, biofilm inclusion, agar) can influence the bacterial stress response and the recoverability of *Salmonella* cells (13, 56, 73, 89, 154). *Salmonella enterica* (serotypes Typhimurium, Enteritidis, Newport, and Infantis) dried on polystyrene 96-well plates exhibited better dehydration tolerance over 12 weeks when the inoculum was grown on Luria-Bertani (LB) agar plates compared to cells grown in broth (73). Likewise, tryptic soy agar (TSA) grown cells of *Salmonella* Enteritidis PT 30 inoculated onto whole almond kernels were more resistant to desiccation compared to tryptic soy broth (TSB) grown cells with reductions of 1.7 and 3.7 CFU per almond for agar grown and broth grown inocula, respectively (154).

Biofilm inclusion inoculation methods have demonstrated improved survival of *Salmonella* compared to cells grown in liquid media (34). *Salmonella* Tennessee cells cultured using the biofilm inclusion method had better recovery of cells after desiccation and gastric passage compared to cells grown in broth (13). The ability of *Salmonella* to form biofilms contributes to its resistance and persistence which makes it very important in processing environments (145). Biofilms are an extracellular matrix that can house single, or multiple bacterial species (13). This matrix is comprised mostly of water and the rest is various extracellular polymers including polysaccharides and glycoproteins (68). The formation of a biofilm enables *Salmonella* to protect itself against thermal processing and sanitation (36). Furthermore, previous research has shown that *Salmonella* has an increased tolerance to desiccation, is more stable, and can be recovered in greater numbers from spices when in a biofilm compared to other methods (13). The ability for *Salmonella* to form a biofilm helps protect the organism against hostile conditions (142). Furthermore, this ability most likely enhances survival of *Salmonella* at water activity levels lower than those hypothesized by the food industry (13). Inactivation resistance of *Salmonella* is important to consider when determining inoculation strategies for process validation studies. A worse case scenario is ideal because inactivation data will provide a conservative estimate of reduction for the industry.

Dry Inoculations

Post-harvest cross contamination is likely to occur between dried surfaces and spices. Cross-contamination of foods and equipment can be attributed to the transfer of dry particles in the air, contaminated equipment, and pests (22, 55). For this reason, wet inoculation may not simulate the physiological and transfer potential in real world situations relevant in spice

validation studies. The development of dry inoculation methods could provide true contamination conditions and reduce the effects of additional moisture.

The introduction of liquid to spices can have an impact on the spice flavor and microbiology quality. Furthermore, it can cause a change in surface properties and water activity (161). This is a challenge when using low water activity foods because the intentional manipulation of water activity cannot always be reversed. In one instance, water was incorporated into dry products that were unable to achieve the original un-wetted water activity when re-dried (121). The volatile compounds associated with spices are susceptible to adulteration or leaching when exposed to liquid. Antimicrobials can also leach out of the spice, which can impact surface microbiota and create an artificially high presence of antimicrobials on the surface (127).

There are a variety of dry inoculation applications including the use of inoculated chalk on pecans, inoculated talc in peanut paste, and inoculated sand on walnuts and almonds (25, 29, 56). The use of chalk can provide adequate recoverability of *Salmonella*, however this method presents difficulty with the separation and collection of chalk residues post inoculation (25). *Salmonella* serovars Anatum, Enteritidis, Oranienburg, Sundsvall, and Tennessee were inoculated onto chalk and then transferred onto pecans to provide an initial recovery of 8.28 log CFU/g (24). Furthermore, wet inoculated cells were less resistant to stress than dry inoculated cells. Dry inocula on talc powder ($a_w \leq 0.55$) containing five strains of *Salmonella* Tennessee provided an initial recovery of greater than 8.3 log CFU/g and demonstrated no significant difference in recovery 30 days after preparation (56). *Salmonella* Enteritidis PT 30 was utilized in two different inoculation methods, wet and dry, to compare the survival of the pathogen on almond and walnut kernels (29). Sand was inoculated at a level of 10 log CFU/g to achieve

populations of 7.6-7.7 log CFU/g and then dried before getting transferred onto nut kernels. The reduction of *Salmonella* was similar for both of the inoculation methods and both of the nut kernels during ambient storage conditions (98 days) (29).

***Enterococcus faecium* as *Salmonella enterica* surrogate**

Surrogate microorganisms are non-pathogenic microorganisms that are used in actual factory or pilot plant settings to validate the efficacy of a process to inactivate target pathogenic microorganisms (100). The ideal surrogate is one that is non-pathogenic, models the inactivation kinetics of the target pathogen, has similar environmental tolerance as the target pathogen, has stable and consistent growth characteristics, is easily prepared to yield high-density populations, can remain stable in an inoculum form until used, and is easily enumerated from native microbiota (124).

The thermal resistance of a target pathogen and the surrogate can be impacted by the composition of the food (159). Many different microorganisms have been used as surrogates for *Salmonella* spp. A popular choice of surrogates in low water activity environments is lactic acid bacteria. *Pediococcus* spp. and *Pediococcus acidilactici* have been used as surrogates in ground-and-formed beef jerky (32) as well as in whole muscle turkey jerky (164) and dried pet food (43), respectively. Six different potential surrogates were used in six ground-and-formed beef jerky commercial process that differ widely in lethality (33). In all six processes, *Pediococcus* spp. and *Pediococcus acidilactici* had appropriate reductions to indicate sufficient lethality (≥5.0 log CFU/g) of *Salmonella* and *E. coli* O157:H7. A commercial turkey jerky process was used to demonstrate sufficient lethality of *Salmonella, E. coli* O157:H7, and the surrogate, *Pediococcus acidilactici* (164). Across all processes, there was no significant difference in the reduction of the three bacteria, demonstrating that *Pediococcus acidilactici* is an effective pathogen for in-

plant validation of turkey jerky processing. The same surrogate was compared to *Enterococcus faecium* NRRL B-2354 to demonstrate their potential as surrogates for seven *Salmonella* serovars (Anatum, Montevideo, Senftenberg 775W, Tennessee, Schwarzengrund, Infantis, and Mbandaka) in dried pet food (43). Dried pet food at moisture levels of 9.1, 17.9 and 27.0% was heated between 76.7 and 87.8°C to determine the *D*-values of the *P. acidilactici, E. faecium,* and *Salmonella* cocktail. It was determined that the *D*-values of both *P. acidilactici* and *E. faecium* were higher than those for the *Salmonella* cocktail, indicating that both *P. acidilactici* ATCC 8042 and *E. faecium* NRRL B-2354 can be used as a surrogates for *Salmonella* in dry pet foods. However, at temperatures below 90°C, the use of *P. acidilactici* is favored over *E. faecium* because it has a smaller *D*-value and lower heat resistance than the latter, therefore, it can be easier to work with to generate lethality values for validation studies (43).

Enterococcus faecium NRRL B-2354 has been used in the food industry for the past 64 years (101). *E. faecium* is a Gram-positive cocci, non-spore-former, that can survive in temperatures between 5-50°C and between pH 4.6-9.9, and is tolerant to high salt and desiccation conditions (65, 95). Certain strains of *E. faecium* have been associated with nosocomial infections; however, recent studies have shown that there is a significant difference between hospital- and community- associated strains of the bacteria. Therefore, *Enterococcus faecium* NRRL B-2354 is considered commensal and non-pathogenic (12, 98).

E. faecium has been used as an appropriate surrogate for *Salmonella* in multiple low water activity foods such as almonds, peanut paste, and extruded carbohydrate-protein meal (26, 32, 83, 88, 148). Almonds were treated using moist-air heating for various time, temperature, and humidity regimens (83). *E. faecium* had mean log reductions 0.6 log and 1.4 log lower than those for *Salmonella* Enteritidis PT 30. Likewise, the *D*-values of the surrogate were about 30

percent larger than those for *Salmonella*, indicating that *E. faecium* NRRL B-2354 is a conservative surrogate for *Salmonella* during the moist-air heating of almonds (83). *E. faecium* is used heavily by the Almond Board of California (ABC) in almond process validation studies for dry-heat processes and moist air or steam processes (83, 148). Peanut paste of four various water activities (a_w 0.3-0.6) and fat levels (47 and 56%) were inoculated with *Salmonella* Tennessee, *Salmonella* Typhimurium DT104, and *Enterococcus faecium* to measure their long term survival (88). The log reductions (CFU/g) of each bacteria were measured monthly over a 1 year period to determine that *E. faecium* demonstrated a higher survivability compared to the *Salmonella* serovars (88). *Enterococcus faecium* is also an appropriate surrogate for *Salmonella* in extruded carbohydrate-protein meal (26). Extrusions of the meal at different temperatures indicated that the temperature necessary in order to achieve a 5-log reduction was 73.7°C and 60.6°C for *E. faecium* and *Salmonella,* respectively. Since the inactivation of *E. faecium* occurred at higher temperatures than *Salmonella,* it is acceptable to use as a surrogate for *Salmonella* in extrusion processes. Research on the efficacy of *Enterococcus faecium* NRRL B-2354 as a surrogate for *Salmonella* spp. in low a_w foods provided a starting point to determine if it would also be a good surrogate for the inactivation of *Salmonella enterica* serovars in whole black peppercorns and cumin seeds.

CHAPTER 3: THE INACTIVATION OF *SALMONELLA ENTERICA* AND SURROGATE *ENTEROCOCCUS FAECIUM* ON WHOLE BLACK PEPPERCORNS AND CUMIN SEEDS USING STEAM

ABSTRACT

The microbiological safety and quality of spices is of concern for regulators and the food industry. Further processing using the vacuum assisted steam (dry steam) is performed for many spices to reduce microbial populations and ensure safe products of high quality. The objective of this research was to compare the effectiveness of a lab-scale steam process for the inactivation of *Salmonella enterica* and its surrogate *Enterococcus faecium* NRRL B-2354 inoculated onto whole peppercorns and cumin seeds. Whole black peppercorns were inoculated with a cocktail of *Salmonella enterica* serovars or *E. faecium* using agar grown cells or within a biofilm, while cumin seeds were inoculated using only the TSA-grown inoculation method. The spices were dried to a_w 0.3-0.5, and held 1 day before processing. Cells were enumerated by serial dilution and plated onto TSA for aerobic plate counts or TSA with a thin overlay of XLT4 for *Salmonella* and BEA for *E. faecium*. Average log reductions of *Salmonella* on peppercorns were 4.8 ± 0.19 and 6.3 ± 0.18 log CFU/g for biofilm inclusion and TSA-grown inoculated spices. Likewise, average log reductions of *E. faecium* was 4.7 ± 0.19 and 5.3 ± 0.18 for the biofilm inclusion and TSA-grown inoculation methods, respectively. The average log reduction of *Salmonella* and *E. faecium* on cumin seeds was 7.3 ± 0.21 and 6.7 ± 0.21 log CFU/g respectively. Implementation of validated processing methods that achieve minimum reduction requirements will reduce risk of pathogen exposure to consumers.

INTRODUCTION

Spices have been used throughout history not only as a way to enhance the flavor in foods. The vast majority of spices consumed in the United States are imported from countries with tropical climates such as India, Mexico, and Vietnam. Microorganisms in spices are attributed to the soil and plants from which they are grown and that survive during the drying process (58). Further contamination of spices can occur during the transportation, packing, and storage of spices.

In the past couple of decades, spices have been linked to many foodborne illness outbreaks. Fourteen outbreaks associated with the consumption of spices occurred between 1973 and 2010 (61). Out of the 14 outbreaks, 10 of them were caused by *Salmonella enterica* serotypes (61). In between the years of 1970 and 2003, 21 recalls of spices took place in the U.S. due to bacterial pathogens; 20 of those recalls were due to *Salmonella* contamination (160). Symptoms of Salmonellosis include nausea, vomiting, diarrhea, fever, abdominal cramps, chills, and headache, and can last 4-7 days.

Difficulties in implementation of good agricultural and manufacturing practices in many developing countries where spices are produced, coupled with enhanced survival in the desiccated state has prompted the development of post-harvest interventions to inactivate *Salmonella* sp. without compromising the quality of the spice. Current practices used by the industry to reduce microbial populations in spices include chemical fumigation, irradiation, and steam-based heat treatments (3). Steam-based heat treatments are a popular option of inactivation because they do not utilize any chemicals; only microscopic water droplets to improve heat transfer in food (153). Steam pasteurization can be effective because when steam condenses, a lot of heat is transferred into the target food, which increases the surface

temperature (81). Furthermore, when the same amount of steam and water are compared at any given temperature, steam has a greater heat capability that can penetrate small cracks and crevices that could provide protection for surface attached microorganisms (82, 118). Various steam treatments have been validated to achieve a 4- to 5-log reduction of *Salmonella* on almonds, almonds, and pistachios without impacting the quality of the nuts (14, 44). The types of steam treatment that are currently utilized for spices are saturated and superheated depending on the available technology and the product getting treated.

Enterococcus faecium NRRL B-2354 as a surrogate for *Salmonella enterica* serovars has proven useful to validate commercial processes for almonds (27, 83, 148, 166), dairy products (10), juice (122), balanced-carbohydrate protein meal (26), and meat (110).

The objective of the study was to evaluate the effectiveness of a lab-scale steam processing apparatus using a vacuum-assisted system at inactivating *Salmonella enterica* on whole black peppercorns and cumin seeds at one pressure (16.9 PSIA) and two commonly used reference temperatures (165°C and 180°C). The validity of *Enterococcus faecium* NRRL B-2354 as a surrogate for inactivation on whole black peppercorns and cumin seeds using the lab-scale steam apparatus was also evaluated. In addition, the effect of inoculum preparation on steam inactivation was also compared for whole black peppercorns.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Three *Salmonella enterica* serovars were obtained from low-a_w foods (Tennessee, K4643 from peanut butter outbreak associated in 2010, Ball ARL-SE-085 from black pepper in 2011, and Johannesburg ARL-SE-013 from dried ginger in 2010) and used to inoculate whole black peppercorns and whole cumin seeds. The survival of *Salmonella* under stressful conditions such

as heat and desiccation can vary greatly among strains within a species. The *Salmonella* serovar Tennessee strain used in this study was isolated from a major outbreak that occurred between August 2006 and July 2007. The outbreak was traced back to *Salmonella* contaminated peanut butter (a_w 0.7) that caused 715 salmonellosis cases spanning across 48 states (140). The strain of *Salmonella* serovar Ball was isolated from black pepper that has been associated with the spice in the EU (11, 156) and provided by the FDA. *Salmonella* servovar Johannesburg was isolated from ginger and also provided by the FDA (61). The combination of these strains are expected to provide an accurate representation of potential contamination that spices could contain and therefore will provide representative inactivation data that spice processors can utilize. *Enterococcus faecium* (NRRL B-2354, ATCC strain #8459) was obtained from American Type Culture Collection (Manassas, VA). Individual stocks were maintained at -80 °C in glycerol stocks. Strains were resuscitated by streaking onto Tryptic soy agar (TSA, Becton Dickinson, Franklin Lakes, NJ) incubated at 37°C for 24 h. One single colony was then transferred onto Xylose-Lysine-Tergitol 4 (XLT4, Becton Dickinson, Franklin Lakes, NJ) for each *Salmonella* strain or Bile Esculine Agar (BEA, Becton Dickinson, Franklin Lakes, NJ) for *Enterococcus faecium* and incubated at 37 °C for 24 h. Following incubation, a sterile 1- μ L loop was used to collect a single colony from each plate (one colony of each strain) and each colony was placed in 10 mL Trypic Soy Broth (TSB) (Becton Dickinson, Franklin Lakes, NJ). The individual cultures were incubated at 180 rpm, 37 °C for 24 h.

Spice varieties and sources

Whole black peppercorns and cumin seeds were provided in bulk by a major national spice processor. Spices were not further processed before arrival.

Inoculation Methods

Whole black peppercorns were inoculated using two preparation methods, as previously described (8). These two methods include inclusion within a native microbiota biofilm surrounding the whole peppercorn or cells grown on TSA plates that were scraped and applied in a small volume of liquid to the peppercorns. Similar methods were used for preparation of *E. faecium* cells grown on TSA (7). Cumin seeds were inoculated using only the TSA agar grown cell preparation method due to observed swelling of the cumin seed husk, potentially allowing the internalization of *Salmonella* using the biofilm preparation method. After inoculation with either preparation method, the spices were arranged in a single layer on sanitized 13 in. x 9 in. aluminum foil covered baking sheets and placed in a biological safety cabinet for 24-48 h until the spices reached the desired a_w at room temperature. Whole peppercorns inoculated using the biofilm inclusion and TSA-grown methods were air dried within a Biosafety cabinet until achieving mean a_w of 0.51 ± 0.20 and 0.33 ± 0.09, respectively. Cumin seeds were dried down to a mean a_w of 0.38 ± 0.12 prior to processing. Water activity of whole peppercorns (5 g) and cumin seeds (4 g) was determined by using an AquaLab 4TE water activity meter (AquaLab, Pullman, WA). Once the desired water activity was reached, the spices were placed in sealed Whirl-Pak bags and held in a desiccator (RH: ~40-45%) containing Drierite desiccant for 24 h before processing.

Biofilm inclusion (48 h incubation of *Salmonella* and *Enterococcus faecium* and seeds in TSB)

This inoculation methodology was adapted from the method used by Aviles et al. (4) to form biofilms on spices instead of silica beads. *Salmonella* strains were incorporated within native microbiota biofilms on the surface of the spice seeds by immersing seeds in inoculum within

Tryptic Soy Broth (TSB) and incubating statically for a total of 48 h. To perform this method, sterile 2L Erlenmeyer flasks were filled with 25 grams of cumin seeds while others were filled with 60 grams of whole peppercorns, the amount needed to cover the bottom of the flask with a single layer of spice. The spices were submerged in 150 mL TSB (to a depth of about 1 cm) along with 5 mL of 24-hour liquid culture (*E. faecium* or *Salmonella* cocktail) and swirled to mix. To create the 24-hour liquid culture, *Salmonella* inoculated tubes each containing a separate strain was combined to create the *Salmonella* cocktail. The flasks were incubated statically at 37 °C for 24 h. The flasks were removed after 24 hours and the TSB was decanted off into a waste bin by covering the flask with sterile gauze to prevent any spice loss. New TSB (150 mL) was added back into each flask and then placed back in the incubator at 37 °C for 24 h. After 24 hours, the TSB was decanted off using sterile gauze with as minimal movement as possible. Sterile 0.1% (w/v) peptone (Sigma-Aldrich, Co., MO) with 0.1% Tween 80 (PW-Tween, Fisher Scientific, Kansas City, MO) was used to wash the peppercorns removing excess nutrients and spent media. The flasks were gently swirled to remove any unattached cells and the liquid was decanted off.

Wet inoculation with *Salmonella* and *E. faecium* cells grown on Tryptic Soy Agar

The following method was adapted from the method developed by the Almond Board of California for inoculation of almonds for process validation (18). Briefly, each strain was spread onto 1- 150 x 15 mm (BD Falcon, Franklin Lakes, NJ) large Tryptic Soy Agar (TSA) (Becton Dickinson, Franklin Lakes, NJ) plates and incubated at 37 °C for 24 h. Cells were harvested from each of the three plates and placed in three separate sterile conical tubes by first applying 5 mL 0.1% (w/v) PT buffer to each plate and scraping colonies into suspension using a sterile cotton swab, and then 4 mL 0.1% (w/v) PT buffer to get any of the remaining cells. For *Salmonella,* the

suspensions were combined in comparable volumes corresponding to similar CFU of each strain; creating a 20 mL liquid culture *Salmonella* cocktail. For *E. faecium,* this was not necessary because only one strain was used. The suspensions were then transferred into 27 oz. sterile Whirl-Pak bags containing either 50 g of dry whole peppercorns or 50 g of dry cumin seeds and hand massaged for 1 minute to evenly coat the spice.

Steam processing using a custom pressure apparatus

A custom steam processing apparatus was developed. An aluminum pressure rated steam sterilizer (All American Model 1925X, Manitowoc, WI) was wrapped in 3" x 120" heating tape (BriskHeat, Columbus, OH) and attached to a valved manifold. The manifold was connected to a steam generator (AmeriVap®, Dawsonville, GA), vacuum pump, compressed gas/air and ambient air supply. The top of the pressure steam sterilizer was modified to accommodate a braided, jacketed steam hose (Tipco, Chantilly, VA) from the supply manifold, a digital pressure gauge (Ashcroft model DG-25-3-1-N-1-NA-M02-L-60#&V, Stratford, CT), additional Pressure Relief Valve, a 4 pair Type T thermocouple hermetic pressure and vacuum rated pass-through set (PFT2NPT-4T, Omega Engineering, Stamford, CT), and an exhaust line leading to the condenser and scrubber. A 3 level scaffold system was made out of PVC pipe, which supported the mesh floors (304 Stainless Steel Mesh, 20x20 mesh, 0.16" wire, McMaster Carr, Atlanta, GA) at each level. This scaffold system was placed inside of the sterilizer to support the spice product during processing. Two muslin drawstring sachets (4 x 6 in.) were filled to a depth of 0.20 ± 0.049 in. for whole black peppercorns and 0.21 ± 0.043 in. for cumin seeds with inoculated spices (50g peppercorns and 40g cumin seeds) were placed on the top two levels of the scaffold system. The heating tape was turned on and set slightly above 350°F. Temperature was measured using the T-type thermocouples and logged using a data logger, (Omega, model RDXL6SD, Stamford,

CT) while pressure was measured using the pressure gauge. Preconditioning in absence of spices was performed first flushing air by a applying a vacuum to 4.7 PSIA followed by using steam injection until a measured temperature of the air achieved a temperature of 220°F and 16.9 PSIA. A final vacuum was pulled to 4.7 PSIA before opening the lid. Once the temperature reached this point, the spice rack holding multiple sachets was inserted. Thermocouples were inserted into the middle of each sachet of spice. Once the lid was closed with the spices inside, the vacuum was pulled to 4.7 PSIA and steam was inserted until the temperature read close to 185°F. The dwell time then occurred for a specified amount of time. Once the time was up, the vacuum was pulled to 4.7 PSIA to prevent any condensation from forming in the kettle and the pressure was slowly released with an air inbleed. The lid of the kettle was quickly taken off, and the spice rack was removed.

Microbiological detection

Enumeration of total aerobic bacteria, *Salmonella* or *Enterococcus faecium* was performed according to the following method. Spice samples (10 g) were transferred into a sterile filter bag along with 90 mL of sterile PT and blended in a lab blender (Interscience BagMixer, Guelph, Ontario) for 60 seconds. The liquid was vacuum filtered through #4 qualitative filter paper (Whatman, GE Healthcare, Pittsburgh, PA) to remove any spice particles. The filtered supernatant was serial diluted in sterile PT buffer and enumerated by plating onto TSA in quadruplicate. Half of the plates were incubated for 3 hours at 25 °C after which an overlay of 7 ml of XLT-4 was applied. The other half of TSA plates provided aerobic plate counts. Spices inoculated with *Enterococcus faecium* were enumerated by plating onto selective media BEA in duplicate and TSA in duplicate for aerobic plate counts. All plates were incubated at 37 °C for 24h before enumeration.

Data and Statistical analysis

Bacterial counts were log transformed prior to statistical analysis. Log reductions were calculated based on subtracting the log reduction for each run from the initial log CFU/g. Initial log counts are reported using the mean log CFU/g and standard deviation while the log reductions are reported using the mean log reduction CFU/g and standard error. To allow comparison between inoculation method, biofilm inclusion and TSA-grown inoculated peppercorns were processed together and compared for three separate runs. Peppercorns and cumin seeds were processed separately. In all processes, *Salmonella* and *Enterococcus* inoculated spices were included. Statistical analyses were performed using JMP (version 11, SAS, Cary, NC) statistical software. A 1-way ANOVA using blocks for each processing day was performed to compare the average log reduction for *Salmonella* and *Enterococcus faecium.* The log reduction of biofilm inclusion and TSA-grown inoculated peppercorns was not compared. For each run, the time in seconds over 165°F and 180°F was determined using the temperature collected by the data logger. A regression analysis was performed for each spice inoculated using the biofilm inclusion or TSA-grown methods and treated with steam.

RESULTS

Whole Black Peppercorns Inoculated using Biofilm Inclusion and TSA-grown Inoculation Methods

Whole black peppercorns inoculated using either the biofilm inclusion method or the TSA-grown inoculation methods were subjected to various durations of steam treatment. On average, the initial levels (before treatment) of *Salmonella* on whole black peppercorns using the biofilm and TSA-grown inoculation methods were 6.9 ± 0.30 and 9.5 ± 0.40 log CFU/g, respectively. The average initial levels of *Enterococcus faecium* inoculated peppercorns were 8.3 ± 0.79 and 8.9 ±

0.43 log CFU/g for biofilm inclusion and TSA-grown inoculation methods, respectively. Inactivation of *Salmonella* on the peppercorns was comparable to *Enterococcus faecium* when treated with steam (Figure 1-4). Peppercorns inoculated with *Salmonella* serovars using the biofilm inclusion and TSA-grown inoculated methods had average log reductions of 4.8 ± 0.19 and 6.3 ± 0.18 log CFU/g, respectively. Using the same inoculation methods, the average log reductions of *Enterococcus faecium* were 4.7 ± 0.19 and 5.3 ± 0.18 for the biofilm inclusion and TSA-grown inoculation methods, respectively. Statistical analysis indicated that there was no significant difference in the log reduction of *Salmonella* or *Enterococcus faecium* biofilm inclusion inoculated peppercorns ($p=0.57$). In contrast, there was a significant difference between the log reduction of *Salmonella* and *E. faecium* on peppercorns when inoculated using the TSA-grown inoculation method ($p=0.0007$). The mean log reduction of *Salmonella* was greater than the log reduction of *Enterococcus faecium* at 6.3 ± 0.18 and 5.3 ± 0.18 log CFU/g, respectively. Results indicated that there was a significant difference in steam processes between replicates for both inoculation methods used on whole black peppercorns.

Two common reference temperatures used in the industry during thermal processing are 165°F and 180°F. A reduction of at least 5-log (CFU/g) of biofilm inclusion inoculated *Salmonella* on black peppercorns was achieved when temperatures remained over 165 °F for approximately 250 seconds and over 180 °F for 225 seconds (Figures 3 and 4). A reduction of at least 5-log (CFU/g) of *Enterococcus faecium* within a biofilm on peppercorns occurred when the spices remained over 165°F for approximately 450 seconds and over 180°F for 400 seconds. Peppercorns inoculated with *Salmonella* using the TSA-grown inoculation method achieved a minimum of a 5-log reduction when steam processed over 165°F for 125 seconds and over 180°F

for 100 seconds. Likewise, an assured 5-log reduction of *Enterococcus faecium* occurred when spices were processed over 165°F for 200 seconds and 180°F for 175 seconds.

Initial aerobic plate counts (APC) of whole black peppercorns inoculated with *Salmonella* were 7.5 ± 0.27 and 9.6 ± 0.43 log CFU/g for biofilm inclusion and TSA-grown inoculation methods, respectively. Whole black peppercorns inoculated with *Enterococcus faecium* had initial APC levels of 8.0 ± 0.29 and 9.2 ± 0.40 log CFU/g for biofilm inclusion and TSA-grown inoculation methods, respectively. The average log reduction of APC was 1.5 ± 0.20 and 2.4 ± 0.08 log CFU/g for biofilm inclusion and TSA-grown inoculated peppercorns, respectively. An assured 5-log reduction of APC did not occur at either 165°F or 180°F when for any peppercorns, regardless of inoculation method (Figures 5 and 6).

Cumin Seeds using the TSA-grown Inoculation Method

Cumin seeds inoculated using the TSA-grown inoculation method with both *Salmonella* and *Enterococcus faecium,* were subjected to various durations of steam treatment. The average starting inoculum of cumin seeds using the TSA-grown inoculation method was 9.16 ± 0.54 and 9.01 ± 0.29 log CFU/g for *Salmonella* and *E. faecium*, respectively. The initial APC levels for *Salmonella* and *E. faecium* inoculated cumin seeds were 9.00 ± 0.26 and 9.22 ± 0.18 log CFU/g respectively. Statistical analysis indicated that there was no significant difference in log reduction of *Salmonella* and *E. faecium* when inoculated onto cumin seeds ($p=0.0822$) (Figure 7 and 8). The mean log reduction of *Salmonella* and *Enterococcus faecium* on cumin seeds was 7.3 ± 0.21 and 6.7 ± 0.21 log CFU/g respectively. The mean log reduction of APC was 5.5 ± 0.34 and 5.9 ± 0.31 log CFU/g for *Salmonella* and *E. faecium* inoculated cumin seeds, respectively.

To achieve a 5-log reduction of *Salmonella, E. faecium,* and APC, cumin seeds needed to remain over the reference temperature of 165°F in the lab-scale steam apparatus for 110, 110, and 560 seconds, respectively (Figure 9). When temperatures remained over 180°F, a 5-log reduction occurred at 65, 80, and 430 seconds for *Salmonella, E. faecium,* and APC respectively (Figure 10).

DISCUSSION

The lab-scale steam apparatus designed for this research utilized a vacuum to improve the penetration of steam into the pores of the spices. Furthermore, the vacuum allowed for an increase in pressure and temperature in the chamber as well minimized cold pockets, which provided a more uniform heat distribution throughout the chamber. The system was designed to create a saturated steam environment. Saturated steam is vapor at the boiling temperature of the liquid (153) which means that when using water, it is when water vapor is at 100°C. At this point, the rate of water vaporization is equal to the rate of condensation. If the temperature of the saturated steam is lowered at constant pressure, condensation will occur. During this phase change, heat is released. Adding heat to the system during this time will change the temperature, pressure, or both. Advantages of saturated steam treatment include safe, clean, inexpensive, pressure can control temperature so temperature can be precisely established in a timely manner, improved product quality and productivity, and a smaller surface area for heat transfer is needed so equipment takes up less space (152).

Superheated steam occurs when water vapor is at a temperature higher than the boiling point (100°C) (153). The degrees that exceed the boiling point are considered the degrees superheat. Adding heat to superheated steam can increase the superheat at constant pressure or change the pressure and temperature at constant volume (153). On the other hand, removing the

heat will cause the temperature to drop to boiling temperature and the vapor will condense. There is a form between saturated vapor and superheated steam called vapor-liquid mixtures, which occur when the quality of steam (percentage of vapor-liquid mixture in the form of saturated vapor) is less than 100%. In this form, water can exist as either saturated liquid or saturated vapor. Since the steam was less than 100% quality, the steam apparatus used in this study produced vapor-liquid mixtures.

Studies in the literature focus on the use of either saturated steam or superheated steam. Therefore, results from this study will be compared to these two forms of steam. The effectiveness of saturated steam and superheated steam at inactivating *Salmonella* Typhimurium biofilms on PVC was investigated (16). Saturated steam treatment (212°F) for 5-30 seconds resulted in a 1.35-3.43 log reduction CFU/coupon whereas superheated steam treatment resulted in a 1.42-5.03 decrease in *Salmonella* cells after treatment for 5—30 seconds. More specifically, a 3.43 log reduction CFU/coupon occurred at 212°F for 30 seconds on PVC material treated with saturated steam. When the same material was treated with superheated steam, a 5.03 log reduction CFU/coupon occurred at 347°F after only 20 seconds. The lab-scale steam apparatus utilizing vapor-liquid mixtures took much longer (about 11.25 times) to achieve a 5-log reduction CFU/g of *Salmonella* biofilms on whole black peppercorns, however, the temperature was about 167°F lower than the temperature of superheated steam. Almonds and pistachios inoculated with *Salmonella* Typhimurium and *Salmonella* Enteritidis PT 30 were treated with saturated steam and superheated steam (14). Saturated steam treatment at 212°F for 15 seconds resulted in a 3.8 and 2.9 log CFU/g reduction for *S.* Typhimurium and *S.* Enteritidis on inoculated almonds, respectively, whereas superheated steam treatment for the same amount of time at 392°F resulted in a 6.5 and 6.7 log CFU/g reduction. Pistachios inoculated with *S.*

Typhimurium and *S.* Enteritidis that were treated using superheated steam at 392°F for 30 seconds had a 6.3 and 6.4 log CFU/g reductions whereas saturated steam treatment at 212°F for the same time interval resulted in a 3.3 and 2.7 log CFU/g reductions, respectively. The temperature for both the saturated and superheated steam treatments were greater than the reference temperature used in the lab-scale steam apparatus, however, the time in seconds required for superheated steam was much less. Since superheated steam takes less time to achieve a 5-log reduction of *Salmonella* serovars on the PVC and nuts, and did not impact the quality of the nuts, it might be more efficient to use this form of steam compared to vapor-liquid mixtures for the treatment of spices.

Spices have the potential to be contaminated by *Salmonella* in the form of a biofilm, which can increase protection against stress. A biofilm is an extracellular matrix comprised of mostly water and various polymers (68). When in this form, *Salmonella* has an increased tolerance to desiccation and can be recovered in greater numbers (13). Encasement of *Salmonella* within a biofilm can increase resistance to various environmental stresses such as heat and therefore protect the cells (34). The inactivation of both *Salmonella* and *E. faecium* inoculated onto whole black peppercorns using the biofilm inclusion method was lower than the inactivation of TSA-grown inoculated cells. Steam treatment to inactivate cumin seeds inoculated with *Salmonella* using the biofilm inclusion method was ineffective. In preliminary experiments, the biofilm inclusion inoculation process caused the cumin seeds to swell. Then, once the spices were dried, it is hypothesized that the cumin seeds internalized the *Salmonella* so that during the steam process, the steam was unable to reach the *Salmonella.* In wet conditions such as those produced during the biofilm inclusion process, *Salmonella* has demonstrated the ability to migrate through almond hulls and shells in order to colonize the kernel (49). In another

low-a_w case, *Salmonella* infiltrated in-shell pecans to reach the kernel and remain viable after drying and during subsequent storage (23). The formation of a biofilm and the presumed internalization of *Salmonella* increased the ability for the pathogen to protect itself from thermal inactivation and made the recovery of *Salmonella* very difficult. Therefore, cumin seeds were not inoculated using the biofilm inclusion method. Despite the decision to not use the biofilm inclusion method to inoculate cumin seeds, the data on the inactivation on whole black peppercorns using this method is valid because a minimum starting inoculum of 7-log CFU/g of *Salmonella* was consistently achieved. Furthermore, inactivation of *Salmonella* could have been attributed to the physiological differences between cumin seeds and peppercorns. Considering it is very plausible that *Salmonella* can form a biofilm on cumin seeds, future research should evaluate the inactivation of cumin seeds inoculated using the biofilm inclusion method.

The American Spice Trade Association suggests that the use of *E. faecium* NRRL B-2354 as a surrogate for *Salmonella enterica* on spices may be beneficial (3). However, the suitability of a surrogate microorganism used for a pathogen must be validated for each product and each process (3). *Enterococcus faecium* NRRL B-2354 has validated as a surrogate for *Salmonella* Enteritidis PT 30 on the surface of almonds subjected to moist air-heating (83). The inoculated almonds were treated for various time, temperature, and humidity parameters. *Enterococcus faecium* had a mean log reduction 0.6 log and 1.4 log lower than those for *Salmonella*, indicating that it would be an acceptable surrogate for steam heating (83). Whole black peppercorns inoculated with *Salmonella* serovars and *Enterococcus faecium* NRRL B-2354 using the biofilm inclusion method did not show a significant difference in reduction using steam and can also be considered a suitable surrogate under these conditions. Cumin seeds inoculated with *Salmonella* and *E. faecium* using the TSA-grown did not demonstrate a significant difference. However,

cells inoculated onto whole peppercorns using the TSA-grown method did show a significant difference. A conservative surrogate should demonstrate a lower log reduction than the target pathogen. Despite the significant difference in reduction, *Salmonella* could serve as a surrogate for *E. faecium* using the TSA-grown inoculation method because the reduction of *Salmonella* was larger than that of *E. faecium.* Based on the findings in this study, *Enterococcus faecium* NRRL B-2354 is a conservative surrogate for *Salmonella enterica* ser. Tennessee, Ball, and Johannesburg inoculated whole black peppercorns and cumin seeds undergoing a validated lab-scale steam processing treatment.

Limitations in the study included the inactivation of *Salmonella* inoculated cumin seeds using the biofilm inclusion method along with the consistency of the lab-scale steam processing apparatus. Future research should focus on the internalization of *Salmonella* in spices. Furthermore, methods to effectively inactivate and recover *Salmonella* once it has been internalized should be evaluated. Further research on lab-scale steam processing units could provide better consistency and results.

FIGURES

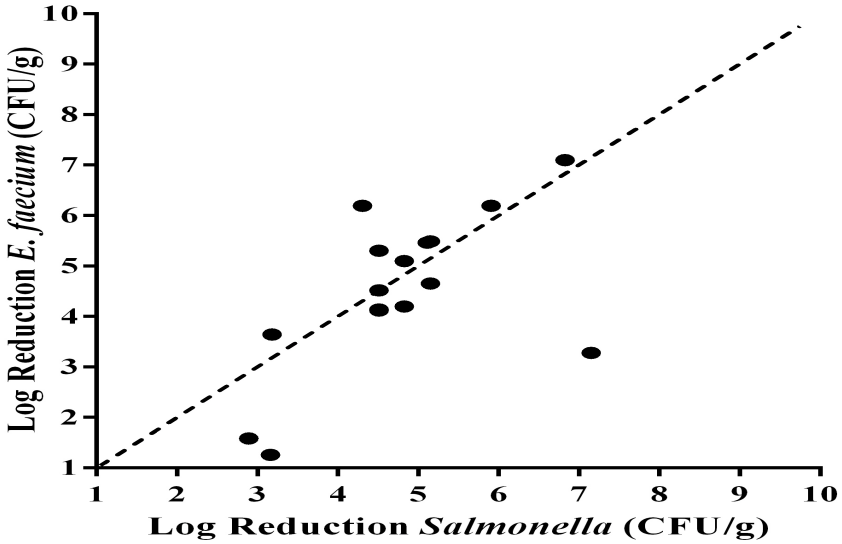

Figure 1. *Log reduction (CFU/g) of Salmonella enterica compared to the log reduction of Enterococcus faecium on whole black peppercorns inoculated using the biofilm inclusion method*

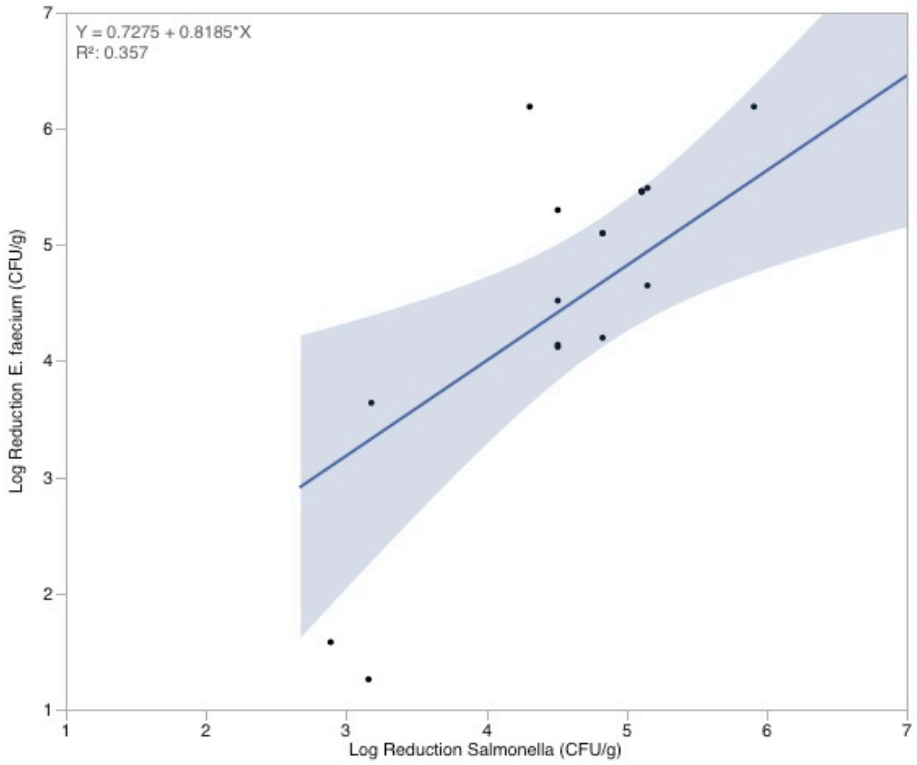

Figure 2. *Linear regression line and R^2 value of the log reduction (CFU/g) of Salmonella enterica compared to the log reduction of Enterococcus faecium on whole black peppercorns inoculated using the biofilm inclusion method. The gray area shows the 95% confidence interval.*

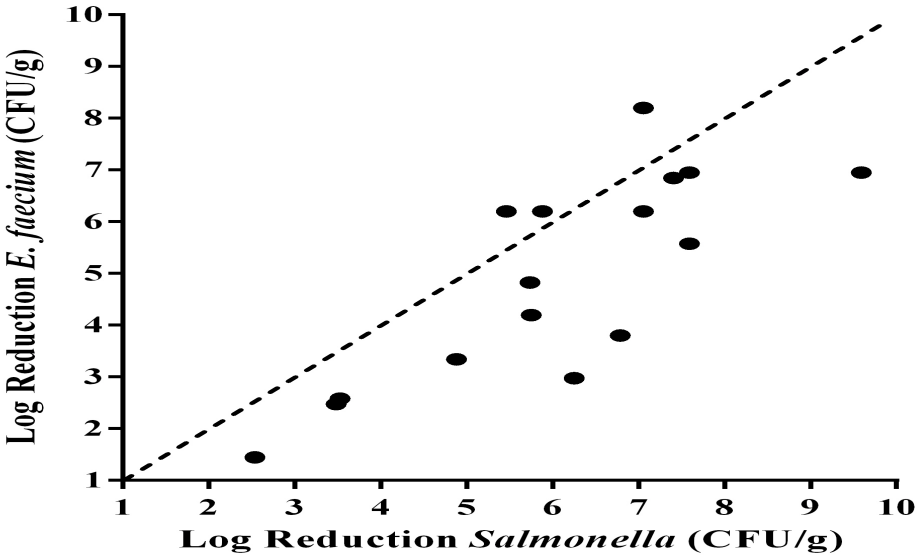

Figure 3. *Log reduction (CFU/g) of Salmonella enterica compared to the log reduction of Enterococcus faecium on whole black peppercorns inoculated using the TSA-grown inoculation method*

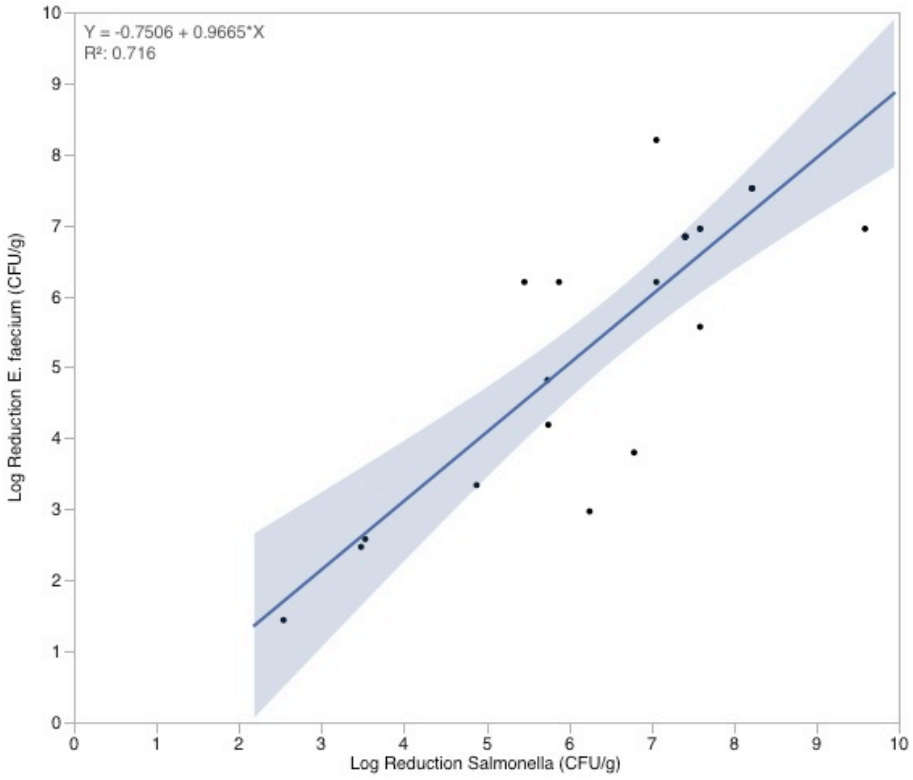

Figure 4. *Linear regression line and R^2 value of the log reduction (CFU/g) of Salmonella enterica compared to the log reduction of Enterococcus faecium on whole black peppercorns inoculated using the TSA-grown method. The gray area shows the confidence interval.*

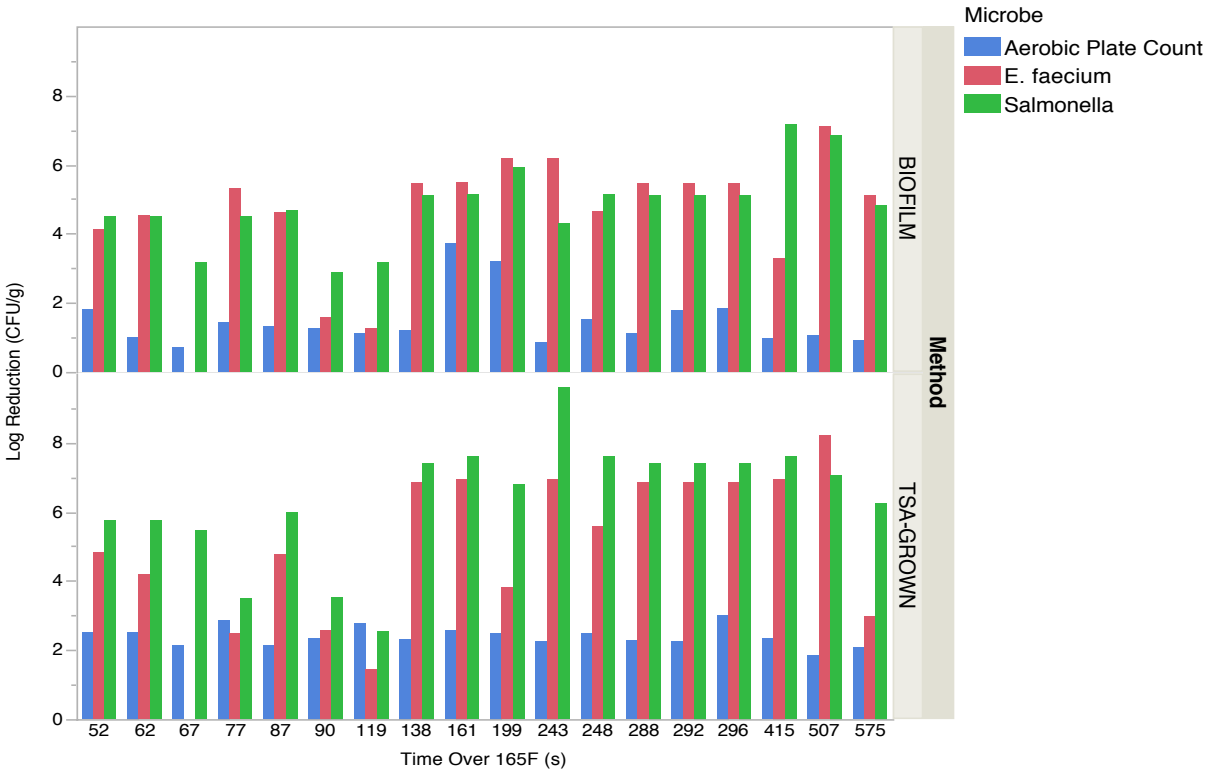

Figure 5. *Log reduction (CFU/g) of Salmonella enterica and Enterococcus faecium inoculated using various methods onto whole black peppercorns processed using a lab-scale steam apparatus associated with varying exposures to temperatures above 165°F. Each bar represents one run.*

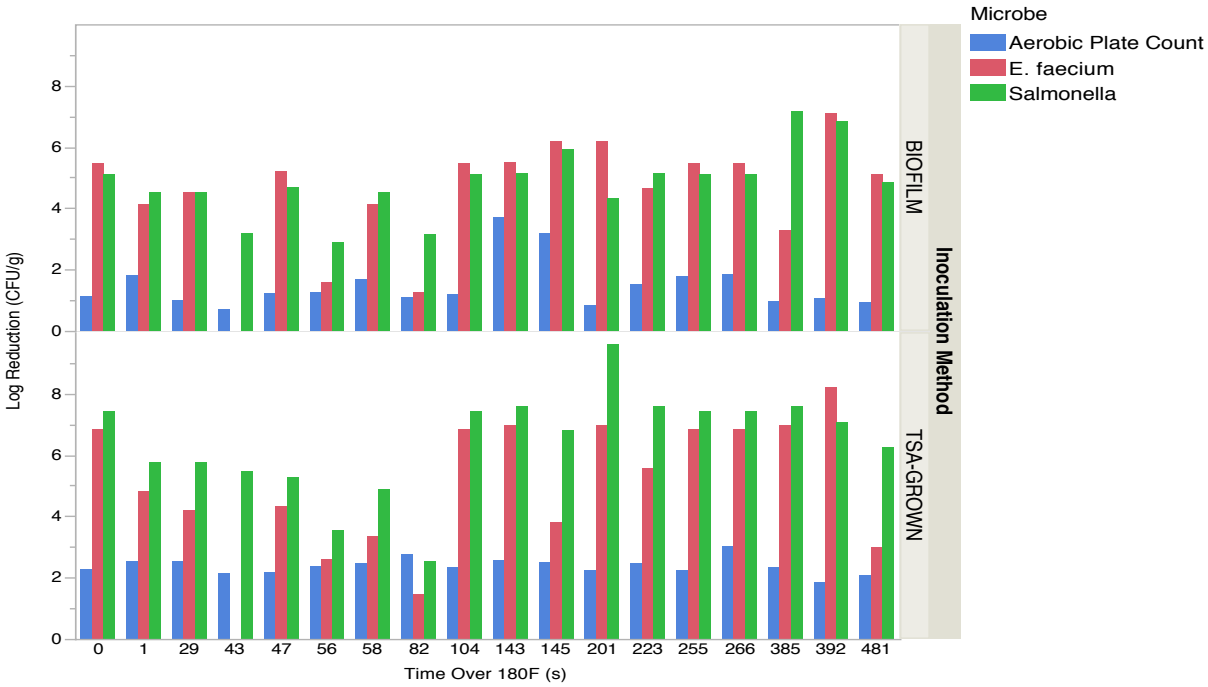

Figure 6. *Log reduction (CFU/g) of Salmonella enterica and Enterococcus faecium inoculated using various methods onto whole black peppercorns processed using a lab-scale steam apparatus associated with varying exposures to temperatures above 180°F . Each bar represents one run.*

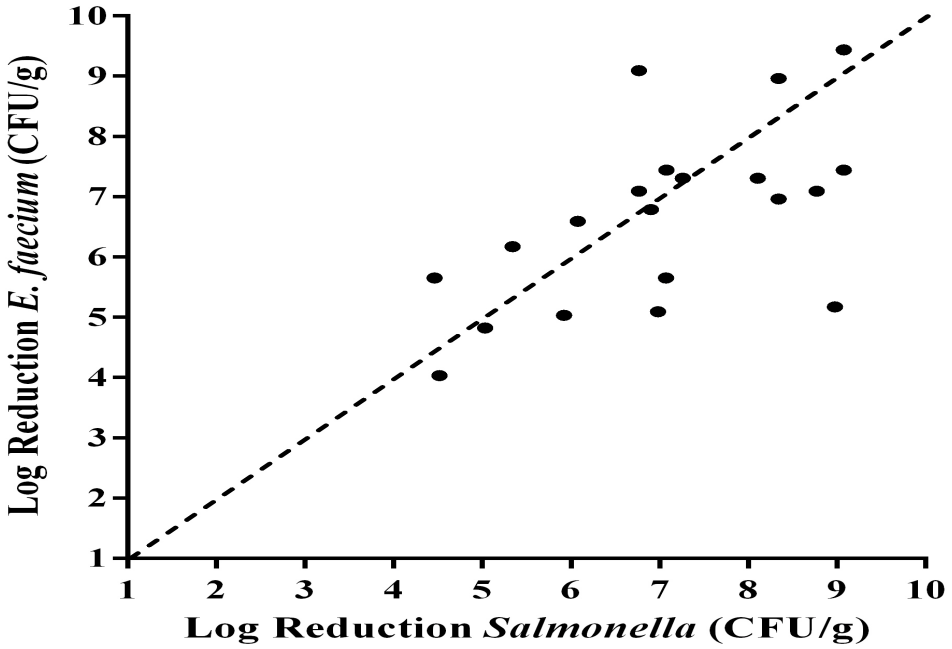

Figure 7. *Log reduction of Salmonella enterica compared to the log reduction of Enterococcus faecium on cumin seeds inoculated using the TSA-grown inoculation method*

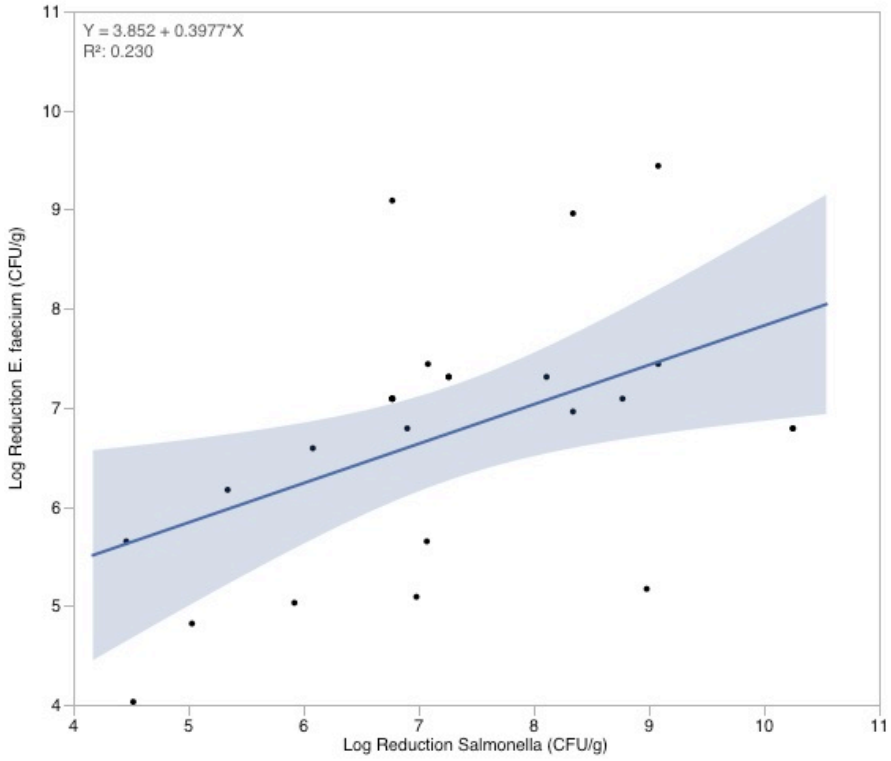

Figure 8. *Linear regression line and R^2 value of the log reduction (CFU/g) of Salmonella enterica compared to the log reduction of Enterococcus faecium on cumin seeds inoculated using the TSA-grown method. The gray area shows the confidence interval.*

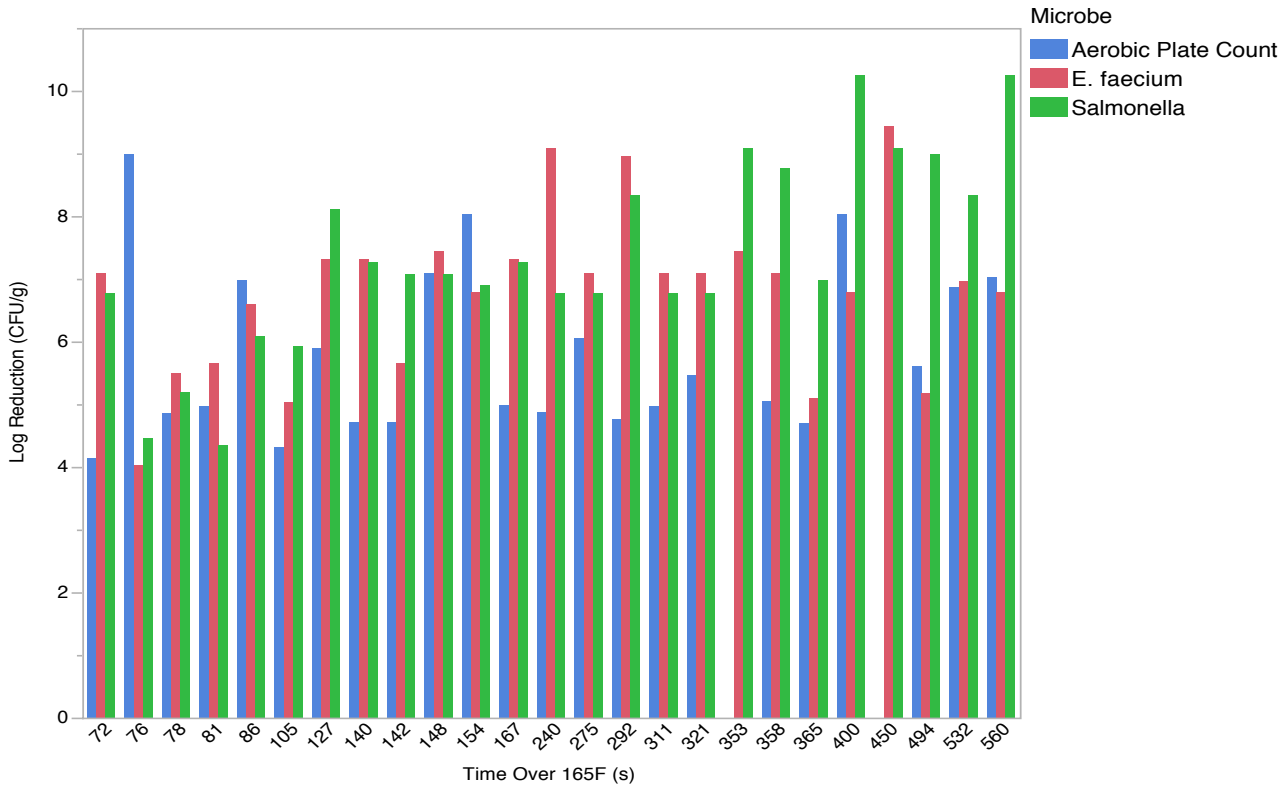

Figure 9. *Log reduction (CFU/g) of Salmonella enterica and Enterococcus faecium inoculated cumin seeds processed using a lab-scale steam apparatus associated with varying exposures to temperatures above 165°F*. Each bar represents one run.

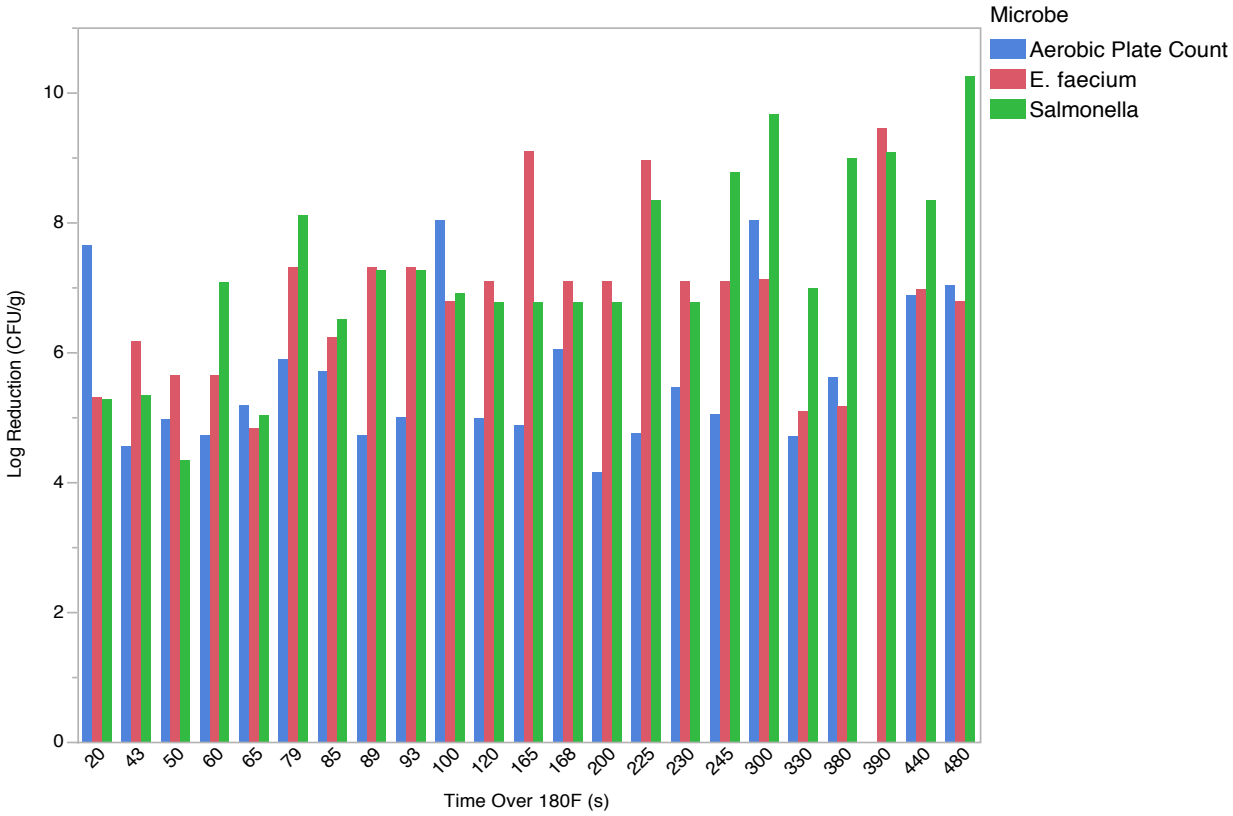

Figure 10. *Log reduction (CFU/g) of Salmonella enterica and Enterococcus faecium inoculated cumin seeds processed using a lab-scale steam apparatus associated with varying exposures to temperatures above 180°F* . Each bar represents one run.

Table 1. *The cubic R^2 value indicating the comparison between the log reduction (CFU/g) of Salmonella and Enterococcus faecium on inoculated spices treated with steam using a cubic model.*

Spice	Inoculation Method	Cubic R^2 Value
Pepper	Biofilm Inclusion	0.643
Pepper	TSA-grown	0.741
Cumin	TSA-grown	0.378

CHAPTER 4: THE INACTIVATION OF *SALMONELLA ENTERICA* AND SURROGATE, *ENTEROCOCCUS FAECIUM,* USING A COMMERCIAL ETHYLENE OXIDE FUMIGATION PROCESS AND IMPROVED RECOVERY METHODS

ABSTRACT

In recent years, the microbiological safety and quality of spices has caused concern for regulators and the food industry. Further processing using the chemical fumigant ethylene oxide (EtO) is performed for many spices to reduce microbial populations and ensure a safe product of high quality. Whole black peppercorns and cumin seeds were inoculated with a *Salmonella* cocktail or *Enterococcus faecium* NRRL B-2345 using TSA-grown cells, and the spices were dried to a_w of 0.30-0.36. Spices were packaged (5 lb) in polywoven bags (n=3 per spice) and shipped to a commercial processor for EtO treatment using FDA approved spice treatment parameters and returned by overnight shipment. Cells were enumerated by serial dilution and plated onto TSA for aerobic plate counts or TSA with a thin overlay of XLT4 for *Salmonella,* or BEA for *E. faecium*. Additionally, supplementation with several compounds (sodium pyruvate) + yeast extract (0.6%) [NaYe], 3,3'-thiodipropionic acid [TDP], glycerophosphate [GP], ATP, guanine [Guan], and magnesium [Mg]) previously shown to improve recovery of damaged *Salmonell*a was performed. EtO fumigation significantly reduced total aerobic plate counts for whole peppercorns and cumin seeds. While ethylene oxide fumigation significantly reduced the mean populations of *Salmonella* and *E. faecium* on whole black peppercorns and cumin seeds ($p<0.05$), the distribution of inactivation of *Salmonella* between bags varied between 2.02-8.34 log CFU/g and 2.62-9.85 log CFU/g for black peppercorns and cumin seeds, respectively. Recovery of *Salmonella* was significantly reduced when plated onto XLT4 alone compared to the overlay method with or without supplementation. *Enterococcus faecium* NRRL B-2354

demonstrated effectiveness as a surrogate for the inactivation of *Salmonella* in whole black peppercorns using ethylene oxide fumigation. However, that was not the case for the inactivation of *Salmonella* in cumin seeds.

INTRODUCTION

Spices are used widely for their flavor enhancing characteristics. Typically, the low water activity of spices restricts the growth of bacteria and fungi that are frequently associated with the spice (132). Black peppercorns and cumin frequently contain between 2-6 log CFU/g, these microorganisms are typically indigenous to the soil and plants from which they are grown and may vary by production region (132). However, the microbiological quality can be further compromised by cross contamination during production, potentially introducing human pathogens (58).

Typical cultivation and harvest practices of spices may lead to contamination with spoilage associated microorganisms or human pathogens resulting in declines in quality of ready to eat or minimally processed foods. Preventing all sources of contamination is impractical, therefore it is important to improve detection and validate methods for inactivation of pathogens on spices. Currently microbial reduction strategies include gas fumigation with ethylene oxide (EtO) or propylene oxide (PPO), irradiation and steam to reduce microbial load.

Based on data from processors, the American Spice Trade Association (ASTA) estimates that between 40-85% of spices imported into the U.S. are treated with EtO each year (3). Ethylene Oxide (C_2H_4O) is a colorless, flammable gas with a sweet smell. Ethylene oxide is mixed with non-flammable gases such as nitrogen and carbon dioxide and is used as a fumigant for sterilizing and disinfecting medical devices and other organic materials (i.e. spices, gums,

starch, flour, yeast, and milk) (147). Studies using ethylene oxide have proven that it can achieve a 90% reduction of aerobic bacterial populations on spices (155).

EtO is a direct alkylating agent of cellular constituents (nucleic acids and functional proteins) of organisms, preventing cellular metabolism and reproduction, resulting in nonviable microbes (31, 72, 116, 125, 128, 135, 150). Death of *Salmonella* serovar Senftenberg 775W, a serotype known for its extreme resistance to thermal processing, displayed first order kinetics when exposed to EtO vapor (117). Many factors contribute to the efficacy of the fumigation treatment: concentration of EtO, temperature, relative humidity of environment, and moisture content. The Food Quality Protection Act Tolerance Reassessment Decision Document (57) was developed by the EPA and includes the tolerances for EtO and its reaction product, ethylene chlorohydrin (ECH) residues, which are 7 and 940 ppm on spices, respectively. All commercial processors of spices must use process parameters during EtO fumigation that will not result in exceeding the residual limits set by the EPA. Since 2008, any spice treated by EtO in the United States must follow the directions on the EtO label (3).

Although EtO has been used for decades, the effectiveness of standard ethylene oxide process parameters against *Salmonella enterica* on whole spices is not readily available in scientific literature. Furthermore, the efficacy of recovery on selective media of *Salmonella enterica* cells injured by Eto, has not been reported. Use of overlay methods as well as addition of nutrients, free radical scavengers and chemical compounds that aid in the repair damaged cellular components have all been reported to improve recovery of sub-lethally stressed *Salmonella* from processed foods (13, 48, 74, 76, 86, 144, 165). Repairing injured cells avoids an underestimation of microbial populations as the cells may repair themselves if returned to a favorable environment such as a ready to eat food (129).

The objective of this research was to examine the effectiveness of commercial treatment of whole black peppercorns and cumin seeds with ethylene oxide on the survival of total aerobic bacteria, *Salmonella enterica, and Enterococcus faecium* NRRL B-2354 as well as the subsequent recovery of sub-lethally injured *Salmonella* after processing.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Three *Salmonella enterica* serovars were obtained from low-a_w foods (Tennessee, K4643 human isolate from 2010 peanut butter associated outbreak, ,Ball ARL-SE-085 isolated from black pepper in 2011, and Johannesburg ARL-SE-013 from dried ginger in 2010).. *Enterococcus faecium* (NRRL B-2354, ATCC strain #8459) was obtained from American Type Culture Collection (Manassas, VA). Individual stocks were maintained at -80 °C in glycerol stocks. Strains were resuscitated by streaking onto Tryptic soy agar (TSA, Becton Dickinson, Franklin Lakes, NJ) incubated at 37°C for 24 h. One single colony was then transferred onto Xylose-Lysine-Tergitol 4 (XLT4, Becton Dickinson, Franklin Lakes, NJ) for each *Salmonella* strain or Bile Esculine Agar (BEA, Becton Dickinson, Franklin Lakes, NJ) for *Enterococcus faecium* and incubated at 37°C for 24 h. Following incubation, a sterile 1-μ L loop was used to collect a single colony from each plate (one colony of each strain) and placed in 10 mL Trypic Soy Broth (TSB) (Becton Dickinson, Franklin Lakes, NJ). The individual cultures were incubated at 180 rpm, 37°C for 24 h.

Spice varieties and sources

Whole black peppercorns and cumin seeds were provided in bulk by a major national spice processor. Spices were not processed before arrival.

Wet inoculation with *Salmonella* and *E. faecium* grown on TSA

The following method was adapted from the method developed by the Almond Board of California for inoculation of almonds for process validation (148). Briefly, each strain was spread onto 1- 150 x 15 mm (BD Falcon, Franklin Lakes, NJ) large Tryptic Soy Agar (TSA) (Becton Dickinson, Franklin Lakes, NJ) plates and incubated at 37 °C for 24 h. Cells were harvested from each of the three plates and placed in three separate sterile conical tubes by first applying 5 mL 0.1% (w/v) PT buffer to each plate and scraping colonies into suspension using a sterile cotton swab, and then 4 mL 0.1% (w/v) PT buffer to get any of the remaining cells. For *Salmonella,* the suspensions were combined in comparable volumes corresponding to similar CFU of each strain; creating a 20 mL liquid culture *Salmonella* cocktail. For *E. faecium,* this was not necessary because only one strain was used. The suspensions were then transferred into 27 oz. sterile Whirl-Pak bags containing either 50 g of dry whole peppercorns or 50 g of dry cumin seeds and hand massaged for 1 minute to evenly coat the spice. After inoculation, both spices were arranged in a single layer on sanitized 13 in. x 9 in. aluminum foil covered baking sheets and placed in a biological safety cabinet for 24-48 h until the spices reached an a_w of 0.3-0.5 at room temperature. Peppercorns and cumin seeds were determined to have an a_w of 0.30-0.36 prior to inoculation. Water activity of whole peppercorns (5 g) and cumin seeds (4 g) was determined by using an AquaLab 4TE water activity meter (AquaLab, Pullman, WA).

Packaging methods

The dried, inoculated spices (40g cumin, 50g peppercorn) were individually packaged within muslin drawstring sachets (4 x 6 in.) and placed within a larger polywoven polypropylene bag that was filled with 5lbs of non-inoculated spice. Each polywoven bag (n=3 per spice) contained sachets of spice inoculated with either *Salmonella* (n=9) or *Enterococcus* (n=9). The bags were packed in insulated boxes and shipped overnight to a commercial processor for EtO treatment.

Prior to treatment the polywoven bags were removed from the shipment box and placed in a full size (4-pallet chamber).

EtO treatment

Spices were treated in a commercial facility using the standard operating procedures typically used by this facility. The 5-lb bags of spices (n=8) were placed into a one pallet capacity chamber and put through five nitrogen pulse cycles (130°F) to remove oxygen as well as increase the temperature in the chamber. Following the nitrogen pulses, ethylene oxide (20% EtO in 80% CO_2) was injected into the chamber (130 ± 3.53°F) and held for 325 min, at 130 ± 7.64°F. The EtO dwell was followed by 21 steam washes (SP=125 ± 7.64°F) and four nitrogen pulse cycles (SP=125 ± 7.07°F) to complete remove residual EtO.

Enumeration methods

Enumeration of total aerobic bacteria, *Salmonella* or *Enterococcus faecium* was performed according to the following method. Spice samples (10 g) were transferred into a sterile filter bag along with 90 mL of sterile PT and blended in a lab blender (Interscience BagMixer, Guelph, Ontario) for 60 seconds. The liquid was vacuum filtered through #4 qualitative filter paper (Whatman, GE Healthcare, Pittsburgh, PA) to remove any spice particles. The filtered supernatant was serial diluted in sterile PT buffer and enumerated by plating onto TSA in quadruplicate. Two of the plates were used for aerobic plate counts while the other 2 TSA plates were overlaid with 7 ml of XLT-4 (*Salmonella*) (86) or bile esculin azide agar (BEA, *Enterococcus faecium)* after a 3 h incubation at 37 °C. All plates were incubated at 37°C for 24 h before enumeration.

Supplementation to improve *Salmonella* recovery

Supplements were tested in combination with overlay using the traditional overlay method described above. TSA was supplemented with sodium pyruvate (Fisher Scientific, Fair Lawn, NJ) (9.09 mmol) + yeast extract (Becton Dickinson, Franklin Lakes, NJ) (0.6%) [SPY], 3,3'-thiodipropionic acid (Acros Organics, NJ) (5.6 mmol)[TDP], glycerophosphate (MP Biomedicals, Solon, OH) (10 mmol)[GP], ATP (Sigma-Aldrich, St. Louis, MO) (8.34 mmol), guanine (Sigma-Aldrich, St. Louis, MO) (64.5 mmol)[GU], and magnesium (Sigma-Aldrich, St. Louis, MO) (52.5 mmol) [MG]. The overlay XLT4 media did not contain any additional supplements and was applied as described above. All plates were incubated at 37 °C for 24 h prior to enumeration.

Statistical Analysis

Bacterial counts were log transformed prior to statistical analysis. Statistical analyses were performed using JMP (version 11, SAS, Cary, NC) statistical software. The effect of EtO fumigation on the log CFU/g reduction of total bacteria, *Salmonella* or *E. faecium* were compared within spice using an ANOVA test followed by a Tukey's post-hoc test. Results are recorded using the standard deviation. Differences among media and supplements used for recovery were examined for level of significance by Tukey's HSD test. $P<0.05$ were considered significant.

RESULTS

Total aerobic plate count (APC).

The mean of the initial total aerobic bacteria of non-inoculated spices was 9.35 log CFU/g and 8.73 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. No dark brown/black colonies indicative of *Enterococcus* appeared on BEA plates from non-inoculated spices. EtO fumigation significantly reduced total aerobic plate counts by 3.42 ± 0.38 log CFU/g and 2.87 ± 0.06 log CFU/g for whole peppercorns and cumin seeds, respectively (results not shown).

Inactivation of *Salmonella* and *E. faecium*

While ethylene oxide fumigation significantly reduced the mean populations of *Salmonella* and *E. faecium* on whole black peppercorns and cumin seeds ($p<0.05$), the distribution of inactivation variable between bags (Figures 1 and 2). In general, reductions of *Salmonella* on peppercorns (6.62 ± 0.62 log CFU/g) were significantly greater than those for *Enterococcus* ($p=0.05$, 2.96 ± 0.62 log CFU/g) (Figure 1). For all inoculated peppercorns samples, the inactivation of *Salmonella* after EtO treatment was greater compared to *E. faecium*. Reductions of *Salmonella* and *Enterococcus* on cumin seeds were not significantly different ($p=0.33$, 4.9 and 4.6 log CFU/g, respectively) (Figure 2). In bag D, the mean log reduction CFU/g of *Enterococcus* was greater than that of *Salmonella* on cumin seeds subjected to EtO.

Recovery of *Salmonella* subjected to EtO treatment using overlay and various supplements

Salmonella inoculated peppercorns treated with ethylene oxide showed no significant differences between supplements and overlay alone but both supported greater ($P < 0.05$) recovery of *Salmonella* compared to XLT4 alone (Figure 3 and 4). For cumin treated with ethylene oxide overlay was the best recovery medium (5.82 CFU/g) with GP (5.73 CFU/g) the most effective supplement (Figure 5 and 6). The overlay was significantly better ($P < 0.05$) than the least effective supplement, ATP (5.44 CFU/g). Overlay and all the supplements were superior to XLT4 alone (4.96 CFU/g).

DISCUSSION

The FDA considers a 5-log reduction as appropriate for risk management purposes for some foods such as almonds (50). EtO fumigation was able to achieve an average 5-log reduction of *Salmonella enterica* inoculated whole black peppercorns. However, not all sachets containing the inoculated spices achieved comparable reductions between the replicate polywoven bags. The sachets were centrally placed in the bags before shipping and were surrounded by spices on each side. However, the sachets were not anchored in place and may have shifted during shipping and handling. Instructions were provided to place each 5-lb polywoven bag flat on the floor of the chamber in a single layer prior to processing. We expect that the exposure to EtO was not uniform between the polywoven bags and therefore had an influence on the reduction of *Salmonella* and *Enterococcus faecium.*

Ethylene oxide must be able to diffuse into pores and/or through packaging materials to be effective (113, 130). The main concern when choosing packaging is not only the heat and gas permeability but also the absorption of EtO and byproducts ethylene glycol and ethylene chlorohydrin (91). In the medical industry, medical papers and nonwoven materials such as Tyvek (manufactured by DuPont) or ultra-high molecular weight polyethylene (UHMWPE)) are used because they are porous and do not absorb EtO residuals due to large surface areas for them to evaporate off the material (113).

Spices are packaged in bulk for ethylene oxide processing using many different materials and sizes. There is no guideline or standard for packaging; however, spices should be placed in breathable packaging that allows for EtO penetration (146). Common materials used for the bulk packaging of spices include materials such as, high-density polyethylene (HDPE) containers or drums, burlap bags, woven polypropylene bags, and cardboard boxes. In similar sterilization

processes using propylene oxide (PPO) fumigation of almonds, it is suggested that bulk containers such as fiberboard bins and unlined boxes are used because gas cannot penetrate plastic bags and metal containers (51). The effectiveness of ethylene oxide sterilization is greatly influenced by the ability for the process to force the gas into the package and contact the product (113). In these experiments we chose a polypropylene woven bag that has good gas penetration, the gas needed to then penetrate the surrounding spices and a highly permeable muslin sachet to contact the inoculated spices within the center of the bags. The differences in the inactivation between the bags likely reflects different locations within the bags and the presence of the spice may have influenced the penetration. Upon return some of the sachets were located at the outer edges of the bags suggesting these sachets were exposed to more gas than the sachets that remained in the middle of the container. Due to company regulations the spices had to arrive by shipment and researchers could not be present for placement or unloading. As a result no data loggers were placed in the product and the temperatures and gas reported are those measured for the chamber itself.

Surrogate microorganisms are utilized by the industry to validate the effectiveness of control points (124). *Enterococcus faecium* NRRL B-2354 has been used as a surrogate for *Salmonella* species in products such as extruded carbohydrate-protein meal, dry roasted almonds, and moist-air convection treated almonds (1, 26, 83). A conservative surrogate should demonstrate a higher resistance to processing than the target pathogen; therefore a smaller log reduction would be expected of the surrogate. In this instance, *Enterococcus faecium* demonstrated smaller log reductions (greater recovery) than *Salmonella* when inoculated onto whole black peppercorns. In contrast, the log reductions were similar and in one replicate larger for *Enterococcus faecium* when inoculated onto whole cumin seeds indicating it is lee suited as a

surrogate for ethylene oxide treated cumin seeds. Differences in the food matrix can influence the effectiveness of a surrogate microorganism. In a wheat-based product heated at two different temperatures (160°F and 200°F), *Enterococcus faecium* NRRL B-2354 demonstrated a lower heat resistance than the target pathogen, *Salmonella* (99, 101, 102). These results indicated that it would not be an effective surrogate for the inactivation of *Salmonella* using this thermal process in wheat-based products. It is important to note that just because whole black peppercorns and cumin seeds are both spices, they have very different properties. Whole black peppercorns are spherical black, to dark brown dark berries and are between 4.25 and 6 mm in size, whereas cumin seeds are oval in shape, have 4-5 ridges, and are typically 5 mm in size (5, 6).

The recovery of *Salmonella* was improved by application of an overlay approach where damaged cells were provided a three-hour period to repair some damage before the overlay of selective media to be applied. Repair of *Salmonella* injured by ethylene oxide treatments was not notable concerning supplementation providing better repair than overlay alone. In fact, no benefit from supplement addition was apparent, as the recovery was comparable to the non-supplemented overlay for peppercorns and cumin seeds. Glycerophosphate recovered the highest number of cells on peppercorns and was also the best supplement for cumin seeds. In contrast, ATP showed limited ability to repair cells injured by ethylene oxide compared to the other supplements and overlay alone but was still significantly higher than XLT4 alone. Validation studies generally require a process to elicit a specific log reduction to be considered successful. If results are obtained using a selective medium like XLT4 a population reduction could be overestimated by a log or more as shown in Figures 3 and 4. This could lead to an erroneous declaration that product is safe and may lead to illness as injured cells that cannot resuscitate themselves on XLT4 will revive themselves if exposed to a nutrient rich environment.

Supplements were selected based upon the mechanism of action of ethylene oxide. The high energy molecule readily alkylates DNA, RNA, and proteins, impeding normal metabolic functions which inhibits reproduction, rendering cells inactive (30). TDP and sodium pyruvate are known to be antioxidants, scavenging reactive oxygen species that may further damage injured cells (115). These supplements were shown to provide greater recovery of heat injured *Salmonella* in egg albumen (74). Glycerophosphate is a metabolic intermediate included to support the metabolism of injured cells and has been shown to mediate 90% recovery of Salmonella injured in hot water bath (48). Guanine, magnesium, and ATP were selected in order to aid in the repair or creation of DNA and RNA. Magnesium (Mg2+) are important in the stability and function of ribosomal subunits (94, 163). Tween 80 combined with Magnesium resuscitated 36% of freeze damaged *Escherichia coli*. ATP was included because it provides energy essential to the DNA replication process. Guanine was selected to replace ethylene oxide adulterated guanine as it has the highest affinity for reaction with EtO (30).

It was expected that the addition of supplements to overlay would facilitate further repair beyond what was conceded with overlay alone. It has been shown, in an assortment of foods subjected to heat and cold shock, that supplemented media aids recovery of injured bacterial cells (48, 74, 115, 119, 151). It has also been noted that overlay techniques can aid the repair of injured cells (76, 87, 144, 165). In this case, the anticipated synergistic effect was not observed when combining overlay and supplementation. Perhaps the overlay alone recuperated those injured cells that were not beyond the point of recovery so that supplements were unnecessary. Due to the experimental methods used, it cannot be determined if supplementation independently would have provided the same recovery effects as overlay alone. The EtO mechanism damages similar cellular components as temperature shock but inactivates them by chemical addition as

opposed to denaturation and puncturing membranes; this could explain why supplements that were proficient aids to recovery of temperature injured cells were not as helpful to cells injured by EtO.

In order to provide appropriate risk management for a process, the FDA suggests achieving a 5-log reduction of a target pathogen for every sample. Treatment of whole black peppercorns using ethylene oxide fumigation resulted in a mean 6.6 ± 0.62 CFU/g log reduction of *Salmonella enterica.* However, a 5-log reduction was not achieved for each sample analyzed (Figure 1). Likewise, the desired 5-log reduction did not occur with *Salmonella* inoculated cumin seeds for every sample (Figure 2). *Enterococcus faecium* inoculated cumin seeds demonstrated a greater log reduction CFU/g than *Salmonella*, indicating that *E. faecium* is not an ideal surrogate for EtO treated cumin seeds. There was not a significant difference in the reduction of *Salmonella* and *E. faecium* inoculated whole black peppercorns. The recovery of *Salmonella* on whole black peppercorns and cumin seeds treated with EtO fumigation was improved when supplements were used as compared to XLT4 alone.

Further research on the inactivation of *Salmonella enterica* inoculated whole black peppercorns and cumin seeds is necessary. Sachets should be set in place within the polywoven bags so that they are unable to shift during shipping and handling. The placement of bags in the EtO chamber during treatment should also be documented. More importantly, the fluid dynamics of ethylene oxide should be further investigated. Further studies should also explore the specific damage that cells experience when subjected to such treatments. Subsequently supplements particular to those damages can be utilized to optimize recovery.

FIGURES

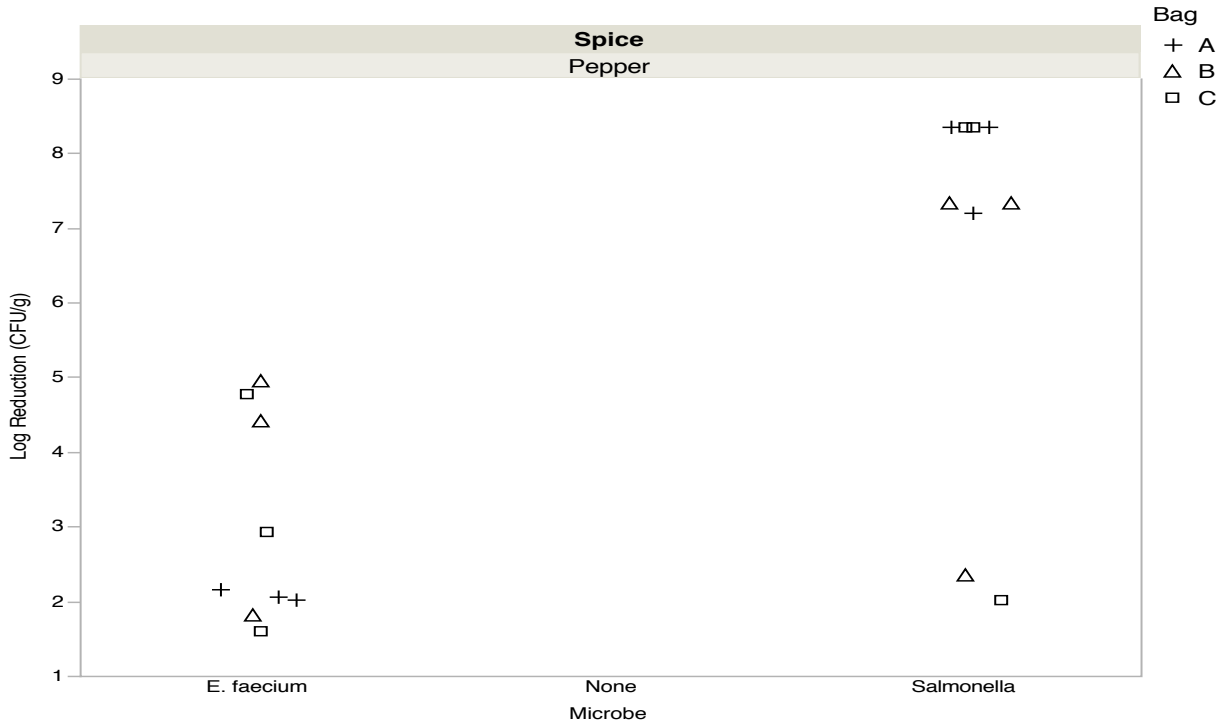

Figure 1. *Log reduction of Salmonella and E. faecium inoculated whole black peppercorns in three different 5-lb bags (A,B,C) each containing 3 replicates per microbe using EtO fumigation. Each symbol represents one sachet located within the 5lb bag.*

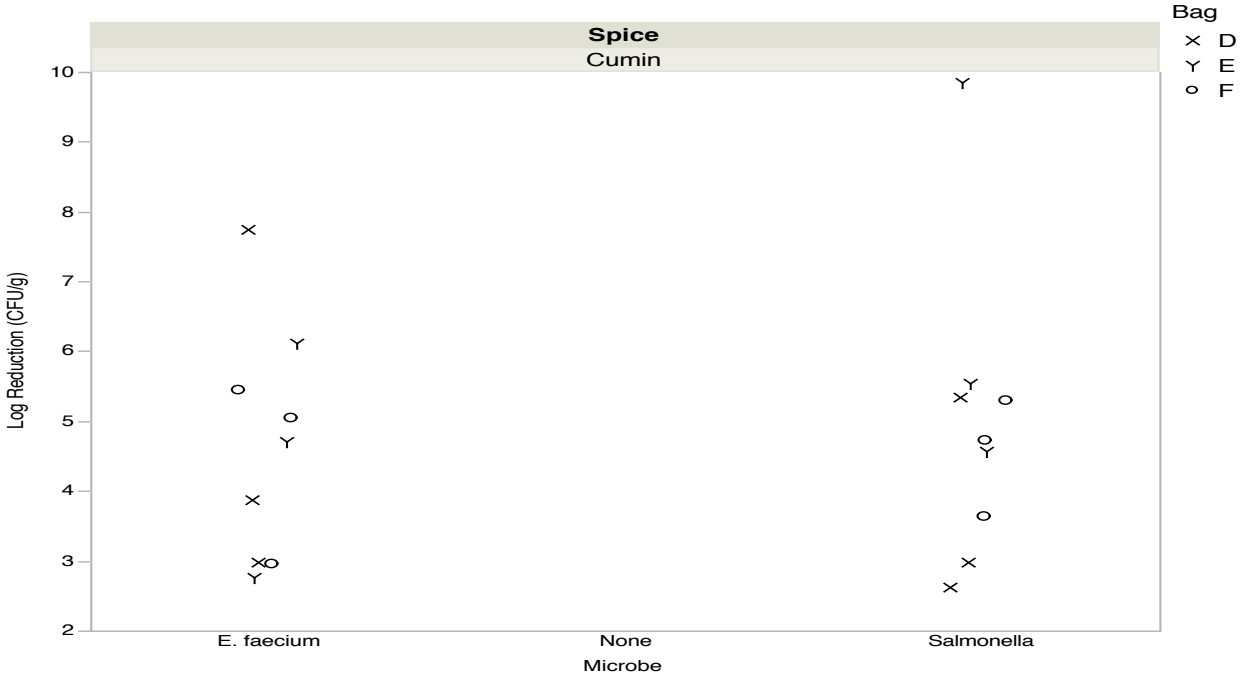

Figure 2. *Log reduction of Salmonella and E. faecium inoculated cumin seeds in three different 5-lb bags (D,E,F) each containing 3 replicates per microbe using EtO fumigation. Each symbol represents one sachet located within the 5lb bag.*

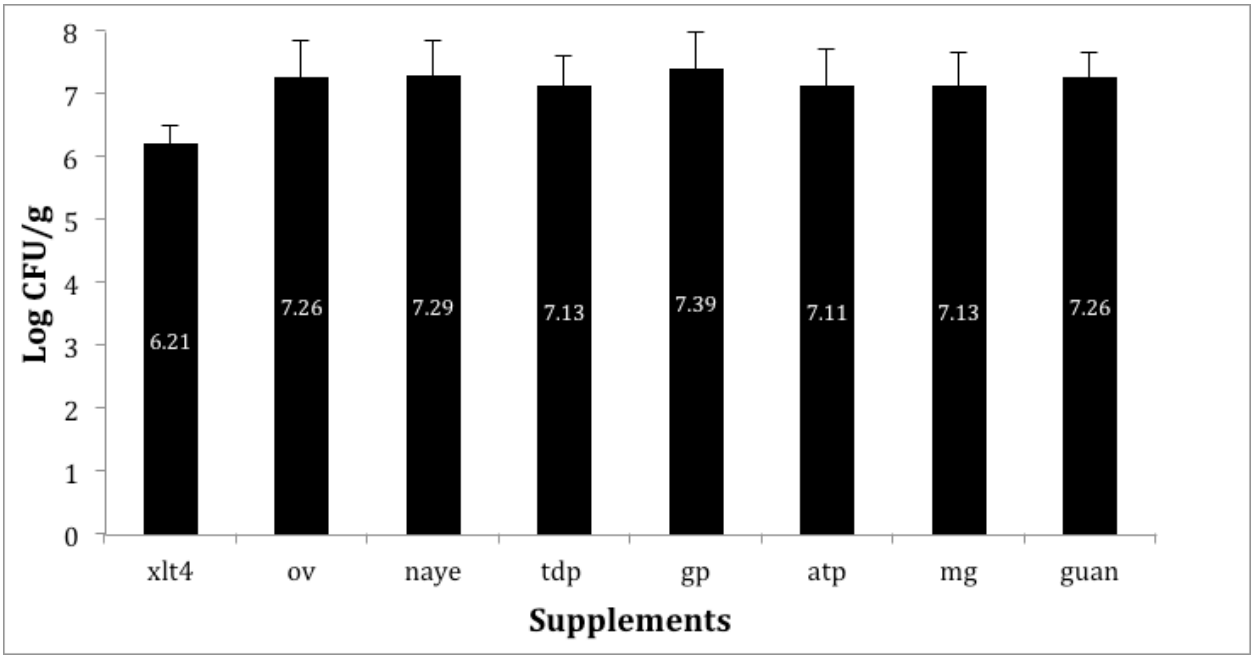

Figure 3. *Comparison of supplements on recovery of Salmonella on peppercorns subjected to ethylene oxide fumigation (n=4), error bars indicate standard deviation from the mean.*

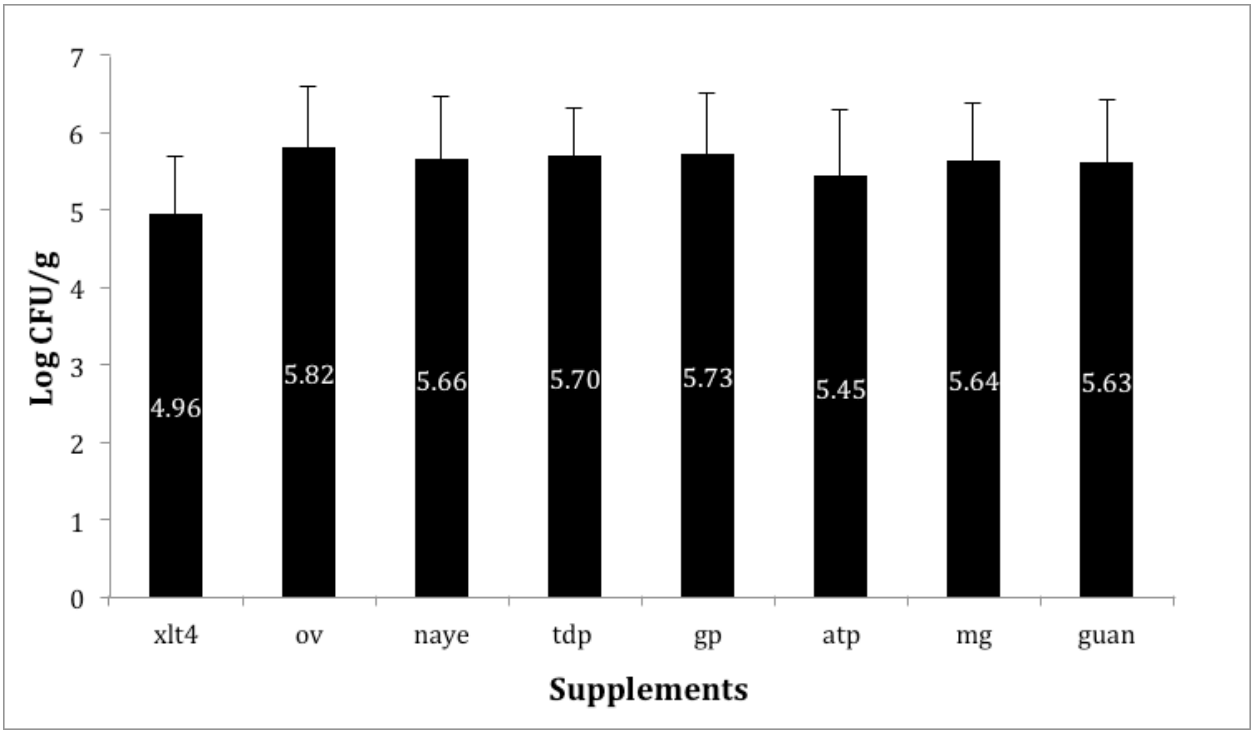

Figure 4. *Comparison of supplements on recovery of Salmonella on cumin seeds subjected to ethylene oxide fumigation (n=4), error bars indicate standard deviation from mean.*

CHAPTER 5: CONCLUSIONS AND FUTURE RESEARCH

The purpose of this research was to determine the effectiveness of a lab-scale steam apparatus and commercial ethylene oxide fumigation treatment on the inactivation of *Salmonella enterica* inoculated whole black peppercorns and cumin seeds. Likewise, the inactivation of *Salmonella* was compared to that of a surrogate microorganism, *Enterococcus faecium* NRRL B-2354, to determine its validity as a surrogate for the pathogen during these processes. The research concluded that *Salmonella* cells inoculated onto whole black peppercorns using the biofilm inclusion method were more resistance to steam treatment than the TSA-grown inoculation method. There was no significant difference in the inactivation of *Salmonella* and *E. faecium* biofilm inclusion inoculated whole black peppercorns. However, there was a significant different in the log reduction of *Salmonella* and *E. faecium* when peppercorns were inoculated using the TSA-grown method. For cumin seeds, there was no significant difference in the reduction of *Salmonella* and *E. faecium* when treated using the lab-scale steam apparatus. Based on these results, *Enterococcus faecium* NRRL B-2354 is a conservative surrogate for *Salmonella enterica* serovars inoculated whole black peppercorns and cumin seeds using these parameters and this lab-scale steam processing equipment.

Whole black peppercorns and cumin seeds were inoculated with a cocktail of *Salmonella enterica* serovars and *Enterococcus faecium* NRRL B-2354 and packaged in 5-lb polywoven polypropylene bags before being treated using a commercial ethylene oxide fumigation process. The research concluded that ethylene oxide fumigation significantly reduced the mean populations of *Salmonella* and *E. faecium* on both whole black peppercorns and cumin seeds ($p<0.05$). However, the inactivation distribution varied between bags. For all peppercorn samples, the inactivation of *Salmonella* was greater than that for *E. faecium.* The reductions of

Salmonella and *E. faecium* on cumin seeds were not significantly different. While inactivation of *Salmonella* and *E. faecium* were similar, the inactivation of *E. faecium* was not consistently lower than *Salmonella* for cumin seeds, which indicates limitations on its applicability as a surrogate for all process parameters and spices.

For future research on the inactivation of *Salmonella* inoculated spices using a lab-scale steam apparatus, modifications should be made to the apparatus and steam generator so that a more consistent process can be achieved. Likewise, an internal pressure gauge connected to a data logger recording at a minimum of one-second intervals should be used. The internalization of *Salmonella* in spices and ways to inactivate the pathogen once it has internalized should also be studied.

To determine the effectiveness of a commercial ethylene oxide fumigation process on the inactivation of *Salmonella* in spices, more replicates should be performed. During these replicates, inoculated spice bags should be set in place and documented to better understand the penetration of ethylene oxide into spices. It would also be beneficial to investigate the gas penetration of common spice bulk packaging materials at various sizes.

References

1. 2007. Guidlines for validation of oil roasting processes, v 1.0, index VG003. *In* Almond Board of California, Modesto, CA.
2. 2010. Investigation of union international food company *Salmonella* Rissen outbreak associated white pepper. *In* California Department of Public Health, Food and Drug Branch, Emergency Response Unit.
3. 2011. Clean, Safe Spices Guidance Document. *In* A.S.T. Association (ed.), ASTA.
4. Date, 2013, Piper nigrum (black pepper). Available at: http://www.kew.org/science-conservation/plants-fungi/piper-nigrum-black-pepper. Accessed February 16, 2016.
5. Date, 2015, Material Data Sheet Black Pepper TGEB- Whole, Heat Treated. Available at: http://www.geraldmcdonald.com/uploads/pdf/M570300.pdf. Accessed, 2016.
6. Date, 2015, Material Data Sheet Cumin Seed Whole. Available at: http://www.geraldmcdonald.com/uploads/pdf/M480100.pdf. Accessed, 2016.
7. Abd, S. J., K. L. McCarthy, and L. J. Harris. 2012. Impact of storage time and temperature on thermal inactivation of *Salmonella* Enteritidis PT 30 on oil-roasted almonds. *Journal of Food Science*. 77:M42-M47.
8. Agres, T. 2015. The cumin scandal: Accidental or fraudulent. *In*, Food Safety & Quality.
9. Andrews, W. H. F., R. S. Silliker, and J. S. Bailey. 2001. Compendium of methods for the microbiological examination of foods.
10. Annous, B. A., and M. F. Kozempel. 1998. Influence of growth medium on thermal resistance of *Pediococcus* sp. NRRL B-2354 (formerly *Micrococcus freudenreichii*) in liquid foods. *Journal of Food Protection*. 61:578-581.
11. Anonymous. Date, 2014, *Salmonella* Ball (presence/25g) in black pepper from Hungary, with raw material from Poland. Accessed, 2015.
12. Arias, C. A., and B. E. Murray. 2012. The rise of the *Enterococcus*: Beyond Vancomycin resistance. *Nat Rev Micro*. 10:266-278.
13. Aviles, B., C. Klotz, J. Eifert, R. Williams, and M. Ponder. 2013. Biofilms promote survival and virulence of *Salmonella* enterica sv. Tennessee during prolonged dry storage and after passage through an in vitro digestion system. *International Journal of Food Microbiology*. 162:252-259.
14. Ban, G.-H., and D.-H. Kang. 2016. Effectiveness of superheated steam for inactivation of *Escherichia coli* O157:H7, *Salmonella* Typhimurium, *Salmonella* Enteritidis phage type 30, and *Listeria monocytogenes* on almonds and pistachios. *International Journal of Food Microbiology*. 220:19-25.
15. Ban, G.-H., S.-H. Park, S.-O. Kim, S. Ryu, and D.-H. Kang. 2012. Synergistic effect of steam and lactic acid against *Escherichia coli* O157:H7, *Salmonella* Typhimurium, and *Listeria monocytogenes* biofilms on polyvinyl chloride and stainless steel. *International Journal of Food Microbiology*. 157:218-223.
16. Ban, G.-H., H. Yoon, and D.-H. Kang. 2014. A comparison of saturated steam and superheated steam for inactivation of *Escherichia coli* O157:H7, *Salmonella* Typhimurium, and *Listeria monocytogenes* biofilms on polyvinyl chloride and stainless steel. *Food Control*. 40:344-350.
17. Banerjee, M., and P. K. Sarkar. 2003. Microbiological quality of some retail spices in India. *Food Research International*. 36:469-474.

18. Bawden, T. 2015. The nuts-for-spices crisis: How a complex supply chain makes tracing dodgy food difficult. *In*, Independent, Independent.
19. Baxter, R., and W. H. Holzapfel. 1982. A Microbial investigation of selected spices, herbs, and additives in South Africa. *Journal of Food Science*. 47:570-574.
20. Bell, C., and A. Kyriakides. 2002. *Salmonella*: a practical approach to the organism and its control in foods. Blackwell Science, Malden, MA; Oxford.
21. Beuchat, L. R. 2009. Behavior of *Salmonella* in foods with low water activity. *In*, IAFP Rapid response symposium, Arlington, VA.
22. Beuchat, L. R., E. Komitopoulou, H. Beckers, R. P. Betts, F. Bourdichon, S. Fanning, H. M. Joosten, and B. H. Ter Kuile. 2013. Low-water activity foods: Increased concern as vehicles of foodborne pathogens. *Journal of Food Protection*. 76:150-172.
23. Beuchat, L. R., and D. A. Mann. 2010. Factors affecting infiltration and survival of Salmonella on in-shell pecans and pecan nutmeats. *Journal of Food Protection*. 73:1257-1268.
24. Beuchat, L. R., and D. A. Mann. 2011. Inactivation of *Salmonella* on in-shell pecans during conditioning treatments preceding cracking and shelling. *Journal of Food Protection*. 74:588-602.
25. Beuchat, L. R., and D. A. Mann. 2011. Inactivation of *Salmonella* on pecan nutmeats by hot air treatment and oil roasting. *Journal of Food Protection*. 74:1441-1450.
26. Bianchini, A., J. Stratton, S. Weier, T. Hartter, B. Plattner, G. Rokey, G. Hertzel, L. Gompa, B. Martinez, and K. M. Eskridge. 2014. Use of *Enterococcus faecium* as a surrogate for *Salmonella enterica* during extrusion of a balanced carbohydrate-protein meal. *Journal of Food Protection*. 77:75-82.
27. Bingol, G., J. Yang, M. T. Brandl, Z. Pan, H. Wang, and T. H. McHugh. 2011. Infrared pasteurization of raw almonds. *Journal of Food Engineering*. 104:387-393.
28. Blackburn, C. d. W., L. M. Curtis, L. Humpheson, C. Billon, and P. J. McClure. 1997. Development of thermal inactivation models for *Salmonella* Enteritidis and *Escherichia coli* O157:H7 with temperature, pH and NaCl as controlling factors. *International Journal of Food Microbiology*. 38:31-44.
29. Blessington, T., C. G. Theofel, and L. J. Harris. 2013. A dry-inoculation method for nut kernels. *Food Microbiology*. 33:292-297.
30. Bohnet, M. 2003. Ullmann's encyclopedia of industrial chemistry. Wiley-VCH, Weinheim.
31. Bommer, J., and E. Ritz. 1987. Ethylene Oxide (ETO) as a major cause of anaphylactoid reactions in dialysis (A review). *Artificial Organs*. 11:111-117.
32. Borowski, A. G., S. C. Ingham, and B. H. Ingham. 2009. Lethality of home-style dehydrator processes against *Escherichia coli* O157:H7 and *Salmonella* serovars in the manufacture of ground-and-formed beef jerky and the potential for using a pathogen surrogate in process validation. *Journal of Food Protection*. 72:2056-2064.
33. Borowski, A. G., S. C. Ingham, and B. H. Ingham. 2009. Validation of ground-and-formed beef jerky processes using commercial lactic acid bacteria starter cultures as pathogen surrogates. *Journal of Food Protection*. 72:1234-1247.
34. Bowman, L. S. 2015. Impacts of inoculation strategy on survival of *Salmonella enterica* and surrogate *Enterococcus faecium* at low water activity on dry peppercorn and cumin seeds. *In*, Food Science and Technology, vol. Master of Science in Life Science. Virginia Tech, Blacksburg, VA.

35. Burnett, S. L., E. R. Gehm, W. R. Weissinger, and L. R. Beuchat. 2000. Survival of *Salmonella* in peanut butter and peanut butter spread. *Journal of Applied Microbiology*. 89:472-477.
36. Carpentier, B., and O. Cerf. 1993. Biofilms and their consequences, with particular reference to hygiene in the food industry. *Journal of Applied Bacteriology*. 75:499-511.
37. CDC. 1993. *Salmonella* serotype Tennessee in powdered milk products and infant formula. p. 516-517. *In* CDC (ed.), Morbidity and Mortality Weekly Report, Canada and United States.
38. CDC. 1998. Multistate outbreak of *Salmonella* serotype Agona infections linked to toasted oats and cereal. p. 462-464. *In* CDC (ed.), Morbidity and Mortality Weekly Report, United States.
39. CDC. 2007. Multistate outbreak of *Salmonella* Tennessee infections linked to peanut butter (final update). *In* CDC (ed.), vol. 2015.
40. CDC. Date, 2010, Multistate outbreak of human *Salmonella* Montevideo infections (final update). Accessed February 25, 2016.
41. CDC. 2010. *Salmonella* Montevideo infections associated with salami products made with contaminated imported black pepper and red pepper. p. 1647-1650. *In*, United States.
42. CDC. April 26, 2015. *Salmonella*. *In* CDC (ed.).
43. Ceylan, E., and D. A. Bautista. 2015. Evaluating *Pediococcus acidilactici* and *Enterococcus faecium* NRRL B-2354 as thermal surrogate microorganisms for *Salmonella* for in-plant validation studies of low-moisture pet food products. *Journal of Food Protection*. 78:934-939.
44. Chang, S. S., A. R. Han, J. I. Reyes-De-Corcuera, J. R. Powers, and D. H. Kang. 2010. Evaluation of steam pasteurization in controlling *Salmonella* serotype Enteritidis on raw almond surfaces. *Letters in Applied Microbiology*. 50:393-398.
45. Cheng-Hsun, and L.-H. Su. Date, *Salmonella*, non-Typhoidal species (*S*. Choleraesuis, *S*. Enteritidis, *S*. Hadar, *S*. Typhimurium). Available at: http://www.antimicrobe.org/new/b258.asp. Accessed February 18, 2016.
46. Craven, P., W. Baine, D. Mackel, W. Barker, E. Gangarosa, M. Goldfield, H. Rosenfeld, R. Altman, G. Lachapelle, J. Davies, and R. Swanson. 1975. International outbreak of *Salmonella* Eastbourne infection traced to contaminated chocolate. *The Lancet*. 305:788-792.
47. Czarra, F. 2009. Spices a blobal history. Reaktion Books Ltd, London.
48. D'Aoust, J. Y. 1978. Recovery of sublethally heat-injured *Salmonella* Typhimurium on supplemented plating media. *Applied and Environmental Microbiology*. 35:483-486.
49. Danyluk, M. D., M. T. Brandl, and L. J. Harris. 2008. Migration of *Salmonella* Enteritidis Phage Type 30 through almond hulls and shells. *Journal of Food Protection*. 71:397-401.
50. Danyluk, M. D., L. J. Harris, and D. W. Schaffner. 2006. Monte Carlo simulations assessing the risk of Salmonellosis from consumption of almonds. *Journal of Food Protection*. 69:1594-1599.
51. Danyluk, M. D., A. R. Uesugi, and L. J. Harris. 2005. Survival of *Salmonella* Enteritidis PT 30 on inoculated almonds after commercial fumigation with propylene oxide. *Journal of Food Protection*. 68:1613-1622.
52. De Boer, E., and E. M. Boot. 1983. Comparison of methods for isolation and confirmation of *Clostridium perfringens* from spices and herbs. *Journal of Food Protection*. 46:533-536.

53. De Boer, E., W. M. Spiegelenberg, and F. W. Janssen. 1985. Microbiology of spices and herbs. *Antonie van Leeuwenhoek*. 51:435-438.
54. Doyle, M. E., and A. S. Mazzotta. 2000. Review of studies on the thermal resistance of *Salmonellae*. *Journal of Food Protection*. 63:779-795.
55. Du, W.-X., M. D. Danyluk, and L. J. Harris. 2010. Efficacy of aqueous and alcohol-based Quaternary Ammonium sanitizers for reducing *Salmonella* in dusts generated in almond hulling and shelling facilities. *Journal of Food Science*. 75:M7-M13.
56. Enache, E., A. Kataoka, D. G. Black, C. D. Napier, R. Podolak, and M. M. Hayman. 2015. Development of a dry inoculation method for thermal challenge studies in low-moisture foods by using talc as a carrier for *Salmonella* and a surrogate (*Enterococcus faecium*). *Journal of Food Protection*. 78:1106-1112.
57. EPA. 2006. Report of the Food Quality Protection Act (FQPA) Tolerance Reassessment and Risk Management Decision (TRED) for ethylene oxide. *In* U.S.E.P. Agency (ed.).
58. Farkas, J. 2000. The microbiological safety and quality of food. Aspen Publishers, Gaithersburg, Md.
59. Farrell, K. T. 1990. Spices, condiments, and seasonings. Chapman & Hall, New York.
60. FDA. 1998. Defect levels handbook. *In* U.S. Food and Drug Administration.
61. FDA. 2013. Draft risk profile: Pathogens and filth in spices.
62. FDA. 2015. Code of federal regulations. *In*, vol. 2. FDA, Department of Health and Human Services.
63. FDA. Date, 2016, Archive for recalls, market withdrawals, & safety alerts. Available at: http://www.fda.gov/Safety/Recalls/ArchiveRecalls/default.htm. Accessed February 27, 2016.
64. Finn, S., O. Condell, P. McClure, A. Amézquita, and S. Fanning. 2013. Mechanisms of survival, responses and sources of *Salmonella* in low-moisture environments. *Frontiers in Microbiology*. 4:331.
65. Fisher, K., and C. Phillips. 2009. The ecology, epidemiology and virulence of *Enterococcus*. *Microbiology*. 155:1749-1757.
66. Foods, T. I. C. o. M. S. f. 1980. 24 - Spices. Academic Press.
67. García, S., F. Iracheta, F. Galván, and N. Heredia. 2001. Microbiological survey of retail herbs and spices from Mexican markets. *Journal of Food Protection*. 64:99-103.
68. Geesey, G. G. 1982. Microbial exopolymers: Ecological and economic considerations. *American Society of Microbiology News*. 48:9-14.
69. Gibson, B. 1973. The effect of high sugar concentrations on the heat resistance of vegetative microorganisms. *Journal of Applied Bacteriology*:365-376.
70. Gieraltowski, L., E. Julian, J. Pringle, K. Macdonald, D. Quilliam, N. Marsden-Haug, L. Saathoff-Huber, D. Von Stein, B. Kissler, M. Parish, D. Elder, V. Howard-King, J. Besser, S. Sodha, A. Loharikar, S. Dalton, I. Williams, and C. B. Behravesh. 2013. Nationwide outbreak of *Salmonella* Montevideo infections associated with contaminated imported black and red pepper: Warehouse membership cards provide critical clues to identify the source. *Epidemiology and Infection*. 141:1244-52.
71. Goepfert, J. M., and R. A. Biggie. 1968. Heat resistance of *Salmonella* Typhimurium and *Salmonella* Senftenberg 775W in milk chocolate. *Applied Microbiology*. 16:1939-1940.

72. Grammar, L. C., Shaughnessy, M. A., Paterson, B.F., Patterson, R. . 1985. Characterization of an antigen in acute anaphylactic dialysis reactions: Ethylene oxide-altered human serum albumin. *Journal of Allergy and Clinical Immunology*. 76:670-675.
73. Gruzdev, N., R. Pinto, and S. Sela. 2012. Persistence of *Salmonella enterica* during dehydration and subsequent cold storage. *Food Microbiology*. 32:415-422.
74. Gurtler, J. B., and J. L. Kornacki. 2009. Comparison of supplements to enhance recovery of heat-injured Salmonella from egg albumen. *Letters in Applied Microbiology*. 49:503-509.
75. Habimana, O., T. Moretro, S. Langsrud, L. K. Vestby, L. L. Nesse, and E. Heir. 2010. Micro ecosystems from feed industry surfaces: a survival and biofilm study of *Salmonella* versus host resident flora strains. *BMC Veterinary Research*. 6:1-10.
76. Hartman, P. A., P. S. Hartman, and W. W. Lanz. 1975. Violet red bile 2 agar for stressed coliforms. *Applied Microbiology*. 29:537-539.
77. Higa, J. 2011. Outbreak of *Salmonella* Rissen associated with ground white pepper: The epi investigation. *In* Department of Public Health, California.
78. Hiramatsu, R., M. Matsumoto, K. Sakae, and Y. Miyazaki. 2005. Ability of shiga toxin-producing *Escherichia coli* and *Salmonella* spp. to survive in a desiccation model system and in dry foods. *Applied and Environmental Microbiology*. 71:6657-6663.
79. Horner, K. J., and G. D. Anagnostopoulos. 1972. Viability of *Staphylococcus aureus*, *Salmonella* Typhimurium and *Salmonella* Senftenberg heated and recovered on a solid medium of controlled water activity. *Journal of the Science of Food and Agriculture*. 23:543-544.
80. ICMSF. 2005. Microorganisms in foods. Kluwer Academic/Plenum Publishers, New York, New York.
81. James, C., E. O. Göksoy, J. E. L. Corry, and S. J. James. 2000. Surface pasteurisation of poultry meat using steam at atmospheric pressure. *Journal of Food Engineering*. 45:111-117.
82. James, C., and S. J. James. 1997. Meat decontamination: The state of the art. MAFF Advanced Fellowship in Food Process Engineering.
83. Jeong, S., B. P. Marks, and E. T. Ryser. 2011. Quantifying the performance of *Pediococcus* sp. (NRRL B-2354: *Enterococcus faecium*) as a nonpathogenic surrogate for *Salmonella* Enteritidis PT30 during moist-air convection heating of almonds. *Journal of Food Protection*. 74:603-609.
84. Joseph, B., S. K. Otta, and I. Karunasagar. 2001. Biofilm formation by *Salmonella* spp. on food contact surfaces and their sensitivity to sanitizers. *Int J Food Microbiol*. 64.
85. Julseth, R. M., and R. H. Deibel. 1974. Microbial profile of selected spices and herbs at import. *Journal of Milk and Food Technology (JMFT)*. 37:414-419.
86. Kang, D.-H., and D. Y. C. Fung. 2000. Application of thin agar layer method for recovery of injured *Salmonella* Typhimurium. *International Journal of Food Microbiology*. 54:127-132.
87. Kang, D. H., and G. R. Siragusa. 1999. Agar Underlay Method for Recovery of Sublethally Heat-Injured Bacteria. *Applied and Environmental Microbiology*. 65:5334-5337.
88. Kataoka, A., E. Enache, D. G. Black, P. H. Elliott, C. D. Napier, R. Podolak, and M. M. Hayman. 2014. Survival of *Salmonella* Tennessee, *Salmonella* Typhimurium DT104, and *Enterococcus faecium* in peanut paste formulations at two different levels of water activity and fat. *Journal of Food Protection*. 77:1252-1259.
89. Keller, S. E., E. M. Grasso, L. A. Halik, G. J. Fleischman, S. J. Chirtel, and S. F. Grove. 2012. Effect of growth on the thermal resistance and survival of *Salmonella* Tennessee and

Oranienburg in peanut butter, measured by a new thin-layer thermal death time device. *Journal of Food Protection*. 75:1125-1130.
90. Keller, S. E., J. M. VanDoren, E. M. Grasso, and L. A. Halik. 2013. Growth and survival of *Salmonella* in ground black pepper (Piper nigrum). *Food Microbiology*. 34:182-188.
91. Kelsey, J. C. 1974. Industrial sterilization, international symposium, Amsterdam 1972. *Journal of Medical Microbiology*. 7:311-311.
92. Kieboom, J., H. D. Kusumaningrum, M. H. Tempelaars, W. C. Hazeleger, T. Abee, and R. R. Beumer. 2006. Survival, elongation, and elevated tolerance of *Salmonella enterica* serovar Enteritidis at reduced water activity. *Journal of Food Protection*. 69:2681-2686.
93. Kirk, M. D., C. L. Little, M. Lem, M. Fyfe, D. Genobile, A. Tan, J. Threlfall, A. Paccagnella, D. Lightfoot, H. Lyi, L. McIntyre, L. Ward, D. J. Brown, S. Surnam, and I. S. T. Fisher. 2004. An outbreak due to peanuts in their shell caused by *Salmonella enterica* serotypes Stanley and Newport-sharing molecular information to solve international outbreaks. *Epidemiology and Infection*. 132:571-577.
94. Klein, D. J., P. B. Moore, and T. A. Steitz. 2004. The contribution of metal ions to the structural stability of the large ribosomal subunit. *RNA*. 10:1366-1379.
95. Klein, G. 2003. Taxonomy, ecology and antibiotic resistance of enterococci from food and the gastro-intestinal tract. *International journal of food microbiology*. 88:123-131.
96. Kneifel, W., and E. Berger. 1994. Microbiological criteria of random samples of spices and herbs retailed on the Austrian market. *Journal of Food Protection*. 57:893-901.
97. Konuma, H., K. Shinagawa, M. Tokumaru, Y. Onoue, S. Konno, N. Fujino, T. Shigehisa, H. Kurata, Y. Kuwabara, and C. A. M. Lopes. 1988. Occurrence of *Bacillus cereus* in meat products, raw meat and meat product additives. *Journal of Food Protection*. 51:324-326.
98. Kopit, L. M., E. B. Kim, R. J. Siezen, L. J. Harris, and M. L. Marco. 2014. Safety of the surrogate microorganism *Enterococcus faecium* NRRL B-2354 for use in thermal process validation. *Applied and Environmental Microbiology*. 80:1899-1909.
99. Kornacki, J. L. 2006. Examples of surrogate validation and use in the industry. *In*, IAFP Annual Meeting, Calgary, Alberta, Canada.
100. Kornacki, J. L. 2011. Indicator organisms: Chaos, confusion and criteria. *In*, Food Safety Magazine, Testing.
101. Kornacki, J. L. 2012. *Enterococcus faecium* NRRL B-2354: Tempest in a teapot or serious foodborne pathogen. *In*, Food Safety Magazine, Bacteria.
102. Kornacki, J. L., and J. Hoorfar. 2012. Hygiene control in the dry food products industry: The roles of cleaning methods and hygienic indicators. *Case studies in food safety and authenticity: lessons from real-life situations*:254-266.
103. Kotzekidou, P. 1998. Microbial stability and fate of *Salmonella* Enteritidis in halva, a low-moisture confection. *Journal of Food Protection*. 61:181-185.
104. Krishnaswamy, M. A., J. D. Patel, and N. Parthasarathy. 1971. Enumeration of micro-organisms in spices and spice mixtures. *Journal of Food Science and Technology*. 8:191-194.
105. Lee, B. H., S. Kermasha, and B. E. Baker. 1989. Thermal, ultrasonic and ultraviolet inactivation of *Salmonella* in thin films of aqueous media and chocolate. *Food Microbiology*. 6:143-152.
106. Lee, S.-Y., S.-W. Oh, H.-J. Chung, J. I. Reyes-De-Corcuera, J. R. Powers, and D.-H. Kang. 2006. Reduction of *Salmonella enterica* serovar Enteritidis on the surface of raw shelled almonds by exposure to steam. *Journal of Food Protection*. 69:591-595.

107. Lehmacher, A., J. Bockemühl, and S. Aleksic. 1995. Nationwide outbreak of human salmonellosis in Germany due to contaminated paprika and paprika-powdered potato chips. *Epidemiology and Infection*. 115:501-511.
108. Lesne, J., S. Berthet, S. Binard, A. Rouxel, and F. Humbert. 2000. Changes in culturability and virulence of Salmonella typhimurium during long-term starvation under desiccating conditions. *International Journal of Food Microbiology*. 60:195-203.
109. Llorente Franco, S., J. L. Gimenez, F. Martinez Sanchez, and F. Romojaro. 1986. Effectiveness of ethylene oxide and gamma irradiation on the microbiological population of three types of paprika. *Journal of Food Science*. 51:1571.
110. Ma, L., J. L. Kornacki, G. Zhang, C.-M. Lin, and M. P. Doyle. 2007. Development of thermal surrogate microorganisms in ground beef for in-plant critical control point validation studies. *Journal of Food Protection*. 70:952-957.
111. Ma, L., G. Zhang, P. Gerner-Smidt, V. Mantripragada, I. Ezeoke, and M. P. Doyle. 2009. Thermal inactivation of *Salmonella* in peanut butter. *Journal of Food Protection*. 72:1596-1601.
112. Margas, E., N. Meneses, B. Conde-Petit, C. E. R. Dodd, and J. Holah. 2014. Survival and death kinetics of *Salmonella* strains at low relative humidity, attached to stainless steel surfaces. *International Journal of Food Microbiology*. 187:33-40.
113. Marsh, K. S. 1997. The Wiley encyclopedia of packaging technology. Wiley, New York.
114. Mattick, K. L., T. J. Rowbury Rj Fau - Humphrey, and T. J. Humphrey. Morphological changes to *Escherichia coli* O157:H7, commensal *E. coli* and *Salmonella* spp in response to marginal growth conditions, with special reference to mildly stressing temperatures.
115. McDonald, L. C., C. R. Hackney, and B. Ray. 1983. Enhanced recovery of injured *Escherichia coli* by compounds that degrade hydrogen peroxide or block its formation. *Applied and Environmental Microbiology*. 45:360-365.
116. Mendes, G. C. C., T. R. S. Brandão, and C. L. M. Silva. 2007. Ethylene oxide sterilization of medical devices: A review. *American Journal of Infection Control*. 35:574-581.
117. Michael, G. T., and C. R. Stumbo. 1970. Ethylene oxide sterilization of *Salmonella* Senftenberg and *Escherichia coli* death kinetics and mode of action. *Journal of Food Science*. 35:631-634.
118. Morgan, A. I., N. Goldberg, E. R. Radewonuk, and O. J. Scullen. 1996. Surface pasteurization of raw poultry meat by steam. *LWT - Food Science and Technology*. 29:447-451.
119. Murthy, T. R. K., and R. Gaur. 1987. Effect of incorporation of tween 80 and magnesium chloride on the recovery of coliforms in VRB medium from fresh, refrigerated and frozen minced buffalo meat. *International Journal of Food Microbiology*. 4:341.
120. Pafumi, J. 1986. Assessment of the microbiological quality of spices and herbs. *Journal of Food Protection*. 49:958-963.
121. Palipane, K. B., and R. H. Driscoll. 1993. Moisture sorption characteristics of in-shell macadamia nuts. *Journal of Food Engineering*. 18:63-76.
122. Piyasena, P., R. C. McKellar, and F. M. Bartlett. 2003. Thermal inactivation of *Pediococcus* sp. in simulated apple cider during high-temperature short-time pasteurization. *International Journal of Food Microbiology*. 82:25-31.
123. Podolak, R., E. Enache, W. Stone, D. G. Black, and P. H. Elliott. 2010. Sources and risk factors for contamination, survival, persistence, and heat resistance of *Salmonella* in low-moisture foods. *Journal of Food Protection*. 73:1919-1936.
124. Ponder, M. 2015. Process validation. *In* Virginia Tech.

125. Poothullil, J., A. Shimizu, R. P. Day, and J. Dolovich. 1975. Anaphylaxis from the product(s) of ethylene oxide gas. *Annals of Internal Medicine*. 82:58-60.
126. Powers, E. M., T. G. Latt, and T. Brown. 1976. Incidence and levels of *Bacillus cereus* in processed spices. *Journal of Milk and Food Technology (JMFT)*. 39:668-670.
127. Prabhakaran Nair, K. P. 2004. The agronomy and economy of black pepper (Piper nigrum L.) —The "King of Spices". p. 271-389. *In*, Advances in Agronomy, vol. Volume 82. Academic Press.
128. Purello D'Ambrosio, F., V. Savica, S. Gangemi, L. Ricciardi, G. F. Bagnato, D. Santoro, S. Cuzzocrea, and G. Bellinghieri. 1997. Ethylene oxide allergy in dialysis patients. *Nephrology Dialysis Transplantation*. 12:1461-1463.
129. Ray, B. 1979. Methods to detect stressed microorganisms. *Journal of Food Protection*. 42:346-355.
130. Rebsdat, S., and D. Mayer. 2000. Ethylene oxide. *In*, Ullmann's Encyclopedia of Industrial Chemistry Wiley-VCH Verlag GmbH & Co. KGaA.
131. Reeves, R. G., C. A. McDaniel, and J. H. Ford. 1985. Organic and inorganic bromide residues in spices fumigated with methyl bromide. *Journal of Agricultural and Food Chemistry*. 33:780-783.
132. Roberts, D., G. N. Watson, and R. J. Gilbert. 1982. Contamination of food plants and plant products with bacteria of public health significance. p. 169-195. *In*, UNITED STATES.
133. Roberts, T. A., J. L. Cordier, L. Gram, R. B. Tompkin, J. I. Pitt, L. G. M. Gorris, and K. M. J. Swanson. 2005. Spices, dry soups, and oriental flavorings. p. 360-391. *In* T.A. Roberts, et al. (ed.), Micro-Organisms in Foods 6 Springer US.
134. Rushdy, A. A., J. M. Stuart, L. R. Ward, J. Bruce, E. J. Threlfall, P. Punia, and J. R. Bailey. 1998. National outbreak of *Salmonella* senftenberg associated with infant food. *Epidemiology and Infection*. 120:125-128.
135. Rutala, W. A., and D. J. Weber. 1999. Infection control: The role of disinfection and sterilization. *Journal of Hospital Infection*. 43, Supplement 1:S43-S55.
136. Santillana Farakos, S. M., J. F. Frank, and D. W. Schaffner. 2013. Modeling the influence of temperature, water activity and water mobility on the persistence of *Salmonella* in low-moisture foods. *International Journal of Food Microbiology*. 166:280-293.
137. Santillana Farakos, S. M., D. W. Schaffner, and J. F. Frank. 2014. Predicting survival of *Salmonella* in low-water activity foods: An analysis of literature data. *Journal of Food Protection*. 77:1448-1461.
138. Scott, V. N., Y. U. H. Chen, T. A. Freier, J. Kuehm, M. Moorman, J. Meyer, T. Morille-Hinds, L. Post, L. Smoot, S. Hood, J. Shebuski, and J. Banks. 2009. Control of *Salmonella* in low-moisture foods: Minimizing entry of *Salmonella* into a processing facility. *Food Protection Trends*. 29:342-353.
139. Shachar, D., and S. Yaron. 2006. Heat tolerance of *Salmonella enterica* serovars Agona, Enteritidis, and Typhimurium in peanut butter. *Journal of Food Protection*. 69:2687-2691.
140. Sheth, A. N., M. Hoekstra, N. Patel, G. Ewald, C. Lord, C. Clarke, E. Villamil, K. Niksich, C. Bopp, T.-A. Nguyen, D. Zink, and M. Lynch. 2011. A national outbreak of *Salmonella* serotype Tennessee infections from contaminated peanut butter: A new food vehicle for Salmonellosis in the United States. *Clinical Infectious Diseases*. 53:356-362.

141. Smith, J. P., D. P. Daifas, W. El-Khoury, J. Koukoutsis, and A. El-Khoury. 2004. Shelf life and safety concerns of bakery products: A review. *Critical Reviews in Food Science and Nutrition*:19-55.
142. Solano, C., B. García, J. Valle, C. Berasain, J. M. Ghigo, C. Gamazo, and I. Lasa. 2002. Genetic analysis of *Salmonella* Enteritidis biofilm formation: Critical role of cellulose. *Molecular microbiology*. 43:793-808.
143. Sotir, M. J., G. Ewald, A. C. Kimura, J. I. Higa, A. Sheth, S. Troppy, S. Meyer, R. M. Hoekstra, J. Austin, J. Archer, M. Spayne, E. R. Daly, and P. M. Griffin. 2009. Outbreak of *Salmonella* Wandsworth and Typhimurium infections in infants and toddlers traced to a commercial vegetable-coated snack food. *The Pediatric Infectious Disease Journal*. 28.
144. Speck, M. L., B. Ray, and R. B. Read. 1975. Repair and enumeration of injured coliforms by a plating procedure. *Applied Microbiology*. 29:549-550.
145. Steenackers, H., K. Hermans, J. Vanderleyden, and S. C. J. De Keersmaecker. 2012. *Salmonella* biofilms: An overview on occurrence, structure, regulation and eradication. *Food Research International*. 45:502-531.
146. Sterigenics. Date, Ethylene Oxide. Available at: http://www.sterigenics.com/services/medical_sterilization/contract_sterilization/ethylene_oxide/sterilization_alternatives__ethylene_oxide.pdf. Accessed February 14, 2016.
147. Steris. Date, 2016, Ethylene Oxide Sterilization. Available at: http://www.isomedix.com/services/eo-sterilization/. Accessed January 28, 2016, 2016.
148. Stevenson, K., K. Ito, L. Beuchat, B. P. Marks, and D. Ashton. 2014. Guidelines for using *Enterococcus faecium* NRRL B-2354 as a surrogate microorganism in almond process validation. *In*, vol. 2015. Almond Board of California.
149. Sumner, S. S., T. M. Sandros, M. C. Harmon, V. N. Scott, and D. T. Bernard. 1991. Heat resistance of *Salmonella* Typhimurium and *Listeria monocytogenes* in sucrose solutions of various water activities. *Journal of Food Science*. 56:1741-1743.
150. Swenberg, J. A., A. Ham, H. Koc, E. Morinello, A. Ranasinghe, N. Tretyakova, P. B. Upton, and K.-Y. Wu. 2000. DNA adducts: Effects of low exposure to ethylene oxide, vinyl chloride and butadiene. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*. 464:77-86.
151. Taskila, S., E. Osmekhina, M. Tuomola, J. Ruuska, and P. Neubauer. 2011. Modification of buffered peptone water for improved recovery of heat-injured *Salmonella* Typhimurium. *Journal of Food Science*. 76:M157-M162.
152. TLV. Date, 2016, Types of steam. Available at: http://www.tlv.com/global/US/steam-theory/types-of-steam.html - toc_2. Accessed February 2, 2016, 2016.
153. Toledo, R. T. 1991. Fundamentals of food process engineering. Van Nostrand Reinhold, New York, New York.
154. Uesugi, A. R., M. D. Danyluk, and L. J. Harris. 2006. Survival of *Salmonella* Enteritidis Phage Type 30 on inoculated almonds stored at -20, 4, 23, and 35°C. *Journal of Food Protection*. 69:1851-1857.
155. Vajdi, M., and R. R. Pereira. 1973. Comparative effects of ethylene oxide, gamma irradiation and microwave treatments on selected spices. *Journal of Food Science*. 38:893-895.

156. Van Doren, J. M., D. Kleinmeier, T. S. Hammack, and A. Westerman. 2013. Prevalence, serotype diversity, and antimicrobial resistance of *Salmonella* in imported shipments of spice offered for entry to the United States, FY2007–FY2009. *Food Microbiology*. 34:239-251.
157. Van Doren, J. M., K. P. Neil, M. Parish, L. Gieraltowski, L. H. Gould, and K. L. Gombas. 2013. Foodborne illness outbreaks from microbial contaminants in spices, 1973–2010. *Food Microbiology*. 36:456-464.
158. VanCauwenberge, J. E., R. J. Bothast, and W. F. Kwolek. 1981. Thermal inactivation of eight *Salmonella* serotypes on dry corn flour. *Applied and Environmental Microbiology*. 42:688-691.
159. Vasan, A., R. Geier, S. C. Ingham, and B. H. Ingham. 2014. Thermal tolerance of O157 and non-O157 shiga toxigenic strains of *Escherichia coli*, *Salmonella*, and potential pathogen surrogates, in frankfurter batter and ground beef of varying fat levels. *Journal of Food Protection*. 77:1501-1511.
160. Vij, V., E. Ailes, C. Wolyniak, F. J. Angulo, and K. C. Klontz. 2006. Recalls of spices due to bacterial contamination monitored by the U.S. Food and Drug Administration: The predominance of *Salmonellae*. *Journal of Food Protection*. 69:233-237.
161. Waje, C. K., H.-K. Kim, K.-S. Kim, S. Todoriki, and J.-H. Kwon. 2008. Physicochemical and microbiological qualities of steamed and irradiated ground black pepper (Piper nigrum L.). *Journal of Agricultural and Food Chemistry*. 56:4592-4596.
162. Weiss, E. A. 2002. Spice crops. CABI Publishing, New York, NY.
163. Wesche, A. M., J. B. Gurtler, B. P. Marks, and E. T. Ryser. 2009. Stress, sublethal injury, resuscitation, and virulence of bacterial foodborne pathogens. *Journal of Food Protection*. 72:1121-1138.
164. Williams, P., W. M. Leong, B. H. Ingham, and S. C. Ingham. 2010. Lethality of small-scale commercial dehydrator and smokehouse/oven drying processes against *Escherichia coli* O157:H7-, *Salmonella* spp.-, *Listeria monocytogenes*-, and *Staphylococcus aureus*-inoculated turkey jerky and the ability of a lactic acid bacterium to serve as a pathogen surrogate. *In*.
165. Yan, Z., J. B. Gurtler, and J. L. Kornacki. 2006. A solid agar overlay method for recovery of heat-injured *Listeria monocytogenes*. *Journal of Food Protection*. 69:428-431.
166. Yang, J., G. Bingol, Z. Pan, M. T. Brandl, T. H. McHugh, and H. Wang. 2010. Infrared heating for dry-roasting and pasteurization of almonds. *Journal of Food Engineering*. 101:273-280.
167. Yang, Y., A. Kumar, Q. Zheng, and H.-G. Yuk. 2015. Preacclimation alters *Salmonella* Enteritidis surface properties and its initial attachment to food contact surfaces. *Colloids and Surfaces B: Biointerfaces*. 128:577-585.