Recovery of *Salmonella* from Steam and Ethylene Oxide-Treated Spices Using Supplemented Agar with Overlay

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SCIENTIFIC ABSTRACT

Salmonella enterica has been associated with several outbreaks due to consumption of low water activity foods including spices. Consequently, to improve microbiological quality, spices are commonly treated before ultimately reaching consumers. These processes may result in sub-lethal injury to cells, which can lead to an underestimation of microbial populations when plating on selective media. Sub-lethally injured cells are difficult to enumerate due to reduced ability to grow on selective microbiological media. Poor recovery of sub-lethally-injured cells may obscure process validation results and lead to overestimation of process effect. Therefore, this work was performed to determine the influence of agar overlay and media supplementation methods on the recovery of *Salmonella* from steam and ethylene oxide treated peppercorns and cumin seeds. Traditional agar overlay allowed recovery of significantly more *Salmonella* ($p < 0.05$) from inoculated peppercorns treated with steam (65.5°C, 15 sec.) than selective media (XLT4) or solid agar overlay. Additional supplementation of the TSA base to contain 3,3'-thiodipropionic acid further improved *Salmonella* recovery from steam treated peppercorns ($p < 0.05$). For peppercorns and cumin seeds subjected to ethylene oxide, neither sodium pyruvate + yeast extract, 3,3'-thiodipropionic acid, glycerophosphate, ATP, nor magnesium enhanced recovery compared to overlay alone but both methods recovered significantly higher numbers than XLT-4 alone ($p < 0.05$) for both cumin seeds and peppercorns.

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GENERAL AUDIENCE ABSTRACT

Salmonella enterica is a pathogenic bacterium that has caused illnesses in several dry foods including spices. Consequently, methods to kill the organism on spices are used. Treatment of cumin seeds and peppercorns with dry steam and ethylene oxide was done in these experiments; these processes can sometimes injure cells without killing them. Injured cells cannot be counted using traditional methods that use only selective media which may lead to an underestimation of population numbers. Therefore, overlay methods and supplementation of media was used to help injured cells repair themselves so more accurate population estimates could be obtained. The traditional overlay method was better at helping cells repair than the solid agar overlay. Furthermore, overlay plus supplementation with 3,3'-thiodipropionic acid and sodium pyruvate + yeast extract aided in improving repair of cells beyond what the traditional overlay alone in peppercorns treated with steam. Supplementation of steam treated peppercorns and both spices treated with ethylene oxide was did not improve repair of cells beyond overlay but all supplementation and overlay improved repair significantly beyond selective media.

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ATTRIBUTION

Robert C. Williams (Food Science & Technology Department at Virginia Tech) served as the major advisor of this project. Dr. Williams provided guidance and support throughout the duration of the research.

Monica A. Ponder (Food Science & Technology Department at Virginia Tech) provided funding for this research as well as direction and support with data analysis and editing of Chapters 1, 2, and 3.

Joseph D. Eifert (Food Science & Technology Department at Virginia Tech) served as a committee member and provided support and knowledge throughout the duration of the research.

Jordan J. Newkirk (Food Science & Technology Department at Virginia Tech) developed protocols and methods for experiments performed in Chapters 2 and 3. Jordan provided the spice samples for investigation in Chapter 3. Jordan also coauthored Chapter 3, specifically sections involving inactivation studies.

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CHAPTER 1: INTRODUCTION AND JUSTIFICATION

Due to foodborne illness outbreaks and foodborne pathogen contamination incidents associated with spices, the U.S. Food and Drug Administration has indicated concern over the safety of spices and has questioned current control measures (FDA, 2013). In addition to spices other low water-activity (LWA) foods have also been associated with foodborne illness outbreaks and contamination incidents. *Salmonella*, One of the most important pathogens associated with LWA foods has been implicated in outbreaks involving spices, peanut butter, dry dog food, nuts, and cereal (CDC, 2014). While *Salmonella* does not typically grow in LWA foods, it may persist for months in LWA environments (Hiramatsu, 2005). Another significant trait of *Salmonella* is increased stress tolerance when exposed to low moisture environments (Gruzdev, 2011).

In recognition of the potential for pathogen presence, spice companies have taken measures to control pathogenic microorganisms in their products. Ethylene oxide (EtO) fumigation, irradiation, and steam treatments are the methods most commonly employed to reduce microbial populations in spices (Leistritz, 1997). In order to show efficacy, these methods must be validated on an individual basis. Parameters such as time, temperature, pressure, and concentration (e.g. ethanol, EtO) need to be carefully measured to ensure adequate control of *Salmonella*. An important component of any validation study is accurate recovery of the viable microorganisms that remain after test treatments. Underestimation of remaining viable pathogen populations may lead to overestimation of the effect of the treatment. Steam, irradiation, and EtO processes can cause sub-lethal injury to some cells, which will reduce recovery when enumerated on traditional selective media. Neglecting these cells will lead to an underestimation of *Salmonella* populations because they can resuscitate if returned to favorable conditions. Special measures must be taken to allow injured cells to resuscitate as a part of the enumeration

procedure. This work was performed in order to identify the best method to recover *Salmonella* from steam and ethylene oxide-treated peppercorns and cumin seeds.

Objectives

I. Determine the influence of three plating methods, traditional overlay, thin agar overlay, and solid agar overlay, on recovery of *Salmonella* on peppercorns and cumin seeds subjected to steam treatments

II. Test the efficacy of media supplemented with 3,3'-thiodipropionic acid, pyruvate + yeast extract, glycerophosphate, mannitol, and lactate to recover *Salmonella* on peppercorn and cumin seeds subjected to steam treatments

III. Test the efficacy of media supplemented with 3,3'-thiodipropionic acid, pyruvate + yeast extract, glycerophosphate, guanine, ATP, and magnesium to recover *Salmonella* on peppercorn and cumin seeds subjected to ethylene oxide treatments

Literature Review

Spice History

Spices are generally defined as the dry parts of plants used to add a desired flavor and aroma to foods. They can come from numerous sections of a plant including its roots, leaves, and seeds (Hirasa and Takemasa, 1998). In early times spices were so valuable it could be used as currency, and kings even accepted it as ransom (Weiss, 2002). Our ancestors in Sumer were using spices at least as early as 3000 BC although at that time spices were valued more for their use as medicine, perfumes, and cosmetics as opposed to their flavor (Weiss, 2002). Most spices require a tropical climate for growth, and it wasn't until the Romans discovered spices via trade with Asia in the 1st century that spices became widely used for their culinary value. From that point, spices spread throughout the Roman Empire and beyond, have been important for trade, and have led to the global spice market in place today (Weiss, 2002).

Peppercorns

Pepper originated in India but trade and exploration has disseminated this spice all around the world. The two major types are long pepper (*Piperaceae longum*) and black pepper (*Piperaceae nigrum*) (Weiss, 2002). The best growing conditions for pepper include an environment with high rain fall and relative humidity with temperatures between 75 and 90°F. These are characteristics typical of tropical regions (Pafumi, 1986). The pepper plant is a vine consisting of dark green leaves and fruiting stems. It is a woody climber reaching to greater than 10 meters (Weiss, 2002). The fruiting stem is a spike containing 50-60 fruits which start green, turn red when ripe, before becoming black after drying. The black skin harbors a single seed known as the peppercorn (Weiss, 2002). Once ripe the spikes are harvested and fermented overnight

making fruit removal easier. Threshing follows harvest and can be done manually by hand, trampling, or mechanically. Blanching can be done in boiling water for two minutes to improve color, remove dust and bacteria followed by sun drying. The "berries" are then graded to remove superfluous matter and sorted by size before being packed and shipped (Peter, 2006).

Cumin

Cumin (*Cuminum cyminum*) is native to the Mediterranean region of Egypt and like pepper, it is dispersed throughout the world today (Peter, 2006). A warmer climate is preferred for growth but it can tolerate cooler environments as well as varying soil types. Extensive rain is not needed for cumin plants to thrive (Peter, 2006). Cumin is a smooth thin plant with blade leaves and white or pink flowers. The fruit is brown, composed of moon shaped ribs (Weiss, 2002). Once the fruit becomes ripe it can be manually or mechanically harvested, followed by cleaning and drying. After desiccation seeds are kept in bulk within bags and kept cool throughout shipping and storage (Weiss, 2002).

Background Microbiota and Contamination

The native microbiota of spices is a reflection of the microorganisms indigenous to the environment in which the spice is grown. Consequently, the background microbiota of each spice varies. Sources of contamination may include soil, dust, insects, feces from animals, as well as unclean irrigation and washing waters (Microbial Ecology of Foods, 2005). Contamination can also be a result of post-harvest practices such as unsanitary processing facilities and handling (Lund and Farkas, 2000). In addition, spices are typically grown in a tropical climate where perpetual high temperature and humidity create a highly advantageous environment for potential microbial populations. The number of microorganisms present on

spice at any moment is greatly dependent upon the drying process and storage conditions as these processes play a critical role in the decline, propagation, and survival of microbial populations (Lund and Farkas, 2000).

The microbial populations that survive on spices are predominantly spore-formers and molds due to the ability to tolerate desiccated environments (Microbial Ecology of Foods, 2005). Of the spore formers *Bacillus* species are most commonly identified (Lund and Farkas, 2000) though pathogenic spore formers *Bacillus cereus* and *Clostridium perfringens* are rarely found in numbers higher than 1,000 CFU/g (De boer, 1985). The most common non-spore-forming bacteria found on spices are coliforms. When identified, coliforms are also found in low numbers, usually around 1,000 CFU/g. *Escherichia coli*, however, are seldom isolated (Parfumi, 1986). *Salmonella enterica* and *Staphylococcus aureus* have been uncommonly identified in spices (Lund, 2000; Parfumi, 1986). Molds most often identified in spices include, *Aspergillus*, *Penicillium*, and *Eurotium* genera (Lund, 2000). From a survey of 125 spices, 79 different mold cultures were isolated. Fifty-two of those identified originated from the aforementioned genera. Over 50% of the population counts fell between 3-5 log CFU/g which is concerning due to the potent toxins produced by certain mold species (De boer, 1983).

Peppercorns are among the spices with the highest rate of contamination (Lund and Farkas, 2000). Of 200 pepper samples evaluated, over 90 percent contained an aerobic plate count of 6 logs CFU/g or greater and mold counts between 3-5 logs CFU/g (ICMSF, 2005; Pafumi, 1986). Cumin samples have also been found to harbor moderate bacterial loads. Of 25 cumin samples assessed, over half contained an aerobic plate count of 5-6 logs CFU/g and mold counts between 2-4 logs CFU/g (Microbial Ecology of Foods, 2005; Pafumi, 1986). A survey of the microbial profile of cumin seeds sold in retail shops in the city of Bombay revealed high aerobic plate

counts ranging from 4 to 8 logs per gram. Among the bacteria present, 50-95% were spore formers consisting mostly of *Bacillus* species. No fungi were found in cumin seed samples examined (Bhat et al, 1987). From spices imported to Japan, Hara-Kuda et al found mean aerobic plate counts of 4.29 and 4.05 logs CFU/g in peppercorn and cumin respectively (Hara-kuda et al, 2005). Peppercorns and cumin seeds evaluated from Mexican markets contained aerobic counts above 5 logs CFU/g 90% of the time (Garcia et al, 2000). Banerjee and Sarkar's results from examinations of retail spices were similar. One hundred percent of peppercorn and 40% of cumin samples inspected contained aerobic counts above 6 logs CFU/g, bacterial spore counts between 2 and 5 logs CFU/g, and mold counts between 1 and 4 logs CFU/g for both spices (Banerjee and Sarkar, 2003).

Salmonella and Spices

In 2014, The United States imported $1.2 billion worth of spices from over 100 countries (Supplier Relations, 2014). Spices are known to have a diverse microbiota including a variety bacteria and fungi though, recently, their association with *Salmonella* has increased (Vij et al, 2006). From 1973 to 2010 there were 14 reported outbreaks attributed to contaminated spices. *Salmonella enterica* was identified as the foodborne pathogen in 10 of the outbreaks; accounting for almost 2,000 illnesses and 2 deaths (Van Doren et al., 2013).

Microbiological sampling of imported spices conducted by the FDA between 2007 and 2009 found *Salmonella* in nearly 7 percent of samples tested; twice the rate of all other foods regulated by FDA (FDA, 2013). From 1970 to 2003 there were 21 recalls of spices due to contamination by pathogens monitored by the FDA. All but one implicated *Salmonella* (Vij et al, 2006).

Both whole peppercorn and cumin seeds have been involved in recalls or outbreaks of Salmonellosis. Moreira et al. (2009) found *Salmonella* in 12 of 66 black pepper samples and 1 of 12 cumin samples from Sao Paulo, Brazil, one of the largest producers of spices in the world. *Salmonella* was found in 8% of 138 cumin samples and 4% of 156 black pepper samples collected by the FDA between 2007 and 2009 (Pathogens and Filth in spice, 2013). Red and black pepper contaminated with *Salmonella* caused 272 illnesses from July 2009 to April 2010 (Gieraltowski, 2013). According to FDA in April, 2014 Sprouts Farmers Market and Frontier Natural Products Co-op recalled several of its products manufactured with organic black peppercorns due to potential *Salmonella* contamination; in 2013 Shabros and Natco did the same for products containing cumin. The spice industry is conscious that products imported from countries with inferior food safety standards may be contaminated with *Salmonella* and that it can persist in low water activity foods.

<u>*Salmonellae*</u>

Salmonella is a Gram negative, facultatively anaerobic, typically motile, non-spore-forming rod; it can cause several illnesses, most importantly typhoid fever and gastroenteritis. *Salmonella* is named after D.E. Salmon who along with T. Smith established it as the etiologic agent responsible for hog cholera in 1886 (Bell and Kyriakides, 2002). Since that time *Salmonella* have caused various outbreaks and its persistence in food continues to be a major concern in human health. Common reservoirs include humans and farm animals including poultry, swine, cattle, birds, and reptiles; the bacterium exists in in the intestinal tract and are shed and transmitted through fecal contact (Baird-Parker et al, 1996).

Every year *Salmonella* is estimated to cause one million illnesses in the United States leading to 19,000 hospitalizations and nearly 400 deaths (CDC, 2014). It is the leading cause of bacterial foodborne illness in the U.S. (Montville et al, 2012). Illness occurs when the organism is ingested in sufficient numbers to survive the acidic environment of the stomach. The infectious dose necessary to cause foodborne illness is generally 6 logs or higher, however in cases involving high fat, low moisture foods such as cheese or chocolate 10 to 100 cells have been known to cause illness (Greenwood and Hooper, 1983). These particular food matrices seem to conceal the *Salmonella* from the harsh acidity of the stomach. Once through the stomach, an infection can proceed only after the organism attaches itself to the intestinal epithelial cells. *Salmonella* infiltrate the epithelial cells and begin to multiply. Symptoms occur when the bacteria become established in the host's tissues; once colonization takes place *Salmonella* produce an enterotoxin causing an inflammatory reaction (Bell and Kyriakides, 2002).The inflammatory response is triggered by the incursion of leucocytes at the infection site. The leucocytes release of prostaglandin causes the inflammation. The subsequent production of enterotoxins by *Salmonella* induce host activation of adenyl cyclase triggering diarrheagenic fluid to flow out of inflamed tissues into the lumen of the intestine (Lund and Baird-Parker, 2000). The symptoms of Salmonellosis are those generally characterized by gastroenteritis including nausea, vomiting, abdominal pain, fever, and diarrhea; these typically develop 12-72 hours after consumption of contaminated food and can last 4-7 days (Golden et al, 2005; CDC, 2015). Death caused by *Salmonella* is rare at less than 1% however the rate is higher among young children, the elderly, and those with compromised immune systems (Sharp, 1990). *Salmonella* can persist in a variety of food vehicles. Some of the foods that have been implicated to cause Salmonellosis include poultry, dairy products, fruit juices, toasted oat cereals, peanut

butter, cantaloupe melons, tomatoes, seed sprouts and more (Curtis and Lawley, 2003). Some of the most notable outbreaks include pasteurized milk and ice cream, both estimated to cause illness in approximately 200,000 people in 1985, and 1994 respectively (Golden et al, 2005).

Salmonella Tolerance to Desiccated Environments

Traditionally, naturally dry foods such as grains, nuts, and seeds were thought to be exempt from prevalence of spoilage and pathogenic bacteria. Moreover, many foods are preserved by drying, *e.g.* fruits and meats or by binding water in jams, jellies, and salted products. The preservation of these foods is not directly related to the amount of moisture but the amount of free water in the food. Microorganisms require a certain amount of water to grow; the water must be in an available form for biological or chemical reactions. This can be described by water activity (a_w), which is the vapor pressure of a substance compared to the vapor pressure of pure water at the same temperature. The ability of *Salmonella* to grow is greatly diminished at water activity below 0.94 (Baird-Parker et al, 1996). However, it can survive in foods at much lower water activities for extended periods of time. *Salmonella* populations generally decrease over time but an adequate number persist to cause illness (Bell and Kyriakides, 2002). Keller et al (2013) showed peppercorns initially inoculated with 8 logs CFU/g that were stored at 40% relative humidity for 8 months decreased by 3-4 logs CFU/g then remained constant at 25 and 35°C. Only a 1-3 log reduction from an initial count of approximately 7 logs CFU/g was demonstrated in almond hulls after drying at 15 and 37 ^{o}C (Uesugi and Harris, 2006). Halva, a traditional Asian confection was inoculated with 7 logs CFU/g of *Salmonella* Enteritidis and stored in air-sealed or vacuum packages at 6 and 18-20 ^{o}C. After 6 days populations decreased approximately 3 logs but afterward remained stable for at least 8 months (Kotzekidou, 1997). *Salmonella* Montevideo survived in dry milk powder (a_w 0.43) for 14 weeks after initial inoculation of 8 log

CFU/g. A 3 log reduction occurred during the first 7 weeks with a total of a 6 log reduction following an additional 7 weeks (Juven et al, 1983). In a model system using paper disks *Salmonella* was inoculated at 7 logs CFU per disk. After drying for 24 hours at 35°C 3-4 logs of *Salmonella* survived. The disks were subsequently stored at 4, 25, and 35°C. *Salmonella* died off after 35 to 70 days of storage at 26 and 35°C but maintained numbers between 3-4 logs when stored at 4°C for up to 2 years (Hiramatsu et al, 2005). Recalls and outbreaks involving *Salmonella* can be attributed to the ability of *Salmonella* to endure virtually indefinitely in low water activity foods.

Recently, the frequency of outbreaks and recalls of LWA foods implicating *Salmonella* as the etiologic agent has increased. In 2011, *Salmonella* Enteritidis was linked to an outbreak in Turkish pine nuts. Consequently, 3 separate companies recalled nearly 30,000 pounds of pine nuts in total from the market. The outbreak involved 42 individuals across 5 states; 2 were hospitalized and no deaths were reported (CDC, 2015). A national brand of peanut butter was recalled in 2012 due to infections associated with *Salmonella* Bredeney. All peanut butter and peanut butter products manufactured between May 1, 2012 and September 24, 2012 were removed from shelves. The outbreak spanned 20 states and of the 39 persons infected 10 were hospitalized; no deaths were reported (CDC, 2015). In 2013 *Salmonella* Montevideo or *Salmonella* Mbandaka was the etiologic agent in illnesses caused by consumption of tahini paste. This outbreak involved 16 people and spanned 9 states, 1 person was hospitalized and 1 death was reported (CDC, 2015). Additional outbreaks of low water activity foods are shown in Table 1. Spices and products using them as an ingredient have also fallen under greater scrutiny as of late. Because of their low water activity and minimal processing *Salmonella* in spices is a risk, especially since they are frequently added to ready-to-eat foods, without further cooking.

Table 1. Selected *Salmonella* outbreaks in low water activity foods for years 2006-2015

Food	**Serotype**	**Reported Illnesses**	**Hospitalizations**	**Deaths**	**Year**
Nut Butter	Braenderup	6	1	0	2014
Chia Powder	Newport, Hartford, Oranienburg	31	5	0	2014
Tahini Paste	Montevideo, Mbandaka	16	1	1	2013
Dry Dog Food	Infantis	49	10	0	2012
Turkish Pine Nuts	Enteritidis	43	2	0	2011
Red/Black Pepper	Montevideo	272	52	0	2010
Peanut Butter	Typhimurium	714	171	9	2008-2009
Peanut Butter	Tennessee	425	71	0	2007

Compiled from CDC's Reports of Selected *Salmonella* Outbreak Investigations (CDC, 2015)

Stress resistance in Low Water Activity Foods

As described previously, low water activity foods were considered very low risk for supporting the growth of pathogenic bacteria. Furthermore, reducing the free water in foods has been used for centuries to control their growth. Conversely, *Salmonella* has proven on numerous occasions that while it may not proliferate in low water activity foods it can persist at levels sufficient to cause illness; consequently, measures have been employed to reduce microbial populations in

these foods. Irradiation, chemical fumigation, and thermal processes are all used as inactivation methods specifically for spices. In some cases hurdle technology is effective in reducing target organism populations. Inauspiciously, *Salmonella* develops resistance to multiple stresses when subjected to a desiccated environment. Desiccated *Salmonella* Typhimurium exhibited significantly increased resistance compared to non-desiccated cells when exposed to the following stresses: ethanol (10 to 30%, 5 min), sodium hypochlorite (10 to 100 ppm, 10 min), didecyl dimethyl ammonium chloride (0.05 to 0.25%, 5 min), hydrogen peroxide (0.5 to 2.0%, 30 min), NaCl (0.1 to 1 M, 2 h), bile salts (1 to 10%, 2 h), dry heat (60, 80, and 100°C, 1 h), and UV irradiation (125 uW/cm^2, 25 min) (Gruzdev et al, 2010). A 3, 8, and 8 log reduction was demonstrated when non-desiccated (10^8 CFU initial population) cells were subjected to 60°C and 80°C and 100°C respectively. Conversely, dehydrated cells had no significant change from their initial population numbers at 60°C and reduced by only 1.5 and 3.1 logs when exposed to 80 and 100°C respectively. In addition, results for inactivation at 100°C revealed that non-desiccated cells were completely inactivated within 10 minutes while 60 minutes of exposure only resulted in the aforementioned 3 log reduction of desiccated cells. Desiccated *S.* Enteritidis, *S.* Newport, and *S.* Infantis displayed comparable resistance to *S.* Typhimurium when subjected to similar stresses (Gruzdev et al, 2010).

Salmonella's resistance to heat under dried conditions has been known for some time now. In the late 1960's early 1970's McDonough and Hargrove (1968) and Dega and others (1972) carried out experiments with *Salmonella* in dried milk powder. McDonough and Hargrove's results show *Salmonella* could not be inactivated (1 log reduction) in 4 and 7% moisture milk powder after 2 hours of treatment at 85°C, on the other hand *Salmonella* was undetectable (4 log reduction, surface plating and enrichment negative) after just 30 minutes of treatment at the same

temperature when the moisture level was 25%. The mean D-value (minutes at 57°C) of *Salmonella* Typhimurium increased from 1.4 to 9.9 to 26.6 with increased milk solid concentrations of 10, 42, and 51% respectively (Dega et al., 1972). The mean $D_{57C\ (min)}$ value of *Salmonella* Montevideo and *Salmonella* Tennessee in sucrose solution (.99 water activity) increased from 1.1 and 0.8 to 75.0 and 35.9 respectively when water activity was decreased to 0.87 (Goepfert, Iskander, and Amundson, 1970). Archer et al (1998) tested the heat resistance of *Salmonella* Weltevreden inoculated into flour. The samples were heated in a hot air oven at 72-74°C. The $D_{74C\ (min)}$ value at a water activity of 0.55 was 50 compared to a D-value of approximately 250 at a water activity of 0.25. Remarkably, the $D_{70C\ (min)}$ value was found to be 720 in milk chocolate (Geopfert and Biggie, 1968). The large sum of data suggests a strong correlation between reduced water activities leading to enhanced thermal resistance in *Salmonella*. Hence, these findings need to be taken into consideration when determining processing parameters for inactivation of pathogens in the industry.

Steam Treatments of Spices and Low Water Activity Foods

Understanding that spices may become contaminated with pathogenic bacteria in route to consumers, companies have begun taking measures to control these pathogens, particularly *Salmonella*. The concern is selecting a method that inactivates pathogens while protecting delicate flavor compounds. Ethylene oxide (EtO) fumigation, irradiation, and steam treatments are the major methods for bacterial reduction in spices (Leistritz, 1997). EtO has been in use since the 1960s and is attractive to companies because it does not required labeling by the FDA and is advertised to reduce microbial populations by 7 logs (Leistritz, 1997; Sterigenics, 2007).

However, it is not allowed in some countries outside the U.S., including those in Europe due to its link to increased cancer risks (Leistritz, 1997). Irradiation via gamma, and electron beam was approved by the FDA in 1988 and is regularly used in the U.S. and abroad because of its superior penetration. It does not introduce moisture, has low impact on flavor compounds, and does not demand special packaging or aeration because it doesn't leave residues (Sterigenics, 2007). However, this technique does require labeling by FDA. Consumer fears of long term effects of irradiation along with banning in certain foreign countries are sources of uneasiness for companies, leading some to use other methods (Leistritz, 1997).

Safety concerns with other processes for spice have led to increased interest in steam treatments. Steam is an accepted treatment for microbial reductions in food around the world. There are two types of methods for steam, wet and dry; because wet processes introduce undesirable moisture dry methods (saturated steam) are preferred for low water activity food (Leistritz, 1997). The heat capacity of steam is much higher than that of water at the same temperature. The efficacy of steam is due to the mass amount of heat transferred to the surface of food when the steam condenses, leading to rapid increases in external temperatures (James et al, 2000). In addition, gas molecules are much smaller than bacteria; therefore, steam can penetrate any cavity or crevice large enough to hide a bacterium (Morgan et al, 1996). Steam pasteurization has shown to be an effective method to reduce pathogenic bacteria in low water activity foods. Chang and others (2010) studied the effect of steam on raw Nonpareil almonds. The almonds were inoculated with *Salmonella* Enteritidis to 7-8 logs CFU/g and processed in a pilot-sized vertical pasteurization machine for 5, 15, 24, 35, 45, 55, and 65 seconds at 95°C (143kPa). Reductions increased with treatment time though only 25 seconds were necessary to achieve a 5 log reduction. A 45 second treatment reduced populations below the detection limit of 0.3 logs

CFU/g (Chang et al, 2010). Another study on almonds by Lee and friends conclude that 65 seconds of steam treatment 93°C provided a 5.76 and 4.10 log reduction of *Salmonella* Enteritidis on Nonpareil and Mission almonds respectively. The $D_{93C\ (sec)}$ were calculated to 12.22 and 16.13 seconds. The expected treatment time to achieve a 5- log reduction based on those findings would be 61.1 and 80.65 seconds for Nonpareil and Mission almonds respectively (Lee et al, 2005). Experiments performed by Ban and colleagues (2014) tested reduction in *Salmonella* Typhimurium biofilms on stainless steel coupons after subjection to saturated and superheated steam. Initial counts were 6.49 logs CFU per coupon. Exposure to saturated steam for 5, 10, 20, and 30 seconds led to survival of 4.73, 4.20, 3.44, and 2.48 logs CFU per coupon respectively. Exposure to superheated steam (150°C) for 5, 10, 20, and 30 seconds led to survival of 4.21, 3.31, and less than detection limit of 1.48 logs CFU per coupon respectively (Ban et al, 2014). Results from Almela and others (2002) indicate that high temperature short time steam treatments are effective to reduce microbial populations on paprika. Paprika subjected to a 6 second process of superheated steam (160°C) reduced total aerobic counts, Enterobacteriacea, coliforms, and yeast and molds from 5, 4, 3, and 4 logs CFU/g to less than the detection limit of 2 logs CFU/g (Almela et al, 2001).

Ethylene Oxide

Steam is an effective method to reduce microbial populations on spices but concerns about loss of volatile organic compounds associated with flavor and changes to color have led some companies to utilize less abrasive methods. An alternative to steam is fumigation with ethylene oxide. Ethylene oxide is the simplest cyclic ether. The bonds within the ring are under

significant stress making it easy to break and extremely reactive. EtO is a highly diffusive alkylating agent that does not necessitate cellular metabolic activity (Phillips and Miller, 1973). This high energy molecule alkylates DNA, RNA, and protein, impeding normal metabolic functions which intern inhibits reproduction, rendering cells inactive (Bohnet and Matthias, 2003). This is the mechanism by which EtO reduces microbial populations; the same is why it is dangerous for humans. Due to the mutagenic nature of ethylene monchlorydrin, EtO is regulated by the EPA who have specified an acceptable residue of no greater than 50 ppm in foods for human consumption. Additional requirements such as preheating the air, reducing initial concentrations of EtO in air and contact times are specified to assure that residue levels do not exceed allowable levels. Though there are concerns with safety, between 40% and 85% of spices in the U.S. are treated by EtO processes each year (ASTA, 2011). Studies conducted by Vajdi and Pereira (1973) showed EtO fumigated black pepper and celery seeds had aerobic microbial reductions of 3.43 and 4.67 logs CFU/gram respectively.

Recovery Methods

There are several recommended methods for recovery of *Salmonella* although most are only concerned with detection as opposed to quantitation of organisms. For example, the FDA Bacteriological Analytical Manual (BAM) recommends pre-enrichment in nutrient and then selective broth, followed by plating on selective media, and confirmation using selective media, serological analysis, or biochemical tests (Andrews and Hammock, 2014). The USDA FSIS Laboratory Guidebook provides similar instructions but also relies on BAX PCR Assay for confirmation as a substitutable for verification using selective media, serological analysis, or

biochemical tests (USDA, 2014). Similarly, the ISO protocol (ISO 6579:2005) endorses a two-step enrichment followed by plating on selective agar with a final confirmation step (International Standards Organization, 2005). When determining the efficacy of inactivation treatments the emphasis is not simply on the presence/absence of *Salmonella* but the difference in population before and after treatment. This requires more measured quantitative approaches.

Enumerating *Salmonella* populations in a food product generally consists of taking a representative sample from the food, plating on selective media, and counting colonies. However, when a processing step is added complications arise as the population will include dead cells, uninjured, normal cells and, cells that survive but are injured. Heat is known to cause damage to the cell wall and membranes as well as DNA, RNA, and proteins; however, an extensive detailed examination of physiological status of the cell is lacking (Lund et al, 2000). Membrane damage to Gram negative bacteria results in loss of lipopolysaccharides and other essential outer membrane members. The deficiency leads to creation of pores in the membrane and loss of permeability control (Hurst, 1977). Degraded protein means nonfunctional enzymes and loss of essential transport mechanisms while impaired DNA and RNA leads to increased mutations and loss of the ability for gene expression. Cells that incur damage are inhibited by selective media. These cells are referred to as viable but non-culturable or sub-lethally injured and can lead to an underestimation of *Salmonella* populations since the injured cells will repair themselves and propagate in food when conditions are favorable. A step to allow injured cells to restore themselves must be added; enrichments, overlay methods, and supplemented media are frequently used.

Enrichment of food is done to increase numbers of pertinent organisms so occurrence of false negative results is reduced (Robinson et al, 2000). Lactose, TSB, and nutrient broths are

commonly used for enrichments although they are customarily used for detection as opposed to quantitative analysis. Quantification can be done if time in enrichment media is tapered in relation to the lag phase of organisms. However, some question if healthy cells are multiplying during this time. Warseck and others (1973) found that freeze injured *Escherichia coli* plated directly on violet red bile agar (VRBA) or deoxycholate lactose agar could not be recovered; on the other hand, enrichment in TSB for 1 hour allowed cells to repair leading to recovery when plating.

Enrichment based methods are effective in detection of *Salmonella* in foods but can take days to implement and is not widely accepted in the quantification of organisms. When sampling food for pathogen isolation, selective media is used to inhibit growth of background microbiota but can also retard growth of injured target organisms. To shorten the time for enrichment steps, while still allowing selectivity of pertinent pathogens, overlay methods were developed. Overlay methods generally consist of a solidified, nonselective media such as TSA which is inoculated with the sample; the cells are allowed to repair themselves for a given time depending on the duration of the lag phase, after which it is overlaid with selective media such as XLD. Speck et al (1975) were among the first to test this method. He concluded that *Escherichia coli* injured by freezing could repair themselves when plated on TSA when given 2 hours prior to overlay (10-12ml) with violet red bile agar. Strantz and Zottola (1989) found that unstressed *Salmonella* plated on Brilliant Green Agar (BGA), *Salmonella*-Shigella agar (SS), Xylose Lysine Desoxycholate Agar (XLD), and Hektoen Enteric Agar (HE) had 1-2 logs less *Salmonella* than TSA. When the culture was spread plated onto TSA, allowed to stand at room temperature for 4 hours, and overlaid with selective media before 24 hour incubation at 37°C, recovery levels were not significantly different than TSA. Wu and Fung (2001) and Kang and Fung (2000) described a

one-step thin agar lay method (TAL) that eliminates the waiting period during repair. In their protocol 25ml of selective media is poured first and allowed to solidify. Following, 14 ml of the nutrient media is poured, which is also allowed to solidify. Finally, the inoculum is plated and incubated. During the incubation period the selective agents slowly diffuse up into the nutrient medium giving time for repair while also providing desired selectivity. Recovery of heat injured (55°C for 15 minutes) *Salmonella* Typhimurium was not significantly different when using TSA, the traditional overlay, or the thin agar layer method while all methods recovered significantly more *Salmonella* than xylose lysine decarboxylase alone (Kang and Fung, 2000). Yan, Gurtler, and Kornacki (2006) further modified the overlay method and defined it as a solid agar overlay method (SOL). They established that the nutrient overlay could be pre-solidified and added directly onto the selective layer where it would then function like the TAL method. Recovery of heat injured (58°C for 6 minutes) *Listeria monocytogenes* was not significantly different when plated on TSA or SOL but recovered significantly higher numbers than enumeration on modified Oxford agar alone (Yan, Gurtler, and Kornacki, 2006).

Cells that are damaged due to stresses commonly result in bacterial populations that necessitate more exacting growth factors (D'aoust, 1977). For this reason adding supplements to media to improve recovery of injured bacteria is common. D'aoust (1977) determined the number of injured cells to be the difference between counts obtained from selective and nutrient media; of 32 supplements added to Levine eosin-methylene bile-salts agar to recover heat injured (48°C for 30 minutes) *Salmonella* Typhimurium, only lactate, mannitol, and glycerophosphate facilitated at least 90% recovery of injured cells. Gurtler and Kornacki (2009) revealed improved recovery of heat injured (53°C for 4 minutes) *Salmonella* in egg albumen when TSA was supplemented with ferrous sulfate (5.82 log CFU/g), 3, 3-thiodipropionic acid (5.67 log CFU/g), and sodium

pyruvate plus yeast extract (5.57 log CFu/g) compared to TSA (5.27 log CFU/g) alone ($p < 0.05$). Jasson et al (2011) found that the rapid detection methods real-time PCR and Vidas Easy SLM were effective when chocolate samples with low levels of healthy and sub-lethally injured cells were enriched in buffered peptone water with 10% milk powder than with buffered peptone water alone. Buffered peptone water supplemented with ferrioxamine E or the enzyme-controlled substrate delivery system EnBase-Flo was shown to improve detection of heat injured (55°C for 15 minutes) *Salmonella* Typhimurium (Taskila et al, 2011).

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CHAPTER 2: RECOVERY OF *SALMONELLA* FROM WHOLE BLACK PEPPERCORNS AND CUMIN SEEDS SUBJECTED TO A STEAM PROCESS

Abstract

Salmonella enterica has been implicated in several outbreaks of foodborne illness associated with the consumption of low water activity (LWA) foods, including spices. Due to high initial load of microbiota and concerns related to human pathogen presence, steam treatments are frequently used to reduce microbial populations. These processes may cause sub-lethal injury to cells which can lead to an underestimation of microbial populations when plating on selective media. Given favorable conditions, these cells repair and can cause harm when ingested. Reduced recovery of injured cells must be taken into account when validating processes for inactivating pathogens on spices. The efficacy of the traditional and solid agar overlay methods along with supplementation of media was examined to improve recovery of *Salmonella* on peppercorn and cumin seeds treated with steam. The traditional overlay (5.50 logs CFU/g) recovered significantly more *Salmonella* ($p < 0.05$) than the solid agar overlay (4.82 logs CFU/g) and XLT-4 alone (4.46 logs CFU/g) from steam treated peppercorns. Supplements (sodium pyruvate + yeast extract, 3,3'-thiodipropionic acid, glycerophosphate, lactate, mannitol, and a combination of all) were incorporated into the traditional overlay to recuperate *Salmonella* on peppercorns and cumin seeds treated with steam. Improved recovery of *Salmonella* from peppercorns was observed when using 3,3'-thiodipropionic acid (6.60 logs CFU/g) and a combination of all the supplements (6.62 logs CFU/g) compared to overlay alone (6.12 logs CFU/g, $p < 0.05$). The best recovery of *Salmonella* from cumin seeds was obtained when using overlay alone ($p < 0.05$). The poorest recovery of *Salmonella* from cumin seeds and peppercorns occurred when using XLT4 alone ($p < 0.05$). When performing validation studies it is important to consider that medium and plating technique influence recovery of injured cells.

Introduction

From 1973 to 2010 there were 14 reported outbreaks attributed to contaminated spices from 9 countries including the U.S. *Salmonella enterica* was identified as the etiologic agent in 10 of those outbreaks, accounting for almost 2,000 illnesses and 2 deaths (FDA, 2013). As a result of these outbreaks, the spice industry has focused attention on interventions to control pathogens. Ethylene oxide (EtO) fumigation, irradiation, and steam treatments are the methods most commonly employed to reduce microbial populations on spices (Leistritz, 1997). When bacteria are exposed to these treatments three major outcomes result. A portion of the bacterial population will survive the process and will maintain normal cellular functions while others will be inactivated. However, some bacteria will be damaged but survive the treatment. These sub-lethally injured cells are viable but have impaired cellular activity (Ray, 1979). To determine if an inactivation process is successful a specific log reduction must be achieved; special measures should be taken to ensure sub-lethally-injured cells are not overlooked.

When validating a treatment process the ability to recover sub-lethally injured target microorganisms is critical to accurately assess the efficacy of the process. Standard methods for detecting *Salmonella* include pre-enrichment, isolation, and confirmation (Andrews and Hammock, 2014). In addition to the time-consuming and costly nature of these methods, enumeration is not possible. Conventional methods for enumeration use harsh selective media that can inhibit the growth of injured cells (Clavero and Beuchat, 1995). Neglecting these cells can lead to an underestimation of *Salmonella* populations since they can repair themselves if returned to favorable conditions, for instance, if added to a ready to eat product or to a meal after the cooking process. This is a common occurrence for spices.

Hartman et al. (1975) and Speck et al. (1975) developed an overlay method that permits a time for repair of injured organisms on non-selective media while providing selectivity for isolation of a target pathogen through agar overlay with selective media. Other researchers have developed improved enumeration methods. A thin agar layer (TAL) method was developed that alleviates the need for the waiting period while still providing time for repair (Kang and Fung, 2000). Yan et al. (2006) developed the solid agar overlay that uses a pre-solidified nonselective medium overlaid onto a selective medium, which does not require temperature control of liquid media like the TAL method.

In validation studies recovery of the organism including injured cells is vital. In the present study *Salmonella* inoculated cumin seeds and peppercorns were treated with steam. Recovery using the traditional overlay method was compared to the solid agar overlay method. Furthermore, to determine if resuscitation of injured *Salmonella* could be further improved, overlay was used with the addition of supplements.

Materials and Methods

Cultures and culture maintenance

A three strain cocktail of *Salmonella enterica* (Ball ARL-SE-085 from black pepper in 2011, Tennessee K4643 from peanut butter in 2010, and Johannesburg ARL-SE-013 from dried ginger in 2010) was used to inoculate whole black peppercorns and cumin seeds. Frozen stock cultures stored at -80°C were streaked separately onto Xylose Lysine Tergitol 4 (XLT4, Becton Dickinson, Franklin Lakes, NJ, U.S.) and incubated at 37°C for 24 hours. An isolated colony

from each plate was transferred into 10ml tryptic soy broth (TSB) (Becton Dickinson, Franklin Lakes, NJ, U.S.) and incubated at 37°C with shaking (180 rpm) for 24 hours.

Preparation of inoculum and inoculation of spices

From each TSB tube, 1ml was spread plated onto large (150mm x 20mm) tryptic soy agar (TSA, Becton Dickinson, Franklin Lakes, NJ, U.S.) plates and incubated at 37°C for 24 hours (2 plates of each strain was sufficient for 100g of spice). Subsequently, 5ml of buffered peptone water (0.1 % peptone, Sigma-Aldrich, St. Louis, MO, U.S.; 0.1 % Tween 80, Fisher Scientific, Kansas City, MO, U.S.) was pipetted onto the TSA plates. Cells were suspended using sterile cotton swabs, and one quadrant was scraped at a time. Suspended cells were then drawn up and pipetted into separate 50ml conical screw cap tubes for each strain. Next, 4 ml of BPW was pipetted onto the plated to remove any remaining cells. The 3 tubes were combined in equal parts to create a *Salmonella* cocktail. Then 20ml of the cocktail was transferred to Whirl-Pak bags containing 50g of each spice. The bags were hand shaken for 1 minute to evenly distribute the inoculum. Afterwards, the spices were spread onto individual baking sheets covered with aluminum foil and allowed to dry to 0.3-0.5 a_w for 24 hours in a biological safety cabinet (BSC). If target water activity was not achieved in the BSC, spices were dried in a desiccator for an additional 24 hours. This preparation achieved initial population on spices of 8.8-9.4 logs CFU/g.

Steam treatment

Once spices reached desired a_w, 50g of each spice was put into muslin drawstring sachets (4x6 in). Thermocouples were placed inside the sachets. The sachets were placed on a metal mesh tray

inside a steam apparatus and treated with steam (150°F, -2 to -5 psi) for 15 seconds using the protocol described by Newkirk et al, 2016.

Media preparation

The traditional overlay was prepared using methods outlined by (Kang and Fung, 2000). Briefly, Tryptic Soy Agar (TSA, 25ml) was poured and allowed to solidify. After inoculum was spread plated, plates were incubated for 3 hours (37°C) before overlay with 7ml of selective medium. Afterwards plates were incubated for 21 hours at 37°C. The solid agar overlay was adopted from Yan et al. (2006). Selective medium (25ml) was poured and allowed to solidify. Nutrient medium was poured and allowed to solidify in a separate plate. The nutrient medium was aseptically transferred directly onto the selective medium; inoculum was spread plated on the nutrient medium and the plates were incubated for 24 hours at 37°C. When using supplements, TSA was supplemented with sodium pyruvate (9.09 mmol) + yeast extract (0.6%) [NAYE], 3,3'-thiodipropionic acid (5.6 mmol)[TDP], glycerophosphate (10 mmol)[GP], lactate (10 mmol) [LAC], or mannitol (100 mmol) [MANN].

Enumeration of *Salmonella* from treated spices

Following treatment, 10g of spice was put into a stomacher bag containing 90ml of BPW. The spice was stomached for 1 minute before being transferred to sterile vacuum flasks with filter paper (20-25 micron particle retention). Serial dilutions using BPW were made as appropriate from the filtrate. Because the traditional overlay was the most efficient to recover *Salmonella* on steam treated peppercorns, this method was used for the remaining experiments investigating supplementation. Dilutions were spread plated on TSA for aerobic plate counts and XLT4 to isolate *Salmonella*. The traditional overlay was used independently and in combination with

supplemented TSA to recover *Salmonella* cells injured by the steam treatment. Plates were incubated for 24 hours at 37°C. Data was recorded following incubation.

Statistical analysis

The experiments were repeated 4 times for peppercorns and 3 times for cumin seeds (each point was averaged from a sample plated in duplicate). Bacterial counts were converted to $\log_{10}$ CFU/g. Analysis of variance (ANOVA) was performed on log CFU/g using JMP Pro 11 statistical software (SAS Institute, Cary, NC). Differences ($p < 0.05$) among treatments were examined for level of significance by Tukey's HSD test.

Results

Recovery of *Salmonella* using overlays and overlay combined with supplements

Salmonella inoculated peppercorns treated with steam were enumerated using XLT4, traditional agar overlay, and solid agar overlay. Recovery of *Salmonella* was increased by 0.7 log CFU/g ($p < 0.05$) when using the traditional overlay compared to the solid agar overlay and XLT4 alone (Figure 1). Significantly larger numbers of *Salmonella* were recovered from peppercorns using traditional overlay in combination with the supplements ALL (6.62 CFU/g) and TDP (6.59 CFU/g) than any other supplement (except NAYE (6.40 CFU/g)), non-supplemented overlay (6.12 CFU/g), and XLT4 alone (5.14 CFU/g) ($p < 0.05$). No significant difference ($P > 0.05$) was found in recovery of *Salmonella* from steam treated cumin seeds between supplements and overlay. Overlay recovered 5.02 CFU/g while the most proficient supplement, NAYE, recovered 5.03 CFU/g. However, both the overlay and supplementation methodologies resulted in

increased recovery ($p < 0.05$) compared to XLT4 alone (3.01 CFU/g) on cumin seeds. Complete results for peppercorns and cumin are shown in Figures 2. and 3. respectively.

Discussion

Steam is an effective approach to inactivate *Salmonella* on a variety of surfaces (Sterigenics, 2015). Steam is a very proficient method of heat transfer (James et al, 2000) which causes damage to cell membranes, proteins and enzymes, and DNA and RNA of bacterial cells (Wu, 2008). Heat treatments have been shown to be effective in reducing pathogens in LWA foods. *Salmonella* Enteritidis populations on almonds were reduced by more than 5 logs when treated with steam (Lee et al., 2006). Paprika subjected to a 6 second process of superheated steam (160°C) reduced total aerobic counts, Enterobacteriace, coliforms, and yeast and molds from 5, 4, 3, and 4 logs CFU/g respectively to less than the detection limit of 2 logs CFU/g (Almela et al, 2001). Steam processes used in this research achieved at least a 3 log reduction for both peppercorns and cumin seeds.

To determine which overlay method to proceed with regarding supplementation the resuscitation capability of the traditional overlay and solid agar overlay were compared. Only steam treated peppercorns were used for these experiments. Recovery between the overlays were expected to be very similar according to results demonstrated by previous studies (Kang and Fung, 2000; Yan et al., 2006). However, the traditional overlay recovered an additional 0.7 logs CFU/g more than the solid agar overlay. This could be due to the 3 hour revival period on nutrient media that the traditional overlay provides. It is difficult to measure how quickly the selective agents in

XLT4 diffuse into the nutrient layer when employing the solid overlay; perhaps this difference can account for the additional recovery of the traditional overlay.

Supplements were selected based on previous studies that used augmented media to repair heat injured *Salmonella* (Gurtler and Kornacki, 2009; D'aoust, 1978). TDP proved to be superior to all other supplements in resuscitation of heat injured *Salmonella* on peppercorns. For unidentified reasons the same was not true for *Salmonella* on cumin exposed to the same treatment; in this case repair using TDP was the least effective of the supplements used, therefore recovery was lower. NAYE was somewhat effective for *Salmonella* recovery from peppercorns and cumin seeds. McDonald et al.(1982) and Rayman et al.(1978) successfully recovered heat injured bacteria using TDP and NAYE. It is suggested that the cell does not necessarily utilize the compounds directly for repair but that the compounds protect cells via reduction of oxidants in the environment that are especially harmful to injured cells. The results for the remaining supplements were erratic though recovery of *Salmonella* was better than when XLT4 was used alone. Lactate, mannitol, and, glycerophosphate were shown to be proficient to repair heat (water immersion, 30 min 48 ^{o}C) injured *Salmonella* Typhimurium (D'aoust, 1977). A similar effect was expected for these experiments. Differences in anticipated recovery could be due to the medium in which the *Salmonella* was inoculated; the difference in heat transfer mechanism could also be a source for unanticipated outcomes. A combination of all the supplements was examined though this preparation didn't show any marked improvement over solitary supplementation indicating there is no collaborative effect gained from using multiple supplements simultaneously.

Conclusions

The use of overlay in combination with supplementation is an effective method to repair injured *Salmonella* on peppercorns treated with steam. For cumin subjected to steam, the overlay alone was as effective as or better than using supplements. For cumin and peppercorns, supplementation and overlay alone produced better recovery than XLT4. Further studies could explore the specific damage cells experience when subjected to these treatments. Subsequently supplements particular to those damages can be utilized to optimize recovery. This study shows that recovery may be improved beyond overlay methods with supplementation and this approach should be considered when validating any inactivation treatment.

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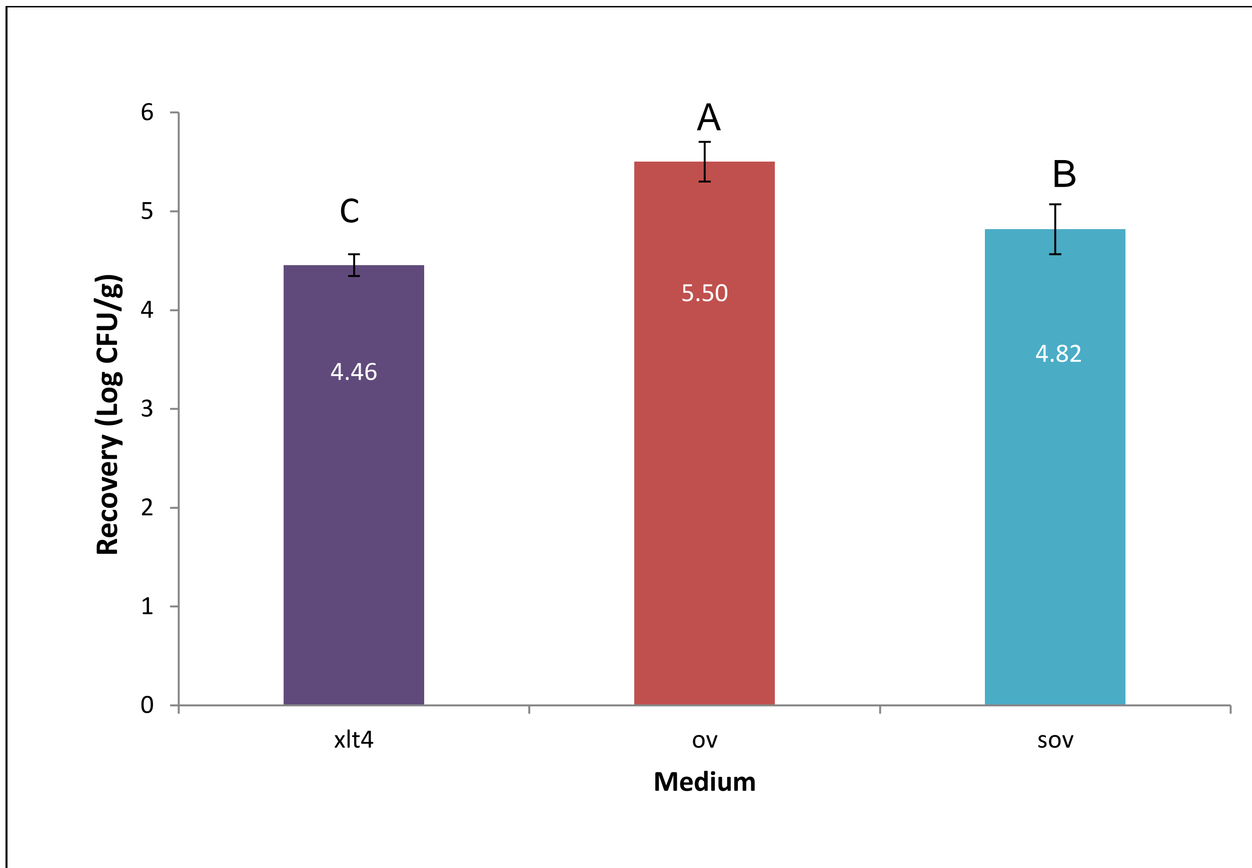

Figure 1. Comparison of overlay methods for recovery of *Salmonella* from inoculated peppercorns subjected to steam treatment. n=4, error bars= standard deviation, letters represent significance level. Figure abbreviations: Xylose Lysine Tergitol-4 (XLT4), Traditional Overlay (OV), Solid Agar Overlay (SOV).

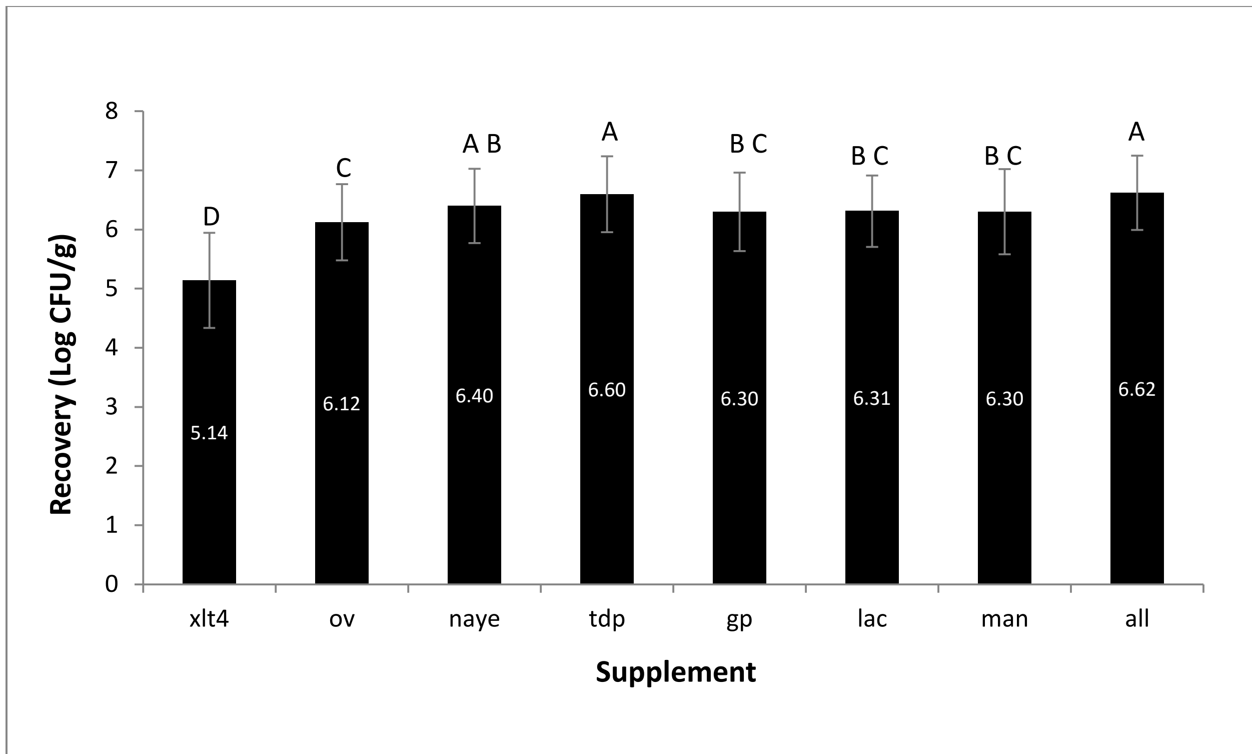

Figure 2. Comparison of supplements for recovery of *Salmonella* from peppercorns subjected to steam treatment. n=4, error bars= standard deviation, letters represent significance level. Figure abbreviations: Xylose Lysine Tergitol-4 (XLT4), Traditional Overlay (OV), Sodium Pyruvate + Yeast Extract (NAYE), 3,3'-Thiodipropionic acid (TDP), Glycerophosphate (GP), Lactate (LAC), Mannitol (MANN) Combination of all supplements (All).

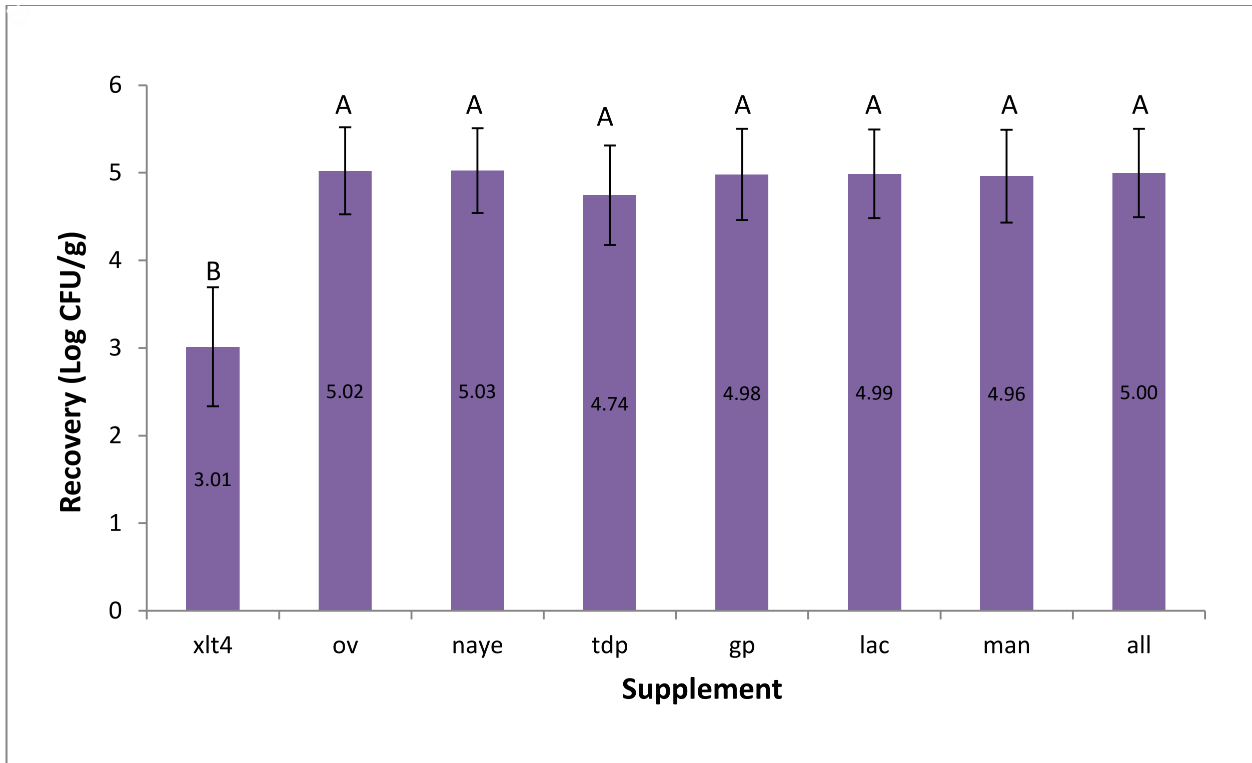

Figure 3. Comparison of supplements for recovery of *Salmonella* from cumin seeds subjected to steam treatment. n=3, error bars= standard deviation, letters represent significance level. Figure abbreviations: Xylose Lysine Tergitol-4 (XLT4), Traditional Overlay (OV), Sodium Pyruvate + Yeast Extract (NAYE), 3,3'-Thiodipropionic acid (TDP), Glycerophosphate (GP), Lactate (LAC), Mannitol (MANN) Combination of all supplements (All).

CHAPTER 3: THE INACTIVATION OF *SALMONELLA ENTERICA* AND SURROGATE, *ENTEROCOCCUS FAECIUM,* USING A COMMERCIAL ETHYLENE OXIDE FUMIGATION PROCESS AND IMPROVED RECOVERY METHODS

Note: Formatted for submission to the Journal of Food Protection

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 agar 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, 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 *Salmonella* 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 known for their flavor enhancing characteristics and antimicrobial properties. Typically, the low water activity of spices restricts the growth of bacteria and fungi that are frequently associated with the spice (35). Black peppercorns and cumin seeds 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 (35). However, the microbiological quality can be further compromised by cross contamination during production, potentially introducing human pathogens (13).

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) (39). Studies using ethylene oxide have proven that it can achieve a 90% reduction of aerobic bacterial populations on spices (43).

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 (7, 14, 27, 31, 32, 36, 41). 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 (28). 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 (12) 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 (4, 9, 15, 16, 18, 37, 45). 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 (33).

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-µL 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 (40). 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 spices. Each polywoven bag (n=3 per spice) contained sachets of spices 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 ± 3.53°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 ± 25.7 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*) (18) 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%) [NAYE], 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 was 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 log CFU/g) with GP (5.73 log 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 log CFU/g). Overlay and all the supplements were superior to XLT4 alone (4.96 log CFU/g)

Discussion

The FDA considers a 5-log reduction appropriate for risk management purposes for some foods such as almonds (10). 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. It is proposed 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.*

As a gas ethylene oxide must be able to diffuse into pores and/or through packaging materials to be effective (34,) 25). 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 (20). 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 (25).

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 (38). 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 (11). The effectiveness of ethylene oxide sterilization is greatly influenced by the ability for the process to move the gas into the package and contact the product (25). In these experiments a polypropylene woven bag was selected 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 reflect different EtO exposure levels within the bags. The presence of the spice itself may also 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 (30). *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 (5, 8, 17). 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 less 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* (22, 23, 24). 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 though 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 (1, 2).

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 a 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 (6). TDP and sodium pyruvate are known to be antioxidants, scavenging reactive oxygen species that may further damage injured cells (26). These supplements were shown to provide greater recovery of heat injured *Salmonella* in egg albumen (15). 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 a hot water bath (9). Guanine, magnesium, and ATP were selected in order to aid in the repair or creation of DNA and RNA. Magnesium (Mg^{2+}) is important in the stability and function of ribosomal subunits (21, 44). 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 (6).

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 (9, 15, 26, 29, 42). Several have noted that overlay techniques can aid the repair of injured cells (16, 19, 37, 45). 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 6.62 ± 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.

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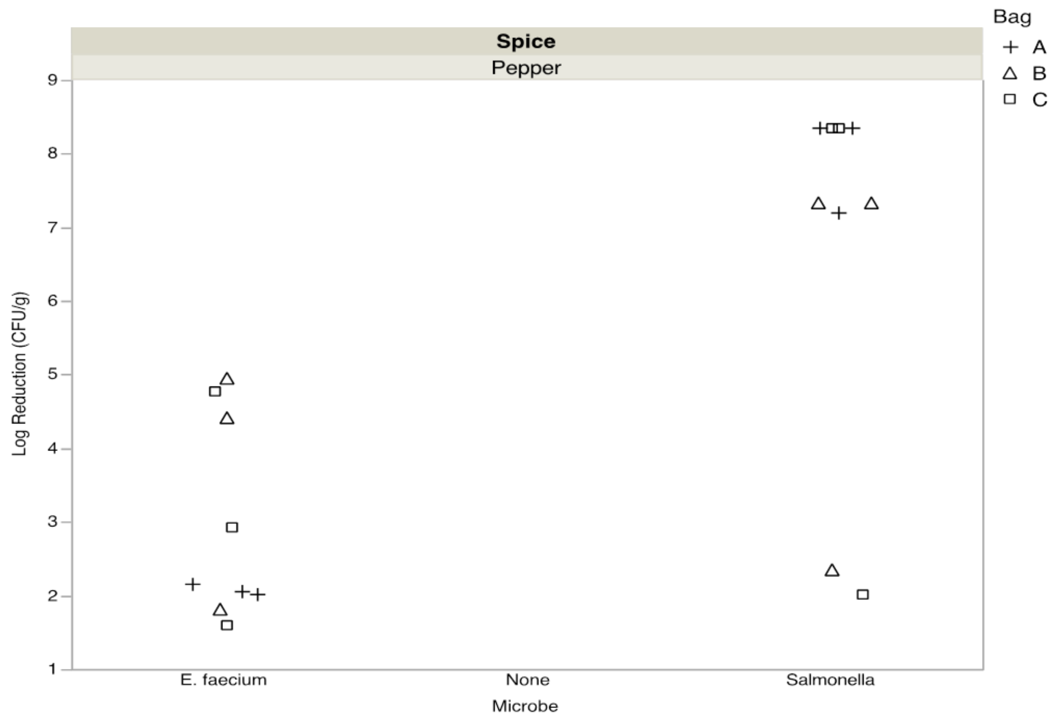

Figure 1. Log reduction of *Salmonella enterica* and *Enterococcus faecium* on whole black peppercorns after treatment with ethylene oxide.

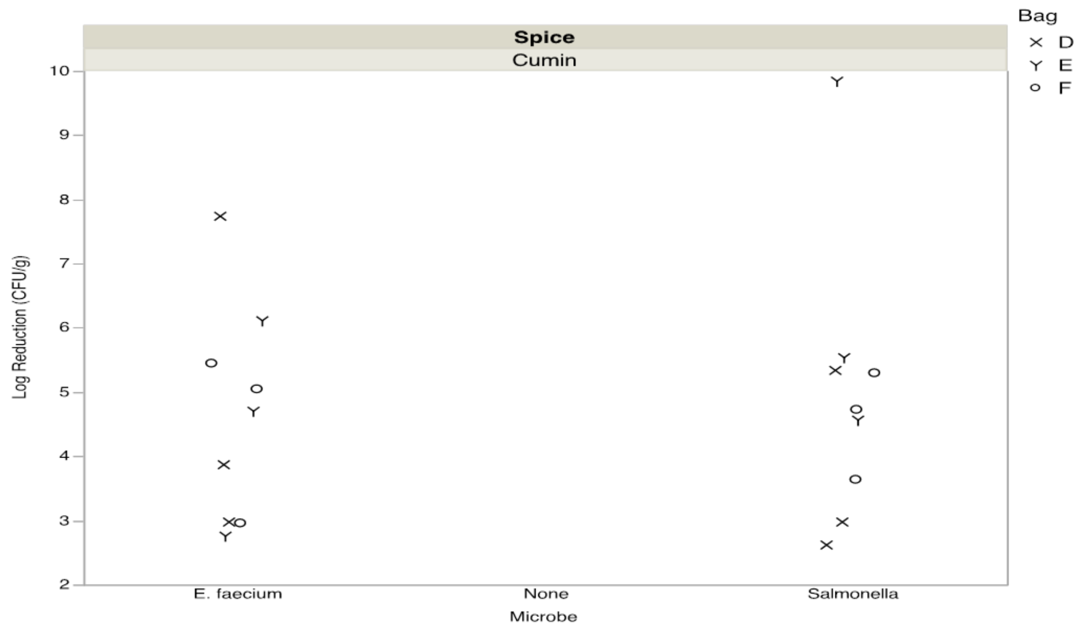

Figure 2. Log reduction of *Salmonella enterica* and *Enterococcus faecium* on cumin seeds after treatment with EtO fumigation.

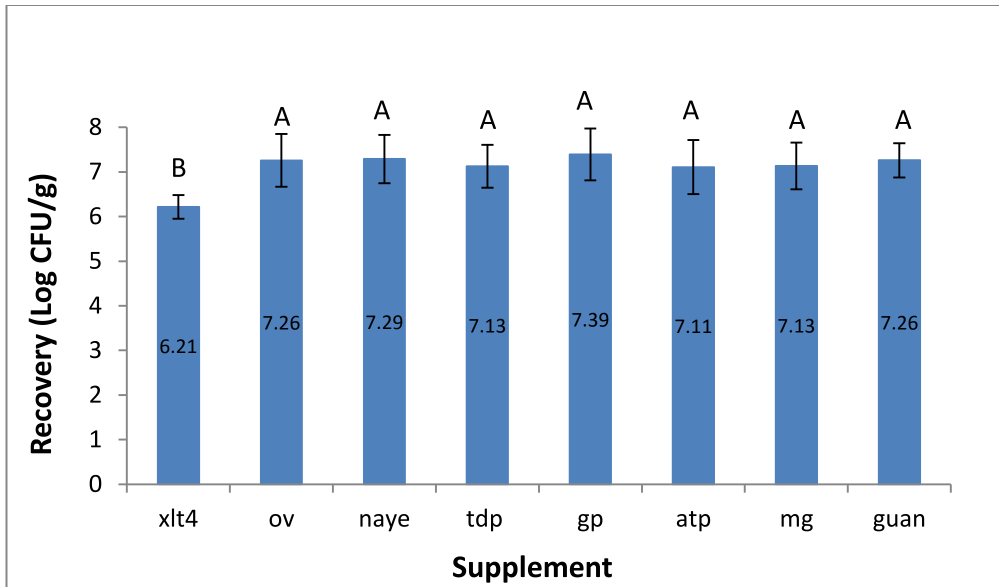

Figure 3. Comparison of media supplements for recovery of *Salmonella enterica* from peppercorns subjected to ethylene oxide fumigation. n=4, error bars=standard deviation. Figure abbreviations: Xylose Lysine Tergitol-4 (XLT4), Traditional Overlay (OV), Sodium Pyruvate + Yeast Extract (NAYE), 3,3'-Thiodipropionic acid (TDP), Glycerophosphate (GP), ATP (ATP), Magnesium (Mg) Guanine (GUAN).

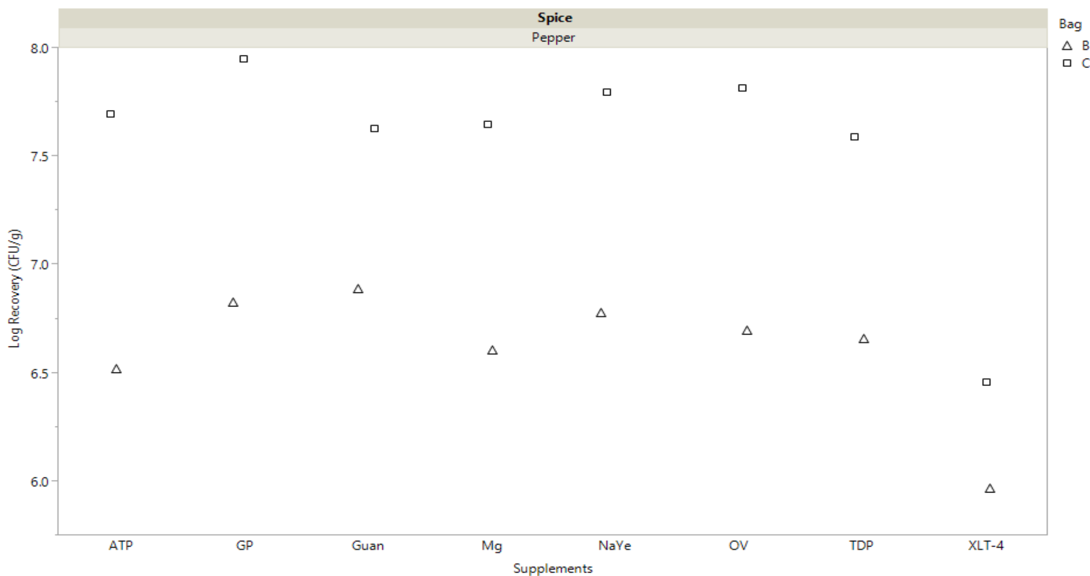

Figure 4. Log recovery of *Salmonella* from peppercorns subjected to ethylene oxide fumigation using various agar supplements.

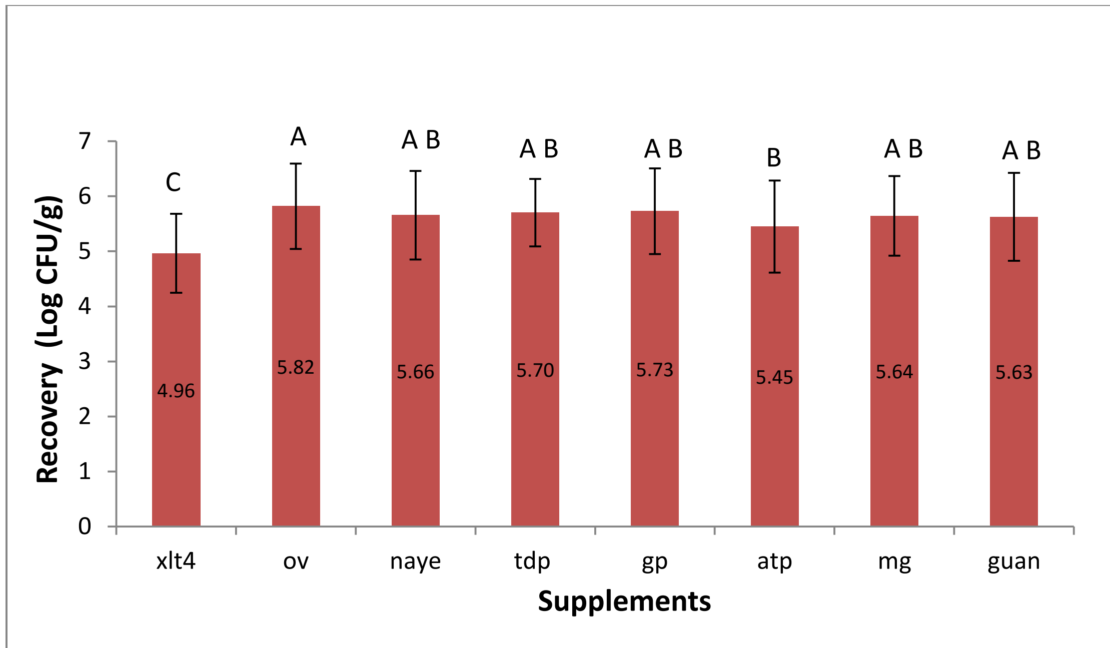

Figure 5. Comparison of media supplements for recovery of *Salmonella enterica* from cumin seeds subjected to ethylene oxide fumigation. n=4, error bars=standard deviation Figure abbreviations: Xylose Lysine Tergitol-4 (XLT4), Traditional Overlay (OV), Sodium Pyruvate + Yeast Extract (NAYE), 3,3’-Thiodipropionic acid (TDP), Glycerophosphate (GP), ATP (ATP), Magnesium (Mg) Guanine (GUAN).

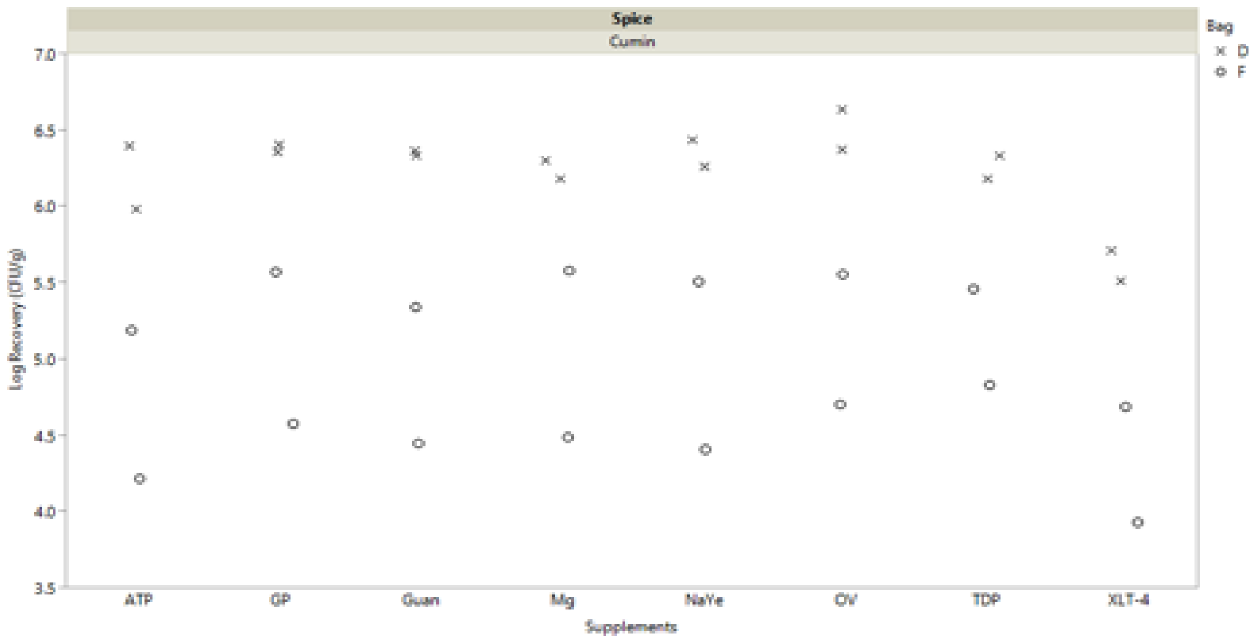

Figure 6. Log recovery of *Salmonella* from cumin seeds subjected to ethylene oxide fumigation using various agar supplements.