Evaluating Methods of Improving Recovery of Sub-lethally Injured *Salmonella* in Low Moisture Foods Treated with Antimicrobial Gas

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A thesis submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements of the degree of

Master of Science in Life Science in Food Science and Technology

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May 4, 2022

Blacksburg, VA

Keywords: *Salmonella*, *Enterococcus*, Spices, Bacterial Growth, Low Water Activity Foods, Food Safety, Chlorine Dioxide, Hydrogen Peroxide

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SCIENTIFIC ABSTRACT

The pathogenic microorganism *Salmonella enterica* has been associated with several outbreaks and recalls of spices, herbs, and seeds. To control these pathogens additional treatment methods, such as fumigation with chlorine dioxide (ClO_2) or hydrogen peroxide (H_2O_2) gas and recovery methods are needed. Recovery methods should accurately quantify all viable cells, even those injured, to prevent overestimation of treatment effectiveness. This study was performed to determine the effect of different recovery media and supplements on the recovery of multiple strains of *S. enterica* and *Enterococcus faecium* NRRL B2354, from chlorine dioxide or hydrogen peroxide treated low moisture foods (LMF) black peppercorns, dried basil leaves, and chia seeds. Also, this study aimed to compare the log reduction of these two microorganisms to evaluate *E. faecium* NRRL B2354 as a surrogate for *S. enterica*. On average, recovery of *S. enterica* was 3.43 log and 4.77 log CFU/g from ClO_2 and H_2O_2 treated LMFs, respectively on the selective media Xylose Lysine Deoxycholate agar, while the average recovery on non-selective media was 4.50 log CFU/g and 5.74 log CFU/g from ClO_2 and H_2O_2 treated LMFs, respectively. The use of non-selective media was correlated with increased recovery compared to selective media. In further studies, addition of sodium pyruvate, ferrous sulfate, or 3'3'-thiodiproionate supplements to MTSAYE did not show increased recovery ($P>0.05$). On each treatment and LMF combination tested, there was no significant difference between the log reduction of *S. enterica* and *E. faecium* NRRL B2354, indicating its suitability as a surrogate under the test conditions.

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GENERAL AUDIENCE ABSTRACT

Spices, dried herbs, and seeds have become popular throughout the world for enhancing the flavor of food, but may also harbor harmful bacteria, including *Salmonella enterica*. It is US federal law under the Food Safety Modernization Act that these foods are safe to eat straight from processors since these foods are typically consumed raw. Novel treatment methods are being tested to kill harmful bacteria on these dried foods without adding water including chlorine dioxide fumigation and hydrogen peroxide fumigation. However, these processes can injure the bacteria without killing them. These injured bacteria might not be counted using traditional means which could lead to overestimating the effectiveness of a treatment. Different media types, used as part of the process to count the number of bacteria in a sample, were tested to determine their effect on recovery of injured *S. enterica* cells. Furthermore, the bacterium *Enterococcus faecium* NRRL B2354 was tested against *S. enterica* to evaluate, if the former, a relatively harmless microorganism, could be used by food processing plants to determine that their treatment processes meets regulatory standards. More injured *S. enterica* cells were recovered from each non-selective media tested, compared to the selective media. Although there isn't a significant difference in injured *S. enterica* recovery between any supplemented non-selective media, any non-selective media recovers more sub-lethally injured cells, and would give more accurate bacterial counts. Results also indicated that *E. faecium* NRRL B2354 is a suitable surrogate to the pathogen *S. enterica* for spices and herbs processed under the same conditions.

ACKNOWLEDGEMENTS

First off, I would like to thank my parents, Marcos and Brunnie, for their unconditional support and for always pushing me to better myself. You have really inspired me throughout my life and have shaped me into the person I am today. I really appreciate you always checking in on me. My sister, Veronica, also has my thanks for being there when I needed someone to talk to. Thank you to all my friends in the food science and technology department, I really appreciate your friendship throughout my time here. Especially for those of us who started during the height of lockdowns, it has been quite a wild experience. Thank you to Kim Waterman, you have been a great help during my time in the lab. Without your help, this project would have been ten times harder. I would also like to give thanks to my committee members, Dr. Eifert and Dr. Strawn, since your knowledge and guidance was crucial in shaping this project and helping me out when I have gotten stuck. Lastly, a big thanks to Dr. Strawn for being a there for me when Dr. Ponder was on sabbatical. Thank you to Dr. Ponder, I really appreciate your guidance throughout my time here and I can't thank you enough for taking me on as your student.

ATTRIBUTION

Multiple contributions were made to this research by the following:

Monica A. Ponder, PhD (Food Science and Technology Department, Virginia Tech): Dr. Ponder is the major advisor on this project. She has provided guidance and funding for the project, as well as assisted with experimental design and statistical analysis. She is a lead author on the manuscript in chapter 2.

Joseph D. Eifert, PhD (Food Science and Technology Department, Virginia Tech): Dr. Eifert acted as a committee member and provided support and guidance throughout the project.

Laura K. Strawn, PhD (Food Science and Technology Department, Virginia Tech): Dr. Strawn acted as a committee member and provided support and guidance throughout the project.

Jeyam Subbiah, PhD (College of Agricultural, Food, and Life Sciences, University of Arkansas): Dr. Subbiah provided guidance and support throughout the project, and assisted and advised on preliminary research and the treatment of LMFs used in the project

Kim Waterman, MS (Food Science and Technology Department, Virginia Tech): Ms. Waterman provided technical support and assistance throughout the project.

Surabhi Wason, MS (College of Agricultural, Food, and Life Sciences, University of Arkansas): Ms. Wason developed and implemented protocols for the inoculation and treatment of LMFs used in the project. She performed the chlorine dioxide and hydrogen peroxide treatments at the University of Nebraska and provided samples.

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CHAPTER 1

Introduction and Justification

Spices have been valued throughout history for their flavor enhancing capabilities. Spice consumption in the United States has increased 60% per capita in the period between 1980 and 2000 *(91)*. With an increased frequency of consumption, the need to ensure a safe product also increases. This is especially critical as the United States Food and Drug Administration classes spices as ready-to-eat products, which are not expected to have any further processing before consumption.

Outbreaks of salmonellosis linked to *Salmonella enterica* in spices and low water-activity foods have been documented in recent history. In the 37-year period between 1973 and 2010, consumption of *S. enterica* contaminated spices and herbs has been linked to 10 different outbreaks and over 1500 illnesses *(87)*. In 2009, 85 cases of salmonellosis were reported in 5 different states. Cases were traced to the consumption of white pepper contaminated with *S. enterica* ser. Rissen *(99)*. A 2010 outbreak of *S. enterica* ser. Montevideo resulted in 272 cases in 44 states and was linked back to *S. enterica* contaminated black and red pepper used to make salami products *(26)*. Additionally, in 1993, Germany experienced a nationwide outbreak of salmonellosis resulting in 1000 cases linked to *S. enterica* ser. Rubislaw, *S. enterica* ser. Saintpaul, and *S. enterica* ser. Javiana on contaminated paprika on potato chips *(43)*. Outbreaks such as these result from contaminated spices being added after typical processing methods that would normally inactivate the pathogen. Foodborne pathogens, such as *S. enterica,* can survive on the surface of foods with low water activity like spices for weeks or months *(58)*. Since no kill step is present, any pathogens on the spices can persist and potentially

result in illnesses. The Food Safety and Modernization Act (FSMA) has implemented a preventative controls rule for spice processors, requiring food facilities to conduct hazard analysis and identification, and then implement preventative controls for those hazards *(82)*.

When processing spices, industry standard methods have the goal of maximized microbial reduction with minimized sensory attribute change. Current methods include chemical fumigation, irradiation, and steam-based heat treatment methods *(4)*. Irradiation treatment can lead to negative effects on taste, smell, color, and texture. This treatment method can also lead to a slight loss of nutrients in the food product *(4)*. Steam treatments can result in significant differences in sensory attributes of whole black peppercorns *(19)*. Chemical fumigation has the advantage of having a minimal impact on flavor and appearance of the treated spice. Sanitizers in gaseous form are also more diffusible than in aqueous form, allowing for more efficiency in eliminating microorganisms on a food surface *(36)*. Common gasses used in chemical fumigation treatment of spices include ethylene oxide and propylene oxide *(4)*. Other gasses beginning to see use include chlorine dioxide gas and hydrogen peroxide gas. These agents are oxidizers, which cause damage to the microbial cell's membrane and proteins, as well as preventing DNA replication *(29)*. Hydroxyl radical attacks on the DNA lead to fragmentation, strand breaks, and damaged bases. This blocks DNA polymerase from replicating DNA and RNA polymerase from transcribing DNA as polymerases are blocked by DNA lesions *(21)*. Oxidative damage on the DNA can also induce base substitution mutations, such as G:C→T:A or A:T→C:G *(57)*. Additionally, protein oxidation can impair enzymatic activity in the microbial cell, and lipid peroxidation can severely damage the cell

membrane, both can lead to microbial cell death *(6)*. However, if a pathogenic cell is only injured by the antimicrobial gas, it may not grow on recovery media but given time and nutrients can repair itself, and potentially may cause illness if consumed. This is important to consider when calculating D Value, or time it takes to reduce the numbers of *S. enterica* by 1.0 log CFU. With recovery methods that do not accurately count all viable microorganisms, it is possible to underestimate the D-value. This D-value underestimation would result in inadequate treatment leading to a potentially unsafe product.

This study was performed to determine the suitability of different plating media for the recovery of *S. enterica* cells from antimicrobial gas treated spices and an herb. This study aimed to compare recovery of *S. enterica* cells using selective/non-selective media types. *S. enterica* cells may be injured during processing, and this may influence growth on selective media. Additionally, this study compared addition of different antioxidants to recovery media to improve the recovery of *S. enterica* cells that have been sub-lethally injured by antimicrobial gas treatment. Rehydration using standard microbiological techniques can inactivate microbial cells due to instantaneous rehydration stress, leading to an underestimation of the pathogenic load *(39)*. This could be due to intracellular vesiculation causing a decreased membrane surface area, bursting the cell when water fills the cell to its original volume *(39)*. Another concern is the accumulation of solutes causing the cell to take in more than its original volume of water, bursting the cell *(39)*. Antioxidants work to neutralize reactive oxygen species by donating an electron to terminate the free radical without becoming one itself *(25)*. The antioxidant supplements chosen for this experiment are sodium pyruvate, ferrous sulfate,

and 3’3’-thiodipropionate. These supplements were added to prevent further oxidative stress in the *S. enterica,* allowing the sub-lethally injured cells to repair and grow on recovery media. Additionally, two of these compounds appear to have additional benefits besides being an antioxidant. Sodium pyruvate was chosen as it is also a source of glucose, which the extra energy source can be used by the cell to aid in its repair. Iron is a key component in biological processes including DNA synthesis *(65)* and it has been shown that iron availability increases *S. enterica* growth and pathogen potential *(38).* In addition to its antioxidant capability, ferrous sulfate was chosen to function as a source of iron for the *S. enterica* microorganisms. These compounds have shown success at improving recovery of *S. enterica* in chlorine dioxide treated egg albumin *(29).*

Additionally, this study aims to compare the recovery of *Enterococcus faecium* NRRL B2354 to a cocktail of *S. enterica* to determine if the *E. faecium* NRRL B2354 is suitable as a surrogate for the pathogen. This research should serve to prevent the overestimation of the reduction of pathogenic microorganisms associated with fumigation interventions.

Objectives and Hypotheses

1. Compare the log CFU reduction of *Salmonella enterica* and *Enterococcus Faecium* NRRL B2354 recovered from spices (black peppercorn, basil leaves, and chia seeds) treated with chlorine dioxide and basil leaves treated with hydrogen peroxide using the different media types MTSAYE, MTSAYE with added sodium pyruvate (MTSAYE-NP), MTSAYE with added ferrous sulfate (MTSAYE-FS), MTSAYE with added 3'3'- thiodipropionate (MTSAYE-TDP) and a selective media XLD.

a. H0: There will be no significant difference in the log CFU of *S. enterica* and *E. faecium* NRRL B2354 on different media types.

b. Ha: There will be a significant difference in the log CFU of *S. enterica* and *E. faecium* NRRL B2354 recovered from media modified with supplemented antioxidants.

c. Ha: There will be a significant difference in the log CFU of *S. enterica* recovered using the non-selective media compared to the selective media.

2. Compare the log CFU reductions of *E. faecium* NRRL B2354 to *S. enterica* on spices (black peppercorn, basil leaves, and chia seeds) treated with chlorine dioxide and on basil leaves treated with hydrogen peroxide to determine the suitability of *E. faecium* NRRL B2354 as a surrogate.

a. H0: There will be no significant difference between the Log CFU of *S. enterica* and *E. Faecium* NRRL B2354 recovered from spices (black peppercorn, basil leaves, and chia seeds) treated with chlorine dioxide. If there is no significant difference between the

two microbes recovered this indicates suitability as a surrogate under the tested conditions.

b. Ha: There will be a significant difference between the Log CFU of *S. enterica* and *E. Faecium* NRRL B2354 recovered from spices (black peppercorn, basil leaves, and chia seeds) treated with chlorine dioxide. *E. faecium* NRRL B2354 would be considered an adequate surrogate if its log reduction values are comparable to or lower than that of *S. enterica*, implying that *E. faecium* NRRL B2354 is harder to kill than *S. enterica*.

Literature Review

Low Water Activity Foods

Water activity (a_w) is defined as "the value of vapor pressure of a food divided by the vapor pressure of distilled water at the same temperature" *(27)*, as solutes are added this water becomes bound. Water activity for pure water is 1.0 under standardized conditions, and as such any water activity measurement in foods will be below 1.0. The Food and Drug Administration (FDA) classifies foods into three categories based on water activity: moist foods with a_w above 0.85, intermediate moisture foods with a_w between 0.85 and 0.60, and low moisture foods with a_w below 0.6 *(81)*. Some low moisture classified foods include dried noodles (a_w 0.50), cookies (a_w 0.30), cereals (a_w 0.20), and crackers (a_w 0.10) *(81)*. Water activity is different from moisture content as different solutes or food components have different affinities for water *(27)*. When the water is bound it is unavailable for uptake and use by the majority of microorganisms *(27)*.

The minimum water activity necessary for growth varies with different microorganisms, with 0.85 a_w considered the cut-off for growth of foodborne pathogens. *(81)*. Moist foods with an a_w above 0.85 require some sort of control or intervention in order to prevent pathogen growth *(81)*. Intermediate moisture foods do not require such controls as their water activity is below that necessary for pathogen growth, however spoilage organisms like yeasts and molds can still be a problem *(81)*. Though pathogenic microorganisms cannot grow at these low water activities, vegetative cells and spores of some bacteria can survive in low water activity foods and ingredients for several months

or even years *(10)*. Survival of only a few cells of a foodborne pathogen, like *Salmonella enterica*, can be a sufficient dose to cause disease *(10)*.

Spices, Herbs, and Seeds

Dried spices, herbs and seeds are low water activity foods, whose per capita consumption has by 60% in the United States in the time period of 1980 to 2000 *(91)*. According to the FDA, spices are defined 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 onion, 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" *(4)*.

Black Peppercorn: Black peppercorns are the dried immature berries of *Piper nigrum (80)*. They are deep brown or black, deep set wrinkled berries and have a characteristic odor and pungent taste when ground *(80)*. To produce black pepper, the fruit is harvested while still unripe, and then the berries are dried in the sun. The berries are dried to a moisture content (wet basis) of 10-12% over the course of 5 to 12 days *(31)*. During this drying time the berries can be potentially contaminated with dirt and dust, and foodborne pathogens can potentially be introduced by rain, animals, and birds *(31)*. Additionally, the hot, humid climate of the growing areas lends itself to rapid microorganism growth *(31)*. The conditions required to grow black peppercorn are found in southeast Asian countries like India, Vietnam, Indonesia, and Malaysia *(63)*. The United States does not have a climate conducive to growing *Piper nigrum*, so the black pepper supply used for consumption is from imported black pepper *(22)*. The industry standard water activity for black peppercorn is below 0.75 *(35)*.

The dried berries are frequently imported into the United States as whole peppercorns, where they can be ground into a powder, or sold as is to allow consumers to grind the peppercorn themselves. Grinding the peppercorns increases product value but can increase surface area and spread contaminants throughout the product. For this fear of adulteration and for the ability to inspect product quality, some consumers would prefer whole peppercorns that they can grind themselves *(31)*. Compounds responsible for the flavor of black peppercorn include oleoresin and piperine *(50)*. However, it has also been shown that the loss of a-pinene limonene and 3-methyl butanal during storage results in a deficit of the pepper notes in the product *(50)*.

Basil: The dried leaves of basil (*Ocimum basilicum*) are classified as a spice but in the fresh form are considered an herb. The highly scented leaves are sold fresh or dried, and its essential oils are used in pharmaceutical and flavoring *(61)*. 1,8 cineol, linalool, citral, methyl chavicol, eugenol, and methyl cinnamate are the key compounds giving basil its aroma, though little basil types have all of these compounds in high quantities *(18)*. In the United States, the region with the largest production of dry leaf products is in California, and the regions with the largest production of oil are in the Eastern states *(61)*. A large quantity of basil consumed in the United States is imported from growers in the Mediterranean region of Europe *(61)*. During growth, the leaves may be contaminated by foodborne pathogens due to transfer from animal excreta, soil, rain or contaminated irrigation water *(61)*. After the plant has been picked mechanically the basil is either dehydrated with forced air dryers, or the essential oil is extracted using steam or solvent *(61)*. Some growers may not be able to economically use forced air dryers and must rely on passive air drying which would continue to expose the product to dust, water, and

animals that could introduce pathogens *(61)*. The industry standard water activity for basil is below 0.75 *(4)*.

Chia Seeds: Chia Seeds are the dry indehiscent fruits of the plant *Salvia Hispanica (17)*. These plants are native to southern Mexico and northern Guatemala *(17)*. Today the world's largest chia producer is Mexico, with chia also being cultivated in Australia, Argentina, Bolivia, Colombia, Guatemala, Peru, and the United States *(37)*. Chia seeds have been valued for their nutritional value, being a good source of proteins, dietary fiber, vitamins, minerals, and antioxidants *(17)*. Chia seeds and oils contain high amounts of tocopherols, phytosterols, carotenoids, and multiple polyphenolic compounds like myricetin, quercetin, and kaempferol; these compounds are responsible for giving chia seeds a high antioxidant activity *(17)*. Chia seeds are also highly hydrophilic, able to absorb 12 times their weight in water *(37)*. Due to this water absorbing property, chia seeds must be harvested with minimal water use to avoid formation of mucilage *(15)*. Preharvest, the seeds can be introduced to microbial contamination from the use of animal manure as fertilizer which could contain pathogenic microorganisms *(98)*. As with any crop grown in open fields, there is also risk of contamination from domestic or wild animals *(98)*. Postharvest, infected farm workers as well as unclean farming equipment and storage vessels all risk contaminating the seed product *(98)*. When processing these seeds, they can be dried in silos with controlled temperature and humidity, however this is dependent on growing area with some growers having to dry seeds in open air *(15)*. The industry standard water activity for chia seeds is below 0.75 *(4)*.

Microbiological Quality and Safety of Spices

The bulk of spices are grown in developing countries where hygiene and sanitation practices differ from developed nations practices *(54)* In addition to inadequate sanitary conditions, the fact that spices are a minimally processed agricultural products means that they are at high risk of foodborne pathogen contamination from the surrounding dirt, dust, birds, animals, and insects in the areas they are grown *(54)*. Spice growers in developing countries with small scale and lower budgets would dry spices on mats or on the ground with little protection from animals and pests *(54)*. In many locations, spice production occurs on small scale farms, and it is common practice for farmers to bring their crops to a local collection point where a broker will gather up the crops and sell them to a processor down the line *(54)*. This combining of small spice lots from various locations increases microbiological diversity in the final lot that moves on to processing *(54)*. As such, there is ample opportunity for contamination with foodborne pathogens both pre-harvest and post-harvest.

Spices typically have water activities below 0.60 when properly dried and stored *(54)*. Due to this low water activity the bacterial growth and mold growth is minimal *(54)*. The American Spice Trade Association (ASTA) recommends storage conditions of less than 60% relative humidity to prevent absorption of moisture *(4)*. Black peppercorns, as well as other spices, are hygroscopic and can pull moisture from the air, and can achieve water activities conducive to bacterial and mold growth in storage conditions with high humidity *(54)*.

The microbiological quality varies from spice to spice. Some spices and herbs like cloves, cayenne, and saroline have low amounts of microorganisms while peppercorns have high amounts *(52)*. Standard plate counts of ground black peppercorn showed microbial loads as high as $2.0*10^8$ CFU/g *(52)*. Coliform levels of <10/g were detected in cumin, garlic powder, mustard seed, nutmeg, parsley, oregano, while parsley has 10-100/g and black peppercorn had $1.1*10^3$g *E. coli (52)*. Spores are prevalent in soil and spices can be exposed to them during growth, and yeast and mold counts showed levels as high as 10^7 CFU/g on cinnamon *(52)*. Levels of the spore forming bacteria *Bacillus cereus* found were generally below <3/g but spiked as high as $1.0*10^5$ CFU/g on samples of black pepper, turmeric, and cumin *(52)*. FDA regulations for ready to eat spices set a microbiological limit for aerobic plate count at $\leq 10^5$/g *(68)*. Total coliforms should be no more than 10/g and Enterobacteriaceae should be $\leq 10^2$/g *(68)*. *S. enterica* and shiga toxin producing *Escherichia coli* should not be detected in the spice product *(68)*. For dried herbs the microbiological limit for aerobic plate count is $\leq 10^6$/g and coliform count must be $\leq 10^4$/g *(68)*. *S. enterica* and shiga toxin producing *E. coli* tests should not be detected in the herb product *(68)*.

From October 1969 to December 2003, twenty recalls monitored by the FDA were due to the presence of *S. enterica (91)*. Additionally, ten outbreaks of *S. enterica* resulted in over 1500 illnesses from the period of 1973 to 2010 as a result of contamination on spices and herbs *(87)*. In one instance, over 640,000 kgs of salami were recalled due to an outbreak of *S. enterica* ser. Montevideo *(99)*. From 2010 to 2018 there have been at least six major outbreaks related to *S. enterica* contaminated spices *(28)*. Recalls of spices due to *S. enterica* contamination are still prevalent. In 2017

Tupperware, Inc. had to recall seasoning packets *(83)* and Spicely Organics recalled tarragon spices because of possible *S. enterica* contamination *(79)*. Sauer brands, inc. needed to recall over 30 spice products in 2020 due to contamination *(85)*. Most recently in 2021, the spice producer McCormick needed to recall 3 different product lines that had been distributed to 32 different states because of *S. enterica* contamination *(67)*. ASTA has identified *S. enterica* as the most common pathogen involved in outbreaks and recalls related to spices and has issued guidance to industry focused on this pathogen *(4)*.

Salmonella and Salmonellosis

S. enterica is a Gram-negative, rod-shaped bacilli capable of causing diarrheal illness in humans *(74)*. There are over 2,500 known serotypes of *S. enterica* though fewer than one hundred are known to cause human illness *(74)*. Serovars *Salmonella Enteritidis, Salmonella Newport,* and *Salmonella Typhimurium* are responsible for the most infections reported to the CDC's laboratory based Enteric disease surveillance system in the year 2016 *(72)*. The pathogen can cause illness in humans or animals, and strains that cause symptoms in animals may not affect humans, and strains that cause symptoms in humans may not affect animals *(74)*. The presence of *S. enterica* has no perceivable effect on the taste, odor, or appearance of the food *(74)*.

Salmonellosis is the infection caused by non-typhoidal serotypes of *S. enterica*. Nontyphoidal *S. enterica* is responsible for an estimated 1.0 million illnesses, 19,000 hospitalizations, and 400 deaths annually *(59)*. The infectious dose of serotypes of *S. enterica* to cause salmonellosis is complicated with multiple factors affecting it. Information from outbreak data place infectious dose ranges from 10^2-10^{11} cells depending on the serovar and vehicle involved *(55)*. Human volunteer studies, in which

different serotypes of *S. enterica* were delivered in milk or buffering solution, determined infectious doses in the ranges of 10^5-10^8 cells depending on strain *(55)*. These human volunteer studies are not necessarily reflective for the entire population as these studies exclude the young, old, and immunocompromised which are populations known to be more susceptible to illness *(55)*. In addition to factors such as age and health status, there may be factors involved in *S. enterica* infectivity associated with the food itself. Outbreaks characterized with a low attack rate and low infectious dose, as determined by the number of cells that were isolated from the food, implies that there is variability in exposure due to unequal distribution of the pathogen in the product *(55)*. One outbreak involved the consumption of ice cream in which a family of three each consumed the equivalent of one cone of ice cream, resulting in each of them falling ill *(92)* Product testing of the ice cream container estimated that the infective dose within the serving was around 28 cells per serving caused illness in a family of three *(92)*. In an outbreak of *S. enterica* ser. Eastbourne, the pathogen was recovered from chocolate balls at a concentration of 2.5 CFU/g *(11)*. *S. enterica's* ability to cause disease can also be enhanced by the alteration of bactericidal activity in the stomach, as reducing stomach acidity can reduce the effectiveness of the stomach's first line of defense *(55)*. Stomach acidity can be reduced by malnutrition, by medicine, or by the buffering effects of food, this can raise pH levels in the stomach to around 5-7 which can be inadequate to inactivate *S. enterica (55)*.

S. enterica can find its way into human digestive tracts via foods contaminated with animal feces *(74)*. This commonly happens as a result of cross contamination, when infected raw meats and poultry come into contact with ready to eat foods *(74)*. *S. enterica*

present on raw meats and poultries can survive if not cooked to a safe internal temperature *(74)*. *S. enterica* can also get into foods via the unwashed hands of a contaminated food handler who may or may not show symptoms of infection *(74)*. Symptoms of salmonellosis include diarrhea, abdominal cramps, fever, chills, headache, nausea, and vomiting *(74)*. These symptoms usually appear within 8 to 72 hours of consumption of contaminated foods and tend to last for 4 to 7 days *(74)*. Though many can recover without treatment from a doctor, salmonellosis can be deadly for infants, pregnant women, the elderly, and those with weakened immune systems in general *(74)*.

In the past, *S. enterica* was traditionally associated with animal products, but recent outbreaks have been related to fresh produce and foods with low water activity, such as spices *(77)*. Over the time period between 1973 and 2010 there have been 10 different recorded outbreaks of *S. enterica* associated with the consumption of spices or fresh herbs *(87)*. An outbreak in 2009 saw eighty-five cases of salmonellosis reported from five different states because of the consumption of white pepper contaminated with *S. enterica* ser. Rissen *(99)*. In 2010, black and red pepper contaminated with *S. enterica* ser. Montevideo was implicated in an outbreak that resulted in 272 cases across forty-four states *(26)*. *S. enterica* continues to be an issue in foodborne safety, with the Centers for Disease Control and Prevention (CDC) having reported no less than 5 outbreaks of *S. enterica* associated with food in the time period of May to July 2021 *(73)*. These outbreaks have resulted in at least 87 cases and 21 hospitalizations across 18 states *(73)*.

Survival of *Salmonella* on Low a_w Foods

S. enterica is the pathogen most commonly implicated in recalls and outbreaks related to spices and other low water activity foods *(4)*. Many low water activity foods

are sold as ready-to-eat foods, and so consumers are not expected to do any further cooking or processing before consumption *(44)*. Thus, the onus is on food processors to eliminate any *S. enterica* potentially residing on the surface of such products. *S. enterica* can survive on dry foods for weeks, months, and even years in some cases *(58)*.

S. enterica shows high survivability on whole black peppercorns. Ground black pepper samples (10 g) were inoculated with 300 µL of an 11 log CFU/mL *S. enterica* cocktail consisting of *S. enterica* serovars Enteritidis PT30, *S. enterica* ser. Oraneinburg, *S. enterica* ser. Anatum, and *S. enterica* ser. Tennessee *(64)*. Samples were stored in jars with desiccant solutions in incubators to achieve the following storage conditions: 25 °C, 33% relative humidity (RH), 25 °C, 97% RH and 37 °C, 33% RH *(64)*. With a starting inoculation of 11 log CFU/g, *S. enterica* populations remained stable, defined as > 5 log CFU/g, stored at 25 °C and 97% RH on black peppercorn samples for 40 days *(64)*. At storage conditions of 25 °C and 33% RH on black peppercorn samples *S. enterica* populations maintained stability for 200 days *(64)*.

S. enterica also has the capability of surviving on fresh basil leaves for extended time periods *(20)*. Leaves on basil plants were inoculated with 8.6 log CFU/g of a solution containing *S. enterica* serovars Reading, Newport, and Typhimurium then stored at room temperature and watered to mimic home storage over 18 days *(20)*. On day 0, a rapid die off occurred with samples showing a concentration of 3.7 log, and on day 18 samples showed a concentration of 2.1 log CFU/g indicating a much lower rate of die off over time *(20)*. Pesto was made with fresh, non-inoculated basil leaves and determined to have a pH of 5.5 and a_w of 0.28 before being inoculated with 3.6 log CFU/g of the *S. enterica* solution and incubated at room temperature and at 4 °C for 4 days *(20)*. At day

0, samples from both storage temperatures showed a concentration of 3.5 log CFU/g and after 4 days of storage the room temperature samples showed around 1 log reduction in concentration while the 4 °C storage samples showed around a 0.6 log reduction in concentration *(20)*. While basil leaves and pesto do not provide an environment suitable for growth, as these products are expected to be eaten fresh there may not be time for *S. enterica* cells to be significantly reduced *(20)*.

Temperature and water activity are key factors affecting inactivation of *S. enterica (89)*. Inoculated dried basil leaves were treated with a Thermal Death Time sandwich system at temperatures of 70 °C, 75 °C, and 80 °C with water activities of 0.40, 0.55, and 0.70 until a 3-5 log reduction in *S. enterica* was achieved *(89)*. At a_w of 0.40, calculated D-values for treatments at 70 °C, 75 °C, and 80 °C were 20.41 min, 9.14 min, and 2.68 min respectively *(89)* For a_w of 0.55 D-values at 70 °C, 75 °C, and 80 °C were 15.02, 6.64, and 1.86 min respectively *(89)*. Lastly at a_w of 0.70 D-values at 70 °C, 75 °C, and 80 °C were 8.77, 3.30, and 1.02 min respectively *(89)*. In dried basil leaves, a lower water activity resulted in a higher calculated D-value at the same temperature thermal treatment.

S. enterica survivability on chia seeds with a a_w of 0.585 has been reported *(23)*. The chia seeds were inoculated with a cocktail containing *S. enterica* strains Enteritidis, Thompson, Typhimurium, Hartford, and Tennessee before being air dried in a biological safety cabinet for 0.5 hours *(23)*. After drying, samples were stored at 20 °C until colonies of *S. enterica* were no longer recovered, with bacterial cell density determined periodically over 150 days *(23)*. Recovery of most serotypes was 3.5 log CFU/g after 80 days storage, but survival of one Hartford strain was greater (4.17 log CFU/g) *(23)*. The

maximum survival duration of each strain varied, with Typhimurium still being detected at 48 days, Enteritidis and Thompson being last detected at 68 days, Tennessee at 135 days, and Hartford still being detected at 150 days of storage *(23)*.

In low water activity, some serotypes of *S. enterica* are reported to have with increased thermal resistance *(58)*. Differences in heat resistance of *S. enterica* for different water activities has been reported for inoculated black peppercorn powder equilibrated to water activities of 0.33, 0.54, and 0.75 *(24)*. These samples were heat treated in Thermal Death Time cells in a water bath at temperatures of 75 °C, 80 °C, and 85 °C for 0.5 to 50 min and then the S. *enterica* inactivation kinetics were modeled to determine treatment times needed for a 5-log reduction *(24)*. At 75 °C the 0.33 a_w and the 0.54 a_w samples had calculated 5-log reduction times of 198 min and 106 min respectively *(24)*. At 70 °C the 0.54 a_w and the 0.75 a_w samples had calculated 5 log reduction times of 272 min and 8 min respectively *(24)* The longer time to achieve 5 log reduction at lower water activities at the same temperature is indicative of resistance to heat treatment at lower a_w *(24)*. The presence of *S. enterica* in low water activity foods is linked to cross-contamination due to poor sanitation practices, substandard facility and equipment design, improper maintenance, poor operational and good manufacturing practices (GMPs), as well as inadequate ingredient control and pest control *(58)*.

Intervention Strategies to Reduce Pathogens on Low Water Activity Foods

The Food Safety Modernization Act (FSMA) requires processors to have a written food safety plan, the primary document guiding the preventative controls in their food safety system *(8)*. The food safety plan requires a written hazard analysis, outline of preventative controls and critical control points, supply chain program analysis, recall

plan, procedures for monitoring implementation of controls, corrective action procedures, verification procedures, and records *(8)*. For low water activity foods, the plan would need to address potential food safety hazards and the preventative controls for these hazards should they be able to cause illness or injury to the consumer; processors should note the possible introduction or presence of bacterial pathogens, like *S. enterica*, from the ingredients or manufacturing environment *(8)*.

Microbial inactivation steps used as controls on low water activity foods include steam treatment, irradiation, and the use of chemical fumigants *(4)*. Steam treatment involves the use of saturated, dry, or superheated steam to reduce the aerobic plate count, as well as load of mold, yeasts, and coliforms on a product anywhere from 1 to 5 logs of reduction *(4)*. Depending on the technology and steam used in treatment, there may be a large change in microbiological reduction and the properties of the food like water activity and organoleptic properties *(4)*. Steam treatment is widely used for microbiological reduction in spices in areas where the use of chemical fumigants like Ethylene oxide or Propylene oxide are not approved, like in Europe *(4)*. Irradiation is a process that exposes foods to radiant energy *(4)*. Ionizing radiation has been approved in the US for the microbial disinfection of foods *(2)*. Irradiation may alter the organoleptic properties of the treated food, but the World Health Organization and the Food and Agricultural Organization of the United Nations has deemed that the treatment do not present any toxicological or nutritional hazard *(4)*. Chemical fumigant treatments involve the use of a chemical gas to inactivate microorganisms on the food product *(4)*. Common compounds used for this purpose include ethylene oxide, chlorine dioxide, and propylene oxide *(4)*.

Ethylene Oxide Gas Treatment of Foods

Ethylene Oxide (EtO) is a flammable, colorless gas that can function as a disinfectant, fumigant, sterilizing agent, and an insecticide *(4)*. Though the majority of the compounds use is in the sterilization of medical equipment, the US spice industry uses ethylene oxide to eliminate pathogenic microbial contaminants in spices *(4)*. An estimated 800,000 pounds of EtO is used by the US spice industry annually for this purpose *(4)*. The advantage of using ethylene oxide for spice treatment is that there is little to no significant impact on the spices flavoring or appearance post treatment *(4)*. Samples of whole black peppercorns treated with ethylene oxide showed little change in sensory qualities post treatment *(19)*. 2.27 kg of spice/herb sample was treated with ethylene oxide per Environmental Protection Agency label specifications; Spices in a chamber were exposed to a mixture of ethylene oxide and air at an ethylene oxide concentration of less than 500 mg/L for a dwell time of less than 6 hours followed by an evacuation of gas from the chamber using no less than 21 steam washes at a minimum 46.11 °C *(19)*. Treated samples were analyzed for appearance and odor via triangle testing for similarity in which a treated spice sample was compared to a control spice sample from the same lot *(19)*. Not enough participants (P values > 0.5) could determine a difference in appearance (P = 0.88) or odor (P = 0.66) between sample and control groups of black peppercorns, implying black peppercorn processed by ethylene oxide retained visual and odor quality post treatment *(19)*.

Additionally, ethylene oxide has been shown to significantly reduce microbial populations when used *(60)*. Ethylene oxide denatures functional proteins, DNA, and RNA of target microorganisms *(48)*. The main mechanism by which this is done is an

alkylation reaction that adds alkyl groups to sulfydryl, hydroxyl, amino, and carboxyl groups *(48)*.

Ethylene oxide gas has been shown to be effective in reducing log CFU/g of *S. enterica* on whole black peppercorns and cumin seeds both in a commercial setting *(49)* and within laboratory settings. Commercially, ethylene oxide treatment significantly ($P < 0.05$) reduces mean populations of *S. enterica* on whole black peppercorn *(49)*. This was tested by taking whole black peppercorns inoculated with *S. enterica* serovars Tennessee, Ball, and Johannesburg and shipping to a commercial processor specializing in ethylene oxide treatment *(49)*. The standard operating procedure for this facility involved five nitrogen pulses at 130 °F in a sealed chamber to reduce oxygen and increase temperature *(49)*. Once this was done, 20% ethylene oxide in 80% CO_2 was injected into the chamber at 130 °F and held for 325 minutes *(49)*. Finally, the process was finished with 21 steam washes and four nitrogen pulse cycles to remove residual ethylene oxide *(49)*. This ethylene oxide treatment resulted in a 6.62 log CFU/g reduction in *S. enterica,* higher than the FDA recommended 5 log reduction for appropriate risk management *(49)*.

In the laboratory, the effectiveness of EtO was improved at higher relative humidity and with elevated temperatures against a 7.51 log cocktail of *S. enterica* ser. Agona 447967, *S. enterica* ser. Montevideo 488275, *S. enterica* ser. Mbandaka 698538, *S. enterica* ser. Tennessee K4643, and *S. enterica* ser. Reading Moff 180418 on whole black peppercorns (a_w 0.5) *(94)*. At different temperature and RH conditions, these samples were treated by placing a layer of whole peppercorns in a petri dish and treating with 100% EtO gas from an EtO cartridge providing a concentration of 735.3 mg/L *(94)*. These inoculated samples were treated at 30% RH and 46 °C, 53 °C, and 60 °C for 180

minutes of exposure time *(94)*. This resulted in log reductions of 3.23, 3.81, and 4.05 log CFU/g respectively *(94)*. Samples were also treated at conditions of 53 °C and 30%, 40%, and 50% RH for an exposure time of 180 min *(94)*. These conditions showed a log reduction of 3.81, 4.74, and 4.92 log CFU/g, respectively, though at 50% RH 4.92 log reduction was achieved after only 20 minutes of exposure *(94)*. It's important to note that the concentration of EtO used in this study exceeds that allowed by the US EPA pesticide label but does illustrate the utility of altering both the temperature and humidity of the chamber to improve *S. enterica* reduction.

However, exposure to ethylene oxide can cause dizziness, nausea, headaches, convulsions, difficulty breathing, and blurred vision *(86)*. Ethylene oxide is also a carcinogen, linked to leukemia and other cancers in both humans and animals *(86)*. Ethylene oxide will also react under ambient conditions with chloride and bromide to create the compounds 2-chloroethanol and 2-bromoethanol, both of which are carcinogenic and mutagenic *(60)*. Due to these harmful effects, the use of ethylene oxide for fumigation is banned in the European Union *(60)*. Processors wishing to access these markets must use alternative processing methods for *S. enterica* reduction.

Chlorine Dioxide Gas Treatment of Foods

Chlorine dioxide (ClO_2) is a compound used to sanitize foods and food contact surfaces. The Food and Drug Administration (FDA) approved the compound for use in washing fruits and vegetables in aqueous form since 1998 *(42)*. The compound is also approved for use in poultry processing provided the concentration does not exceed 3 parts per million (ppm) *(1)*. Chlorine dioxide gas has been approved by the FDA for use in the medical field as a sterilant used on medical devices and instruments *(3)*. Chlorine dioxide

is an oxidizer, which can cause damage to the cellular membrane and prevent protein synthesis *(29)*. Base substitution mutations to the DNA can result from oxidative damage, for example G:C→T:A or A:T→C:G *(57)*. Additionally, lipid oxidation can compromise the cell membrane, leading to microbial cell death *(6)*. When comparing the bactericidal properties of chlorine dioxide to chlorine (HOCl), it was found that 5 ppm chlorine dioxide was equally as effective as 34 ppm chlorine: showing nearly a seven times stronger effect *(45)*. Additionally, chlorine dioxide has a higher solubility, a lower response time, is effective at a broad pH range, and does not react with ammonia to create chloramines *(14)*. An aqueous sanitizer will have low penetration ability and thus low effectiveness at pathogen inactivation, and to overcome this disadvantage a gaseous form of chlorine dioxide can be used *(42)*.

Chlorine dioxide gas has shown effectiveness for reducing *E. coli* O157:H7, *S. enterica* ser. Typhimurium, and *Listeria monocytogenes* on lettuce leaves. A chlorine dioxide gas generating sachet was placed in a treatment chamber with a lettuce sample and 10 mL of water to maintain high humidity, though the exact humidity was not reported *(42)*. The amount of chlorine dioxide gas produced in this system was 1.45, 2.26, and 2.93 ppm after 30 minutes, 1 hour, and 3 hours, respectively *(42)*. After an exposure time of 3 hours, *E. coli* O157:H7 showed a log reduction of 6.9 log colony forming units per gram (CFU/g) *(42)*. A 5.3 log CFU/g reduction of *S. enterica* ser. *Typhimurium* was achieved after 1 hour of treatment time and a 5.0 log CFU/g reduction of *L. monocytogenes* was observed after only 30 minutes of treatment time *(42)*. Treated samples and untreated controls were placed in sterile zip lock bags and stored at 4 °C for 18 days to determine if there is a visual effect of chlorine dioxide gas on lettuce quality

(42). No visible quality difference between untreated control lettuce leaves and leaves that had undergone chlorine dioxide treatment was observed *(42)*.

Chlorine dioxide gas fumigation is also effective at inactivating pathogens on washed carrots. After using an ultrasonic nebulizer to create 100, 200, 400, and 400 ppm of chlorine dioxide gas, inoculated carrot samples were placed in the treatment chamber for contact times of 5 minutes, 10 minutes, and 30 minutes *(14)*. A correlation between treatment time, concentration of chlorine dioxide gas and the bacterial reduction of *S. enterica* ser. Typhimurium, *E. coli* O157:H7, and *L. monocytogenes* was observed. The most effective treatment was 400 ppm chlorine dioxide gas with a contact time of 30 minutes, resulting in a log reduction of 2.3, 2.4, and 2.1 log for *S. enterica* ser. Typhimurium, *E. coli* O157:H7, and *L. monocytogenes*, respectively *(14)*.

Higher gas concentration, longer contact time, and higher relative humidity increased the effectiveness of chlorine dioxide gas at inactivating pathogens on black peppercorn. 1200g of whole black peppercorn inoculated with a cocktail containing 7-8 log CFU/g of *E. coli* O157:H7, *L. monocytogenes,* and *S. enterica* were treated with 290 ppm ClO_2 r over 2.5 hours *(56)*. Gas treatment on black peppercorn achieved max log reduction of 2.7, 2.3, and 2.3 for *E. coli* O157:H7, *S. enterica,* and *L. monocytogenes*, respectively *(56)*. However, increasing the exposure of chlorine dioxide increases the log reduction of pathogens. With an exposure of 8039 ppm ClO_2 per hour, this resulted in increased log CFU/g reductions to 7.2, 6.0, and 7.3 for *E. coli* O157:H7, *S. enterica,* and *L. monocytogenes*, respectively *(13)*. For *S. enterica*, higher relative humidity and contact time increased reduction on whole black peppercorns inoculated with a *S. enterica* cocktail consisting of *S. enterica* ser. Agona, *S. enterica* ser. Montevideo, *S. enterica* ser.

Mbandaka, *S. enterica* ser. Tennessee, and *S. enterica* ser. Reading *(95)*. At 60%, 70%, and 80% RH, whole black peppercorns treated with 5 ppm ClO_2 for 300 minutes had 1.5, 2.5, and 3.4 log CFU/g reductions of *S. enterica*, respectively *(95)*. At the same relative humidity, increases in gas concentration corresponded to an increase in *S. enterica* inactivation, and a 5-log reduction of *S. enterica* was only achieved in both spice types at the most extreme condition of treatment: 80% RH, 15 ppm ClO_2, and 300 minutes treatment time *(95)*.

There are some hinderances to the use of chlorine dioxide gas in food processing. Chlorine dioxide in its gaseous state cannot be transported or stored due to the explosive nature of its radical forms *(14)*. Also, in order to generate chlorine dioxide gas a sophisticated apparatus operated by a trained technician is required *(14)*. In order to have practical applications in the food industry, a sanitizing method must be simple and inexpensive *(42)*.

Hydrogen Peroxide Gas Treatment of Foods

Hydrogen peroxide is a potent oxidant being evaluated for use in fumigation treatment of foods. It has been used in the past as a biocide for disinfection, sterilization, and antisepsis *(46)*. This compound must be able to produce radicals that attack the essential components of the microbial cell, including cell membranes and deoxyribonucleic Acid (DNA) *(46)*. Possible radical variants include the superoxide anion ($O_2\bullet^-$), peroxide ($O_2\bullet^{-2}$), and hydroxyl radical (•OH), each possessing an unpaired electron making them highly reactive *(12)*. Hydroxyl radical damage to DNA includes strand breaks, damaged bases, and fragmentation. This kind of damage if not repaired, prevents DNA replication and transcription as DNA polymerase and ribonucleic acid

(RNA) polymerase are blocked by the DNA lesions *(21)*. Bacterial cells have options to repair this damage to improve survival, including chaperones to refold unfolded proteins, and special enzymatic systems to repair compromised amino acids *(62)*.

However, the presence of catalases and peroxidases in microorganisms can increase their tolerances to hydrogen peroxide as these compounds break down hydrogen peroxide into harmless water and carbon dioxide intracellularly *(46)*. Analysis of the *S. enterica* ser. Typhimurium genome showed five genes encoding enzymes that would degrade hydrogen peroxide: the alkyl hydroperoxide reductases AhpC and TsaA, and the three catalases KatE, KatG, and KatN *(30)*. A mutant that did not possess these reductases and catalases was unable to survive or proliferate in macrophages generating hydrogen peroxide *(30)*. The presence of multiple gene types allows *S. enterica* to survive under the conditions of a macrophage *(30)* Exposure to 1 mM hydrogen peroxide did not affect the plating recovery of a wild type strain of *S. enterica* at two hours after exposure *(30)*. Considering this information, it is likely that hydrogen peroxide treatments of foods will require higher concentrations with longer contact times to achieve a targeted 5 log reduction.

A gaseous form of hydrogen peroxide would be used over the aqueous form because, as shown with chlorine dioxide, the gaseous form would have better penetration of the surface crevices of the product within a container than the aqueous form and enhance pathogen inactivation within crevices of foods *(42)*. Hydrogen peroxide gas can be seen as a less harmful to human health alternative to other chemical fumigants like chlorine dioxide gas or ethylene oxide gas because hydrogen peroxide breaks down into water and oxygen *(16)*.

Hydrogen peroxide fumigation used on lettuce samples successfully inactivated the pathogens *S. enterica* ser. Typhimurium, *E. coli* O157:H7, and *L. monocytogenes*. Hydrogen peroxide was mixed with sterile distilled water on a vol/vol basis to make 1%, 3%, 5% and 10% solutions *(7)*. These aqueous solutions were then aerosolized in a hydrogen peroxide vapor generator and used to treat the inoculated samples for contact times of 2, 4, 6, 8, and 10 minutes *(7)*. The treatment with the most effectiveness was 10% hydrogen peroxide vapor with a contact time of 10 minutes, resulting in reduction of 3.12, 3.15, and 2.95 log CFU/g for *S. enterica* ser. Typhimurium, *E. coli* O157:H7, and *L. monocytogenes*, respectively *(7)*. Additionally, treatment with hydrogen peroxide gas did not significantly ($P > 0.05$) affect the quality (color and texture) of the treated lettuce even after 7 days of storage *(7)*.

CHAPTER 2: COMPARISON OF THE EFFECT OF DIFFERENT MEDIA ON THE RECOVERY OF *SALMONELLA ENTERICA* AND *ENTEROCOCCUS FAECIUM* NRRL B2354 FROM WHOLE BLACK PEPPERCORNS, BASIL LEAVES, AND CHIA SEEDS

Introduction

Spices have been an important part of cuisine throughout history, often used to increase the flavor of foods. The Food and Drug Administration classifies spices as ready to eat products. This is important, as it means that there is no pathogen inactivating cook step on the consumer side. Many outbreaks of Salmonellosis have been linked to contaminated spices. A 2017 outbreak of *Salmonella enterica* occurred in Germany, Luxembourg, Greece, and the Czech Republic which resulted in 40 cases *(28)*. Another major outbreak occurred in Sweden, where an outbreak of *S. enterica* ser. *Enteritidis* from a dried spice mix resulted in 174 cases *(33)*. From 2010 to 2018 there have been at least 6 major outbreaks related to *S. enterica* contaminated spices *(28)*. There have been at least 4 recalls of spice products in the period from 2017 to 2021 involving multiple different companies *(79, 83-85)*. The most recent of these involved the spice manufacturer McCormick, which recalled 3 different blended spice products that had been distributed to 32 different states *(67)*.

The Food Safety Modernization Act Preventive Controls for Human Foods rule mandates that spice manufacturers must implement pathogen interventions on these ready to eat products *(82)*. Some such strategies include irradiation, steam treatment, or chemical fumigation *(4)*. The most common chemical approved for spice fumigation is ethylene oxide, but it is hazardous to human health in certain concentrations. The Environmental Protection Agency (EPA) regulates the use of ethylene oxide *(76)*. The EPA maintains strict guidelines on the use of ethylene oxide, including limiting residue

tolerances to less than 7 ppm ethylene oxide and 340 ppm of ethylene chlorohydrin, a byproduct of ethylene oxide use *(76)*. Dried basil cannot be treated with ethylene oxide because treatment forms levels of ethylene chlorohydrin that are far beyond acceptable levels *(27)*. The use of ethylene oxide is also banned in Europe for health reasons *(60)*. Newer chemical compounds, like chlorine dioxide and hydrogen peroxide, are being investigated for use to replace ethylene oxide, thus it is important these treatments are validated to be effective at inactivating *S. enterica*.

Chlorine dioxide (ClO_2) is an oxidizing compound that has been used to sterilize medical equipment traditionally *(3)*. It is also currently approved for aqueous use on fresh produce *(42)*. It will not react with nitrogen to make chloramines *(53)*. Gaseous ClO_2 is being evaluated as it will not add moisture to low a_w foods and has increased penetration ability through the rough surface of the spice product itself, compared to aqueous ClO_2 *(53, 93)*. Hydrogen peroxide is another oxidizing compound used as a biocide for disinfection and sterilization *(46)*. H_2O_2 is safer than ethylene oxide or ClO_2, as it breaks down into oxygen and water *(16)*. Use of this compound also has minimal effect on the sensory characteristics of a food *(7)*.

A cell is sub-lethally injured when that cell is exposed to a chemical or physical stress and that damage does not kill the microorganism *(96, 97)*. A lethal stress may kill some cells but not all, and a moderate stress may leave a range of healthy to injured to dead cells *(96)*. These cells that are sub-lethally injured suffer a loss of cell function that may be temporary, and frequently undergo a stress adaptation *(96)*. A metabolic injury causes the inability of cells to grow on minimal media *(96)*. While a structural injury would mean that the cell is unable to grow and survive on recovery media containing

selective agents, like sodium deoxycholate *(96)*. Injury like this can be repaired under the right conditions and given ample time *(97)*. Selective agents in media may inhibit the ability for sub-lethally injured cells to repair *(97)*. Sub-lethally injured cells may cause inaccurate results when enumerating on recovery media. The FDA's Bacterial Analytical Manual (BAM) recommends the use of selective media when enumerating *S. enterica (5)*. A processor using these media types may be unable to accurately account for sub-lethally injured *S. enterica* cells in their process validation because selective media might not recover them all.

In this study *S. enterica* and *Enterococcus faecium* NRRL B2354 inoculated black peppercorn, dried basil leaves, and chia seeds were treated with chlorine dioxide or hydrogen peroxide. Different recovery media and supplements were also evaluated to compare their recovery of sub-lethally injured cells. Recovery of *S. enterica* was compared with *E. faecium* NRRL B2354 to determine the latter's suitability as a surrogate organism.

Materials and Methods

Spice inoculation and processing

Collaborators at the University of Nebraska-Lincoln used five different strains of *S. enterica* (*S. enterica* Agona 447967, *S. enterica* Reading Moff 180418, *S. enterica* Tennessee K4643, *S. enterica* Montevideo 488275, *S. enterica* Mbandaka 698538) to make a cocktail used to inoculate spice samples. These strains were chosen by because of their implications in low moisture food outbreaks or for their high thermal

resistance properties. The procedure used for inoculation of black peppercorns, basil leaves, and chia seeds is described by *(94)*. *(90)*, and *(40)* respectively.

S. enterica Agona 447967, *S. enterica* Montevideo 488275 and *S. enterica* Mbandaka 698538 were obtained from the United States FDA, Office of Regulatory Affairs (FDA, ORA) Regional Laboratory in Jefferson, AR. The strains have been linked with foodborne outbreaks caused by toasted oats cereal *(69)*, black and red pepper *(71)*, and sprouts *(32)*, respectively. *S. enterica* Reading Moff 180418 (FDA Culture Collection, Bedford Park, IL) has been identified by the FDA as being associated with cumin *(78)*. *S. enterica* Tennessee K4643 was obtained from the University of Georgia, Athens and has been linked with a peanut butter recall *(70)*.

E. faecium NRRL B2354 was evaluated as a potential non-pathogenic surrogate for *S. enterica* cocktail, as the microorganism showed suitability as a surrogate for *S. enterica* with similar treatments, and was obtained from the United States Department of Agriculture, Agricultural Research Service (USDA, ARS; Peoria, IL). Inoculum preparation was done at the University of Nebraska-Lincoln and was performed by Surabhi Wason. Strains were grown overnight individually in trypticase soy broth with added 0.6% (w/w) yeast extract then spread plated on tryptic soy agar with added 0.6% (w/w) yeast extract and incubated at 37˚ C for 24 hours. Then the bacterial lawns were harvested using 3 mL of 0.1% (w/w) buffered peptone water and a *S. enterica* cocktail was produced by mixing in equal proportions. This procedure was repeated for the preparation of *E. faecium* NRRL B2354.

Inoculation of Samples

Inoculation and treatment of low moisture foods (LMF) were performed at the University of Nebraska-Lincoln by Surabhi Wason. Black peppercorns and dried basil leaves originating from three different lots were obtained pre-sterilized from McCormick Inc. (Baltimore, MD). A mix of black and white chia seeds (Organic chia seeds, BetterBody foods, Utah, USA) originating from different lots were purchased. Upon receival of spice samples, black peppercorns were refrigerated at 4˚ C and dried basil leaves and chia seeds were held at ambient conditions until further use. Initial water activity of LMF samples was confirmed using a dew point water activity meter (Model: 4TE, Meter Group; 25°C). Moisture content of LMF samples was measured using a halogen moisture analyzer (Model: HR73, Mettler Toledo).

To inoculate LMF samples, 300g of a given spice, herb, or seed was packed into a plastic bag individually. Prepared *S. enterica* cocktail or *E. faecium* inoculum were sprayed over a thin layer of LMF sample in the bag. Bags containing inoculated LMF samples were hand massaged for 5 minutes and then hand shaken for 5 minutes to ensure uniformity of bacterial inoculum. All LMF samples were then spread over sanitized stainless-steel trays and held in a custom designed relative humidity equilibrium chamber *(41)*. To equilibrate samples to their native water activities, relative humidity was set to 55% for black peppercorn and dried basil leaves and set to 53% for chia seeds.

Chlorine Dioxide Treatment

LMF samples were subjected to chlorine dioxide treatment as described by *(90)* at the University of Nebraska. A Minidox-M system (ClorDisys) was used to generate chlorine dioxide gas, monitor and maintain concentration, and maintain RH throughout treatment. A polypropylene chamber with dimensions (L x W x H = 0.73 x 0.44 x 0.68

m) was obtained from ClorDiSys Solutions, Inc. (Branchburg, NJ). A temperature and RH inducer (Model 6621, Testo, Titisee-Neustadt, Germany) was installed on the chamber to monitor temperature and RH. Humid air was added to the chamber via pipe using an ultrasonic humidifier (EE-5301, Crane, Itasca, IL, USA). Two fans (38HX82; Grainger, China) were placed at opposite ends of the chamber to circulate chlorine dioxide gas.

The chlorine dioxide gas sterilization process involves a pre-condition phase, a conditioning phase, a charging phase, an exposure phase and an aeration phase. During pre-conditioning, target relative humidity is achieved. The conditioning phase involves holding this target RH for a desired amount of time. Chlorine dioxide gas is generated in the charging phase by passing chlorine gas through sodium chlorite cartridges to a set level before being introduced to the chamber. Gas concentration is held throughout the exposure phase. The final phase removes chlorine dioxide gas via an aeration cycle before the chamber door will unlock for safety.

Two g of inoculated LMF sample were placed in the chamber packed in heat sealed paper bags. Black peppercorn samples were weighed and placed into a petri dish for treatment with chlorine dioxide gas. Treatment was conducted with 70% RH and a gas concentration of 10 mg/L with a 2-hour exposure. Following aeration, LMF samples were removed from the chamber, packed up, and sent to Virginia Tech for enumeration.

Hydrogen Peroxide Treatment

Hydrogen peroxide treatment of spices and seeds were performed at the University of Nebraska. A treatment chamber made of polystyrene with the dimensions

(L x W x H = 0.35 X 0.30 X 0.27 m) was fitted with a vaporized hydrogen peroxide (VHP) generator (Bioquell L-2, Bioquell UK Ltd). A closed loop was made by attaching the inlet and outlet hoses of the generator to opposite sides of the chamber with a camlock connection. Hydrogen peroxide gas sterilization process has a conditioning phase, a gassing phase, a dwelling phase, and an aeration phase. In the conditioning phase, temperature an RH are conditioned to stable target values. After a pre-set conditioning time, the gassing phase begins. Liquid hydrogen peroxide is pumped at a set rate onto a hot surface, producing VHP. VHP is circulated with the airstream into and out of the treatment chamber continuously by the supply hoses for a set amount of time. A dwell phase of no injection of VHP was set to increase residence time of VHP in the chamber. Finally, filtered air was circulated in the chamber for at least 1 hour to remove VHP in the aeration phase. Following this, the chamber could be safely opened to retrieve samples.

Treatment was conducted with an injection rate of 3 g/min and an air flow rate of 10 m^3/h at a temperature of 40˚C. Dried basil leaf samples were exposed for 5 minutes with a dwell time of 30 minutes. Following removal from treatment chamber, dried basil leaf samples were packed up and shipped to Virginia Tech for enumeration.

Media Preparation

The selective media Xylose Lysine Deoxycholate (XLD) (Becton Dickinson, Franklin Lakes, NJ) was prepared per the manufacturer's instruction. The nutrient supplement media designated MTSAYE was created by combining Tryptic Soy Agar (TSA) (Becton Dickinson, Franklin Lakes, NJ) powder (40 g/L), with 6g/L of yeast extract (Remel Inc, San Diego, CA), 0.75g/L of ammonium iron citrate (Sigma-Aldrich,

St. Louis, MO), and 0.3g/L of sodium thiosulfate (Fisher Scientific, Kansas City, MO) in 1L of deionized water. This solution was then brought to a boil before being autoclaved at 121˚C for 15 minutes. The solution was then poured (20 mL) into petri dishes and allowed to solidify.

MTSAYE-NP media was made using Tryptic Soy Agar (TSA) (BD) powder (40 g/L), with 6g/L of yeast extract (Remel Inc, San Diego, CA), 0.75g/L of ammonium iron citrate (Sigma-Aldrich, St. Louis, MO), and 0.3g/L of sodium thiosulfate (Fisher Scientific, Kansas City, MO), and 1g/L sodium pyruvate (Fisher Scientific, Kansas City, MO) then mixed in 1L of deionized water before boiling and autoclaving.

MTSAYE-FS media was made using Tryptic Soy Agar (TSA) (BD) powder (40 g/L), with 6g/L of yeast extract (Remel Inc, San Diego, CA), 0.75g/L of ammonium iron citrate (Sigma-Aldrich, St. Louis, MO), and 0.3g/L of sodium thiosulfate (Fisher Scientific, Kansas City, MO), and 1g/L ferrous sulfate (Fisher Scientific, Kansas City, MO).

MTSAYE-TDP media was made using Tryptic Soy Agar (TSA) (BD) powder (40 g/L), with 6g/L of yeast extract (Remel Inc, San Diego, CA), 0.75g/L of ammonium iron citrate (Sigma-Aldrich, St. Louis, MO), and 0.3g/L of sodium thiosulfate (Fisher Scientific, Kansas City, MO), and 1g/L of 3’3’- thiodipropionate (Acros Organics, Carlsbad, CA). All media plates were prepared ahead of time and stored at 4˚C before use. All plates were returned to room temperature and any condensate allowed to evaporate before plating.

Enumeration of *S. enterica* and *E. faecium* NRRL B2354 from Spice Samples

Upon receival of spices from University of Nebraska Lincoln, 5g of spice sample would be put into a stomacher bag containing 45 mL of neutralizing buffer for black peppercorn and basil leaf samples, and 145 mL of neutralizing buffer for chia seed samples. The spice was stomached for 1 minute at a speed setting of 1 in a lab blender (Bagmixer 400, Interscience, Guelph, Ontario). The liquid diluent for all samples except chia seed samples were then vacuum filtered through #4 qualitative paper to remove spice particles. The filtrate was then serially diluted in sterile neutralizing buffer out to a 10^{-6} dilution, then enumerated by plating onto MTSAYE, MTSAYE-NP, MTSAYE-FS, MTSAYE-TDP, and XLD (Becton Dickinson, Franklin Lakes, NJ), in duplicate on final plate dilutions ranging from 10^{-1} through 10^{-7}. XLD media was only used for *S. enterica* inoculated samples. All plates were incubated at 37˚C for 48 hours before enumeration. The limit of detection was 1 log CFU.

Statistical Analysis

The experiment was repeated three times for each spice sample/inoculation combination on different days using freshly prepared spices. For each replicate, duplicate plating was performed, and the average reported. Bacterial counts were log transformed, and for each spice/treatment/media combination the log CFU/g of treated samples were subtracted from log CFU/g of untreated samples to obtain a log CFU/g reduction for use in statistical analysis. Analysis was performed using JMP (version 11, SAS, Cary, NC).

To compare the effect of different media types on *S. enterica* log reduction, the log reduction CFU/g values of *S. enterica* plated on MTSAYE, MTSAYE-NP, MTSAYE-FS, MTSAYE-TDP, and XLD from each spice type were compared. The log reduction values were compared within each spice type, but not between spice types. An

ANOVA was run to determine if there was a significant ($P<0.05$) difference between log reduction values and Tukey's HSD test was used to determine which log reduction values were different.

To compare the effect of different media types on *E. faecium* NRRL B2354 log reduction, the log reduction CFU/g values of *E. faecium* NRRL B2354 plated on MTSAYE, MTSAYE-NP, MTSAYE-FS, and MTSAYE-TDP from each spice type were compared. The log reduction values were compared within each spice type, but not between spice types. An ANOVA was run to determine if there was a significant ($P<0.05$) difference between log reduction values and Tukey's HSD test was used to determine which log reduction values were different.

To compare the log reductions of the two microorganisms, the log reduction CFU/g of *S. enterica* plated on MTSAYE was compared to the log reduction CFU/g of *E. faecium* NRRL B2354 plated on MTSAYE from each spice type. An ANOVA was run followed by Tukey's HSD test to determine if there was a significant ($P<0.05$) difference in the log reduction of each microorganism.

Results

Media Supplements

On whole black peppercorn samples treated with ClO_2 the average log reductions of *S. enterica* plated on each media type were 2.31, 2.21, 2.43, 2.49, and 3.43 log CFU/g from MTSAYE, MTSAYE-NP, MTSAYE-FS, MTSAYE-TDP, and XLD, respectively. On average, the log reduction of *S. enterica* was 1 log greater when plated on XLD compared to the non-selective media types (figure 1). This indicates that the recovery of

S. enterica on XLD was reduced in comparison to MTSAYE-NP (P=0.04). Average log reductions of *E. faecium* NRRL B2354 plated on different media were 2.18, 2.00, 2.16, and 2.23 log CFU/g from MTSAYE, MTSAYE-NP, MTSAYE-FS, and MTSAYE-TDP, respectively. These log reduction values were not significantly different (P=0.18) from each other regardless of media type (figure 2).

On basil leaves samples treated with ClO_2, the average log reduction of *S. enterica* was 2.16, 2.07, 2.31, 2.43, and 2.88 log CFU/g from MTSAYE, MTSAYE-NP, MTSAYE-FS, MTSAYE-TDP, and XLD, respectively. There was a significant difference (P=0.01) between the log reduction of *S. enterica* on XLD and the non-selective media MTSAYE-NP, as well as between XLD and MTSAYE (Figure 3). On average, the log reduction of *S. enterica* was 0.5-0.6 log greater when plated on XLD compared to the non-selective media types. The average log reduction of *E. faecium* NRRL B2354 plated from different media were 1.63, 1.72, 1.79, and 1.97 for MTSAYE, MTSAYE-NP, MTSAYE-FS, and MTSAYE-TDP, respectively. These log reduction values showed no significant difference (P=0.82) from each other (figure 4).

For chia seeds samples treated with ClO_2 the average log reduction of *S. enterica* was 2.03, 2.07, 2.00, 2.12, and 2.53 log CFU/g for MTSAYE, MTSAYE-NP, MTSAYE-FS, MTSAYE-TDP, and XLD, respectively. There was not a significant difference in the log reduction of *S. enterica* (P=0.41) between the media types. The average log reduction of *E. faecium* NRRL B2354 was 1.98, 1.96, 2.02, and 2.17 log CFU/g from MTSAYE, MTSAYE-NP, MTSAYE-FS, and MTSAYE-TDP, respectively. There was no significant difference (P=0.96) between the log reduction on any of the media types (figure 5 & 6).

The results of H_2O_2 treated basil leaves, showed an average log reduction of *S. enterica* from different media of 0.44, 0.50, 0.42, 0.45, and 1.01 log CFU/g from MTSAYE, MTSAYE-NP, MTSAYE-FS, MTSAYE-TDP, and XLD, respectively. There was a significant difference between the log reduction of *S. enterica* on XLD and all non-selective media tested (P=0.01). On average, the log reduction of *S. enterica* was 0.5 log greater when plated on XLD compared to the non-selective media types (figure 7). The average log reduction of *E. faecium* NRRL B2354 was 0.62, 0.63, 0.61, and 0.70 log CFU/g from MTSAYE, MTSAYE-NP, MTSAYE-FS, and MTSAYE-TDP, respectively. There was no significant difference (P=0.98) between the log reduction on any of the different media types tested (figure 8).

S. enterica vs *E. faecium* NRRL B2354 recovery

For ClO_2 treated black peppercorns, the average log reduction of *S. enterica* plated on MTSAYE was 2.31 log CFU/g and *E. faecium* NRRL B2354 plated on MTSAYE was 2.18 log CFU/g. For ClO_2 treated basil leaves, the average log reduction of *S. enterica* plated on MTSAYE was 2.15 log CFU/g and *E. faecium* NRRL B2354 plated on MTSAYE was 1.63 log CFU/g. For ClO_2 treated chia seeds, the average log reduction of *S. enterica* plated on MTSAYE was 2.03 log CFU/g and *E. faecium* NRRL B2354 plated on MTSAYE was 1.98 log CFU/g. For H_2O_2 treated basil leaves, the average log reduction of *S. enterica* plated on MTSAYE was 0.44 log CFU/g and *E. faecium* NRRL B2354 plated on MTSAYE was 0.62 log CFU/g. The results of the analysis showed that the log reductions of both microorganisms on ClO_2 treated black peppercorn, basil leaves, chia seeds, and H_2O_2 treated basil leaves were not significantly different (P = 0.67, 0.11, 0.91, and 0.41 respectively).

Discussion

Antimicrobial gas residuals on the spices could inhibit the ability of injured *S. enterica* cells to repair and grow. Addition of antioxidants such as 3'3'-thiodipropionate, sodium pyruvate, and ferrous sulfate, which could scavenge oxidative compounds and may prevent further stress to the cells *(25)*. Sodium pyruvate and ferrous sulfate additionally provide glucose and iron respectively for the injured cells to use. Iron availability has been shown to increase *S. enterica* growth potential *(38)*.

These particular supplements were selected because they individually proved effective at increasing recovery of heat injured *S. enterica* from egg albumen *(29)*. TSA alone recovered an average of 5.27 log CFU/mL *(29)*. The addition of 1.0g/L ferrous sulfate increased recovery to an average 5.82 log CFU/mL *(29)*. Adding 1.0g/L 3'3'-thiodipropionate increased recovery to an average 5.67 log CFU/mL and adding 1.0g/L of sodium pyruvate increased recovery to 5.53 log CFU/mL *(29)*. The addition of these compounds individually to TSA significantly increased ($P<0.05$) recovery of *S. enterica* from egg albumin compared to plating on TSA alone *(29)*. The addition of these individual compounds did not cause much of a significant difference in the recovery *S. enterica* compared to each other, only the recovery of sodium pyruvate supplemented TSA and the recovery of ferrous sulfate supplemented TSA had a significant difference ($P<0.05$) *(29)*. This is similar to the results seen in this study, where the media with antioxidant supplements were comparable in log reduction. However, in this study the average log reduction values of *S. enterica* plated on MTSAYE were comparable to the other supplemented, non-selective media which was not the case with recovery from heat treated egg albumin. Recovery there was 5.39 log CFU/mL and was significantly

($P<0.05$) less than the recovery of *S. enterica* from TSA supplemented with the other antioxidants *(29)*. This discrepancy could be because yeast extract shows antioxidant properties *(66)*. The yeast extract would work to neutralize residual chlorine dioxide or hydrogen peroxide on the treated product and reduce stress on injured cells so they can repair easier.

Sodium thiosulfate and ammonium iron citrate are included in the MTSAYE formula to make the resulting media differential for *S. enterica*. The microorganism breaks down thiosulfate into sulfite and H_2S gas, which then the H_2S reacts with the ferric ions in ammonium iron citrate to produce a black precipitate in the center of *S. enterica* colonies *(47)*. Typically, eTSAYE, a modified TSA with esculin hydrate, is used to differentiate *E. faecium* colonies, but in this experiment that microorganism was plated on MTSAYE. Black precipitate centers still occurred in the *E. faecium* colonies, though appearing fainter than those of *S. enterica* colonies on MTSAYE.

In this experiment, an increase in the repair and recovery of sub-lethally injured cells would be facilitated by the added antioxidants. This was not the case as there was little significant difference between the log reduction of the different non-selective media types. Results for recovery of acid-treated *S. enterica* recovered from beef that also evaluated antioxidant supplements showed comparable results, with no significant difference between media types *(34)*. Perhaps the concentrations of the added supplements that was enough to improve *S. enterica* recovery on heat treated egg albumen were not enough to influence recovery on antimicrobial gas treated spices. For log reductions of *S. enterica* recovered from chia seeds, this could be explained by chia seed's properties of high antioxidant activity *(17)*. The inherent antioxidant activity in the

seed could have already neutralized oxidizer residuals, so that further antioxidant supplement becomes unnecessary. In general, this experiment found that ClO_2 treatment had one log higher reduction of *S. enterica* than H_2O_2 treatment for dried basil leaves, implying that the ClO_2 treatment is more effective.

The selective media XLD was chosen for testing as the FDA's bacterial analytical manual recommends XLD as a media choice for recovering *S. enterica* from spices *(5)*. XLD has sodium deoxycholate to inhibit gram-positive microorganisms and includes xylose and lysine which cause a pH shift induced color change when *S. enterica* ferments them *(9)*. Log reduction of *S. enterica* was consistently higher when plated on XLD for each spice and treatment combination tested. This is consistent with findings for acid-treated *S. enterica* recovered from beef *(34)*. In that study *S. enterica* inoculated beef samples were to 30 minutes of a salt and acid treatment then plated on different media with or without a 1.0g/L sodium pyruvate supplement *(34)*. The number of *S. enterica* recovered was on average 10 log CFU/g from TSA, 10.1 log CFU/g from TSA with sodium pyruvate, and 8.8 log CFU/g from XLD *(34)*. On average there was a 1.2 log CFU/g difference between the selective media and the non-selective media *(34)*, matching the average of 1.0 log CFU/g difference in recovery between selective and non-selective media in this study. Additionally, the non-selective media TSA and TSA with added sodium pyruvate showed no significant difference in recovery *(34)*, matching what was seen in this study. It is possible that the selective nature of XLD inhibits the repair and growth of sub-lethally injured *S. enterica*.

In this study, standard deviations for log reductions of *S. enterica* plated on XLD were higher compared to the standard deviations of the log reduction of *S. enterica* plated

on the non-selective media types. Considering that the same serial dilutions were used and the two medias were inoculated at the same time it seems likely that the greater variability in the recovery was due to presence of damaged cells that were unable to grow on the XLD media. When treated with the antimicrobial gas, there is variability in how many cells become sub-lethally injured. Since XLD has a lower capacity to recover sub-lethally injured cells, the variability of log reduction of *S. enterica* plated on XLD can be explained by the difference in ratio of healthy cells to sub-lethally injured cells because of treatment. The existence of such variability may be another reason to avoid the use of XLD when sub-lethally injured cells are expected.

There have been studies indicating that *E. faecium* NRRL B2354 has been considered a suitable surrogate for *S. enterica* for other foods subjected to different inactivation treatments. However, due to differences in food product and treatment processes it is important to evaluate surrogate capability on a case-by-case basis *(75)*. For example, in the case of ethylene oxide treated cumin seeds, *E. faecium* NRRL B2354 showed higher log reduction values than *S. enterica*, indicating that *E. faecium* NRRL B2354 would not be a good surrogate for this process *(49)*. Whereas in radio frequency heating treatment of *S. enterica* and *E. faecium* NRRL B2354 inoculated cumin, results show that *E. faecium* NRRL B2354 has a higher D-value, indicating that it would be suitable for use as a surrogate in this process *(51)*. Despite involving the same microorganism and spice, the fact that the treatment process is different changes the suitability of the surrogate microorganism. Surrogate organisms should be non-pathogenic substitutes for pathogenic target organisms and should be more robust than the pathogen under target conditions *(75)*. Other studies have also indicated suitability of

E. faecium NRRL B2354 to be a surrogate for heat treated black peppercorn *(88)* and ethylene oxide treated black peppercorn *(94)*.

In this experiment, there was no significant difference between the log reduction of microorganisms for any treatment/spice combination tested, with the log reduction of *E. faecium* NRRL B2354 being lower than *S. enterica* for all chlorine dioxide treatments. The results of this experiment support the capability of *E. faecium* NRRL B2354 to function as a surrogate for *S. enterica* for chlorine dioxide treated spices. This agrees with studies that have found that *E. faecium* NRRL B2354 has lower inactivation levels than *S. enterica* after chlorine dioxide treatment on inoculated black peppercorns and cumin seeds *(95)*. In that study, inactivation levels on chlorine dioxide treated black peppercorn for *S. enterica* were around 2-3 log CFU/g and for *E. faecium* NRRL B2354 around 1-2 log CFU/g *(95)*, and in this study were on average 2.31 and 2.18 log CFU/g respectively for the same treatment conditions and recovery media. The D-values of *E. faecium* NRRL B2354 are 1.2-1.9 times greater than that of *S. enterica* for chlorine dioxide treated basil leaves *(90)*, further supporting the conclusion that the microorganism is a suitable surrogate for that dried herb. There is little information evaluating *E. faecium* NRRL B2354 as a surrogate for *S. enterica* on hydrogen peroxide treated spices. In this study, there was no significant difference between the log reductions of the two microorganisms on hydrogen peroxide treated basil leaves. This indicates that the *E. faecium* NRRL B2354 would be suitable as a surrogate for these treatment conditions.

Conclusions

This research indicates that the recovery of sub-lethally injured *S. enterica* cells are inhibited by the use of XLD media during enumeration. Researchers studying the effect of antimicrobial gas fumigation should use a non-selective but differential media when enumerating for the most accurate results. While antioxidant supplement had little effect in this experiment, further research should be performed to ascertain if differing concentrations of supplements would improve recovery. Further study with different low moisture food and treatment combinations will be useful to determine if specific media formulations work better depending on the treatment type. This study also indicates that *E. faecium* NRRL B2354 is suitable as a surrogate for *S. enterica*. Though further study on its use as a surrogate for hydrogen peroxide treated spices would be encouraged. Low moisture food manufacturers using these treatment methods will benefit from being able to use the non-pathogenic surrogate in challenge studies and validation.

Figures

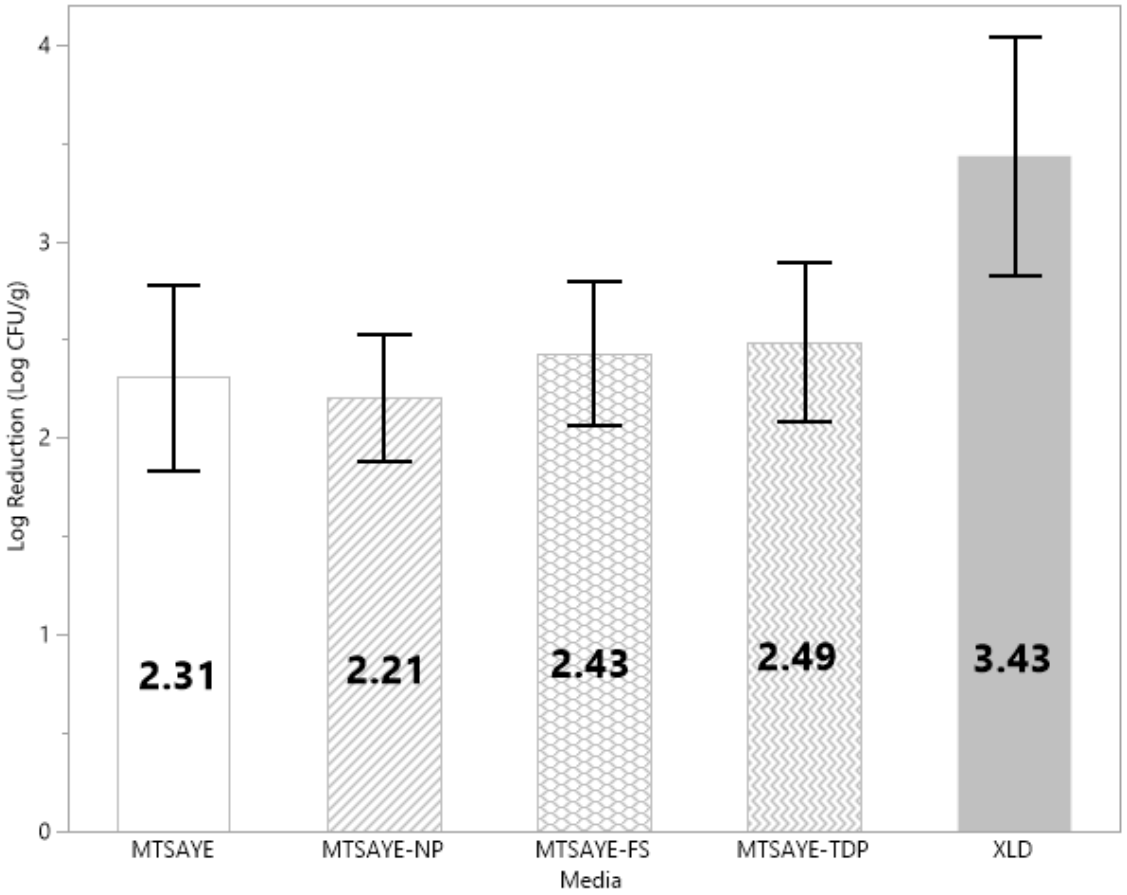

Figure 1. Comparison of the mean log reduction of *S. enterica* (CFU/g) plated on different media formulations recovered from whole black peppercorns treated with ClO_2 fumigation (n=3). Each error bar represents one standard deviation from the mean. Figure abbreviations: Modified TSA with Yeast extract (MTSAYE), MTSAYE with added Sodium Pyruvate (MTSAYE-NP), MTSAYE with added ferrous sulfate (MTSAYE-FS), MTSAYE with added 3'3'-thiodipropionate (MTSAYE-TDP), Xylose Lysine Deoxycholate (XLD)

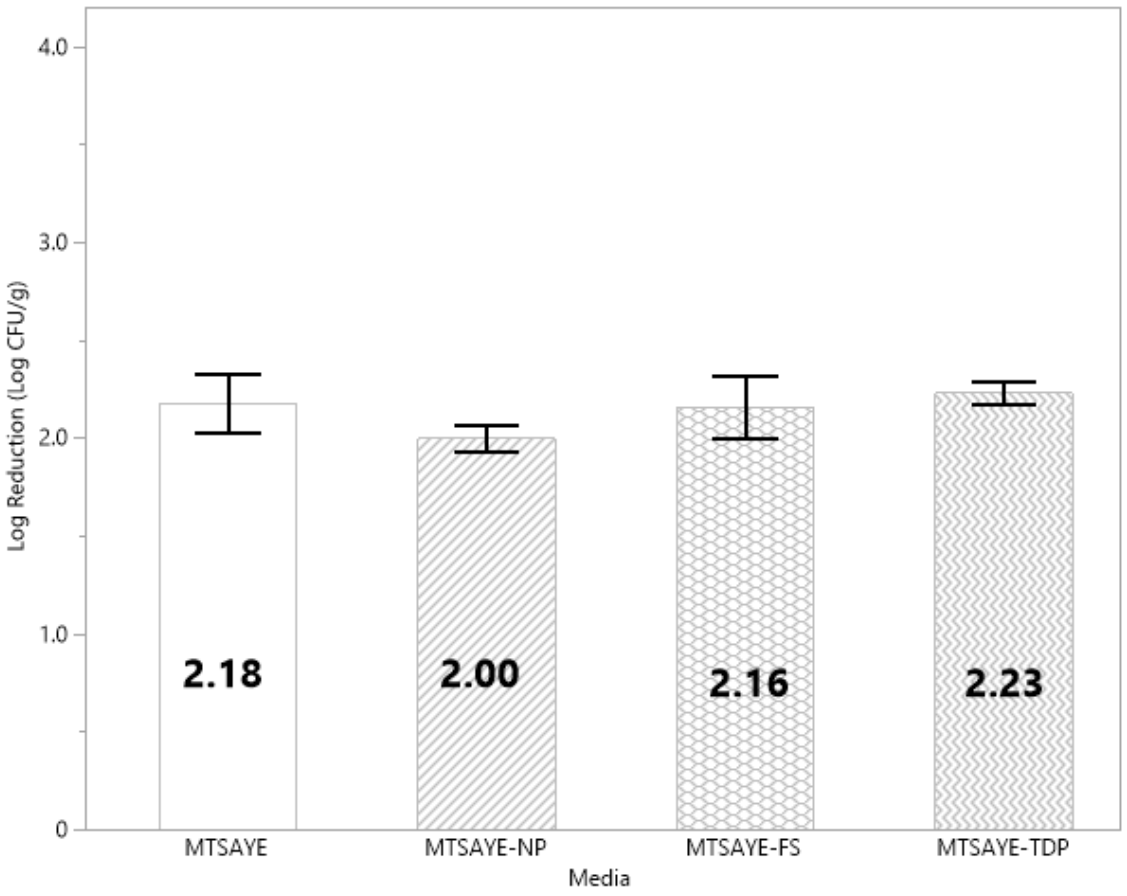

Figure 2. Comparison of the mean log reduction of *E. faecium* (CFU/g) plated on different media formulations recovered from black peppercorn treated with ClO_2 fumigation (n=3). Each error bar represents one standard deviation from the mean. Figure abbreviations: Modified TSA with Yeast extract (MTSAYE), MTSAYE with added Sodium Pyruvate (MTSAYE-NP), MTSAYE with added ferrous sulfate (MTSAYE-FS), MTSAYE with added 3'3'-thiodipropionate (MTSAYE-TDP)

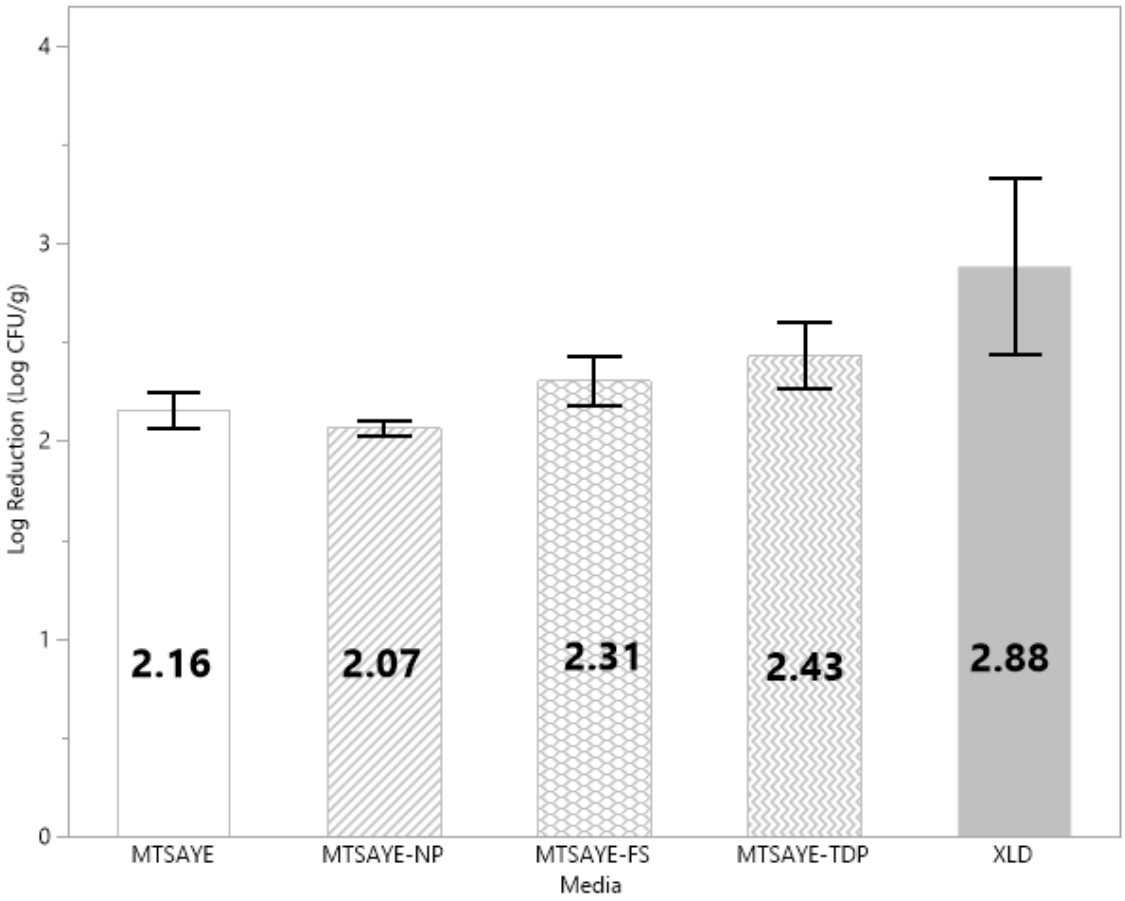

Figure 3. Comparison of the mean log reduction of *S. enterica* (CFU/g) plated on different media formulations recovered from basil leaves treated with ClO_2 fumigation (n=3). Each error bar represents one standard deviation from the mean. Figure abbreviations: Modified TSA with Yeast extract (MTSAYE), MTSAYE with added Sodium Pyruvate (MTSAYE-NP), MTSAYE with added ferrous sulfate (MTSAYE-FS), MTSAYE with added 3'3'-thiodipropionate (MTSAYE-TDP), Xylose Lysine Deoxycholate (XLD)

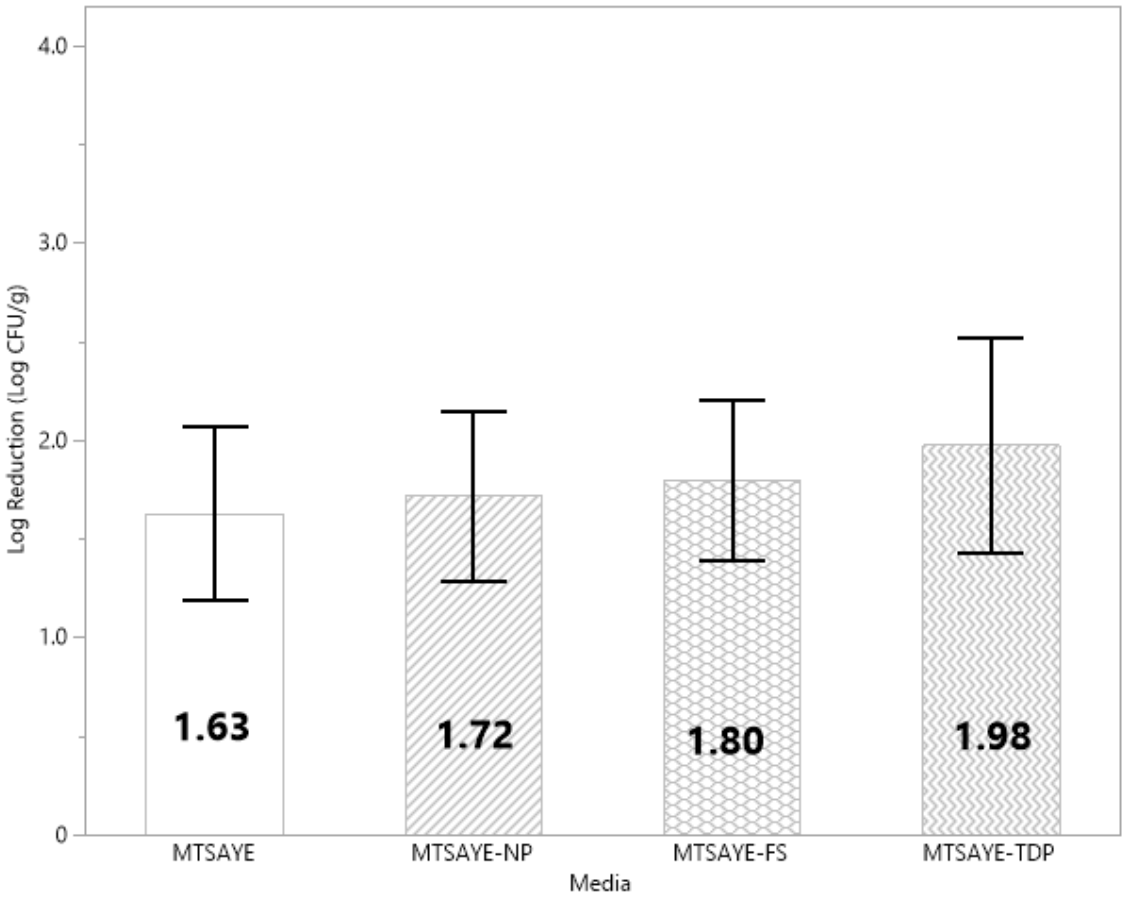

Figure 4. Comparison of the mean log reduction of *E. faecium* (CFU/g) plated on different media formulations recovered from basil leaves treated with ClO_2 fumigation (n=3). Each error bar represents one standard deviation from the mean. Figure abbreviations: Modified TSA with Yeast extract (MTSAYE), MTSAYE with added Sodium Pyruvate (MTSAYE-NP), MTSAYE with added ferrous sulfate (MTSAYE-FS), MTSAYE with added 3'3'-thiodipropionate (MTSAYE-TDP)

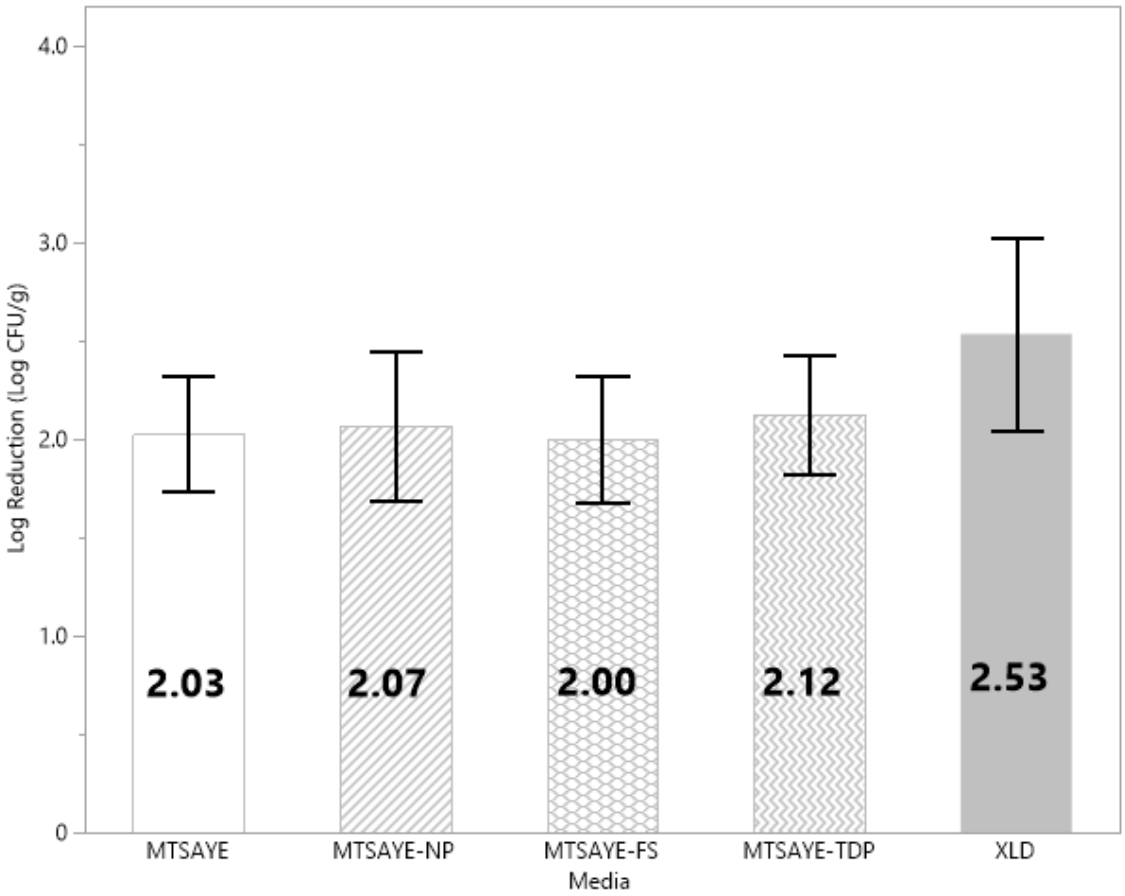

Figure 5. Comparison of the mean log reduction of *S. enterica* (CFU/g) plated on different media formulations recovered from chia seeds treated with ClO_2 fumigation (n=3). Each error bar represents one standard deviation from the mean. Figure abbreviations: Modified TSA with Yeast extract (MTSAYE), MTSAYE with added Sodium Pyruvate (MTSAYE-NP), MTSAYE with added ferrous sulfate (MTSAYE-FS), MTSAYE with added 3'3'-thiodipropionate (MTSAYE-TDP), Xylose Lysine Deoxycholate (XLD)

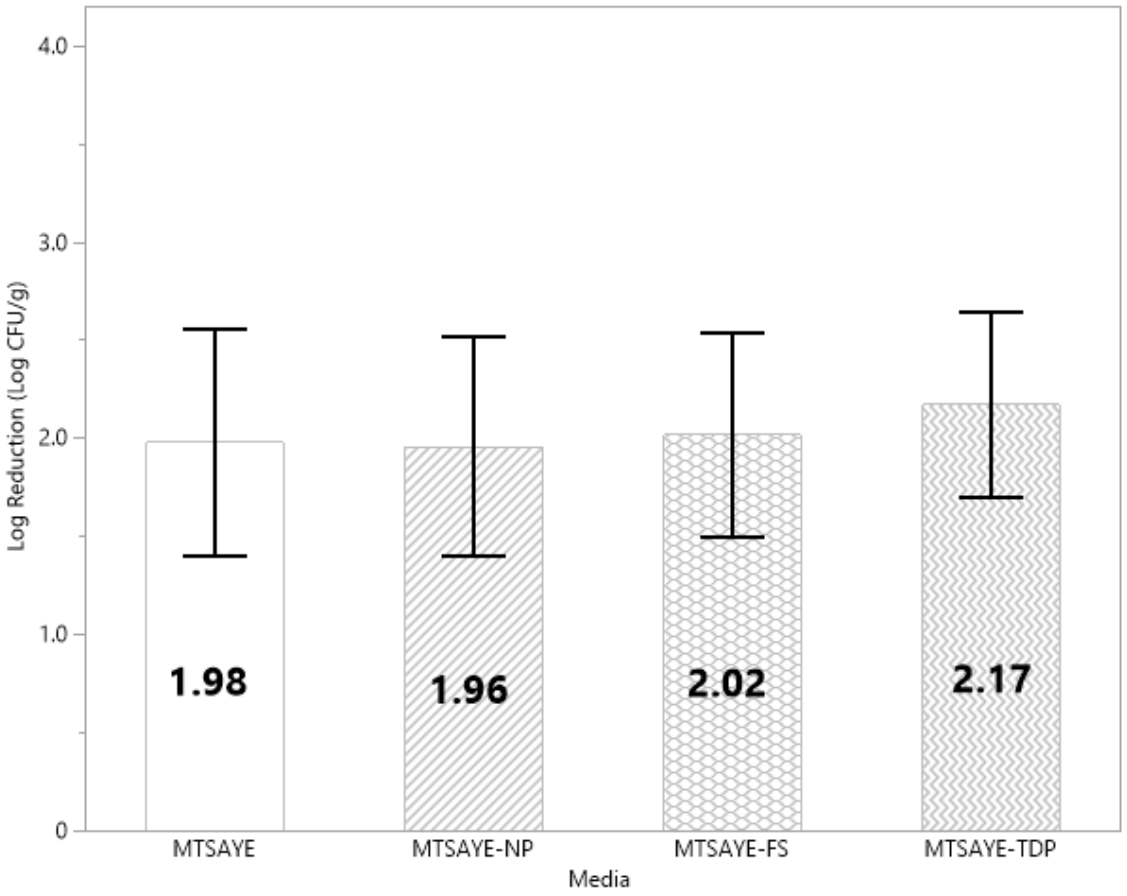

Figure 6. Comparison of the mean log reduction of *E. faecium* (CFU/g) plated on different media formulations recovered from chia seeds treated with ClO_2 fumigation (n=3). Each error bar represents one standard deviation from the mean. Figure abbreviations: Modified TSA with Yeast extract (MTSAYE), MTSAYE with added Sodium Pyruvate (MTSAYE-NP), MTSAYE with added ferrous sulfate (MTSAYE-FS), MTSAYE with added 3'3'-thiodipropionate (MTSAYE-TDP)

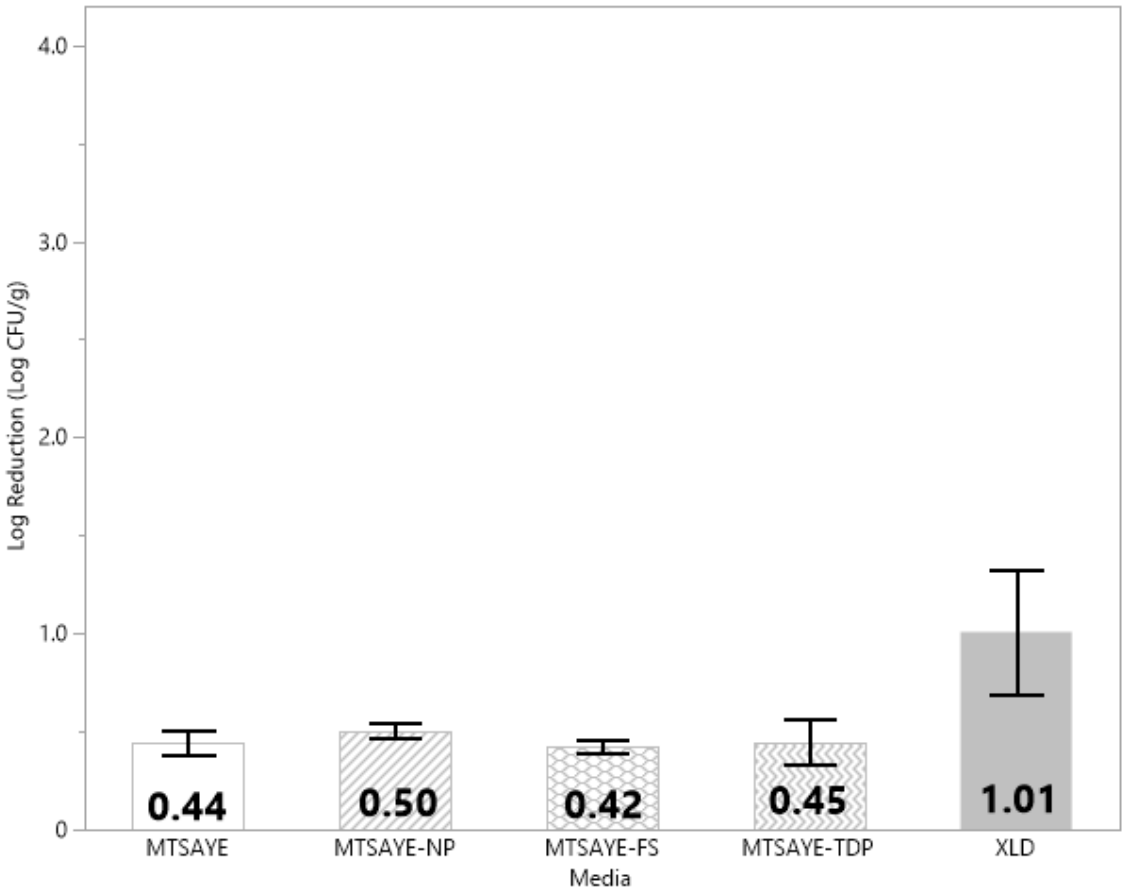

Figure 7. Comparison of the mean log reduction of *S. enterica* (CFU/g) plated on different media formulations recovered from basil leaves treated with H_2O_2 fumigation (n=3). Each error bar represents one standard deviation from the mean. Figure abbreviations: Modified TSA with Yeast extract (MTSAYE), MTSAYE with added Sodium Pyruvate (MTSAYE-NP), MTSAYE with added ferrous sulfate (MTSAYE-FS), MTSAYE with added 3'3'-thiodipropionate (MTSAYE-TDP), Xylose Lysine Deoxycholate (XLD)

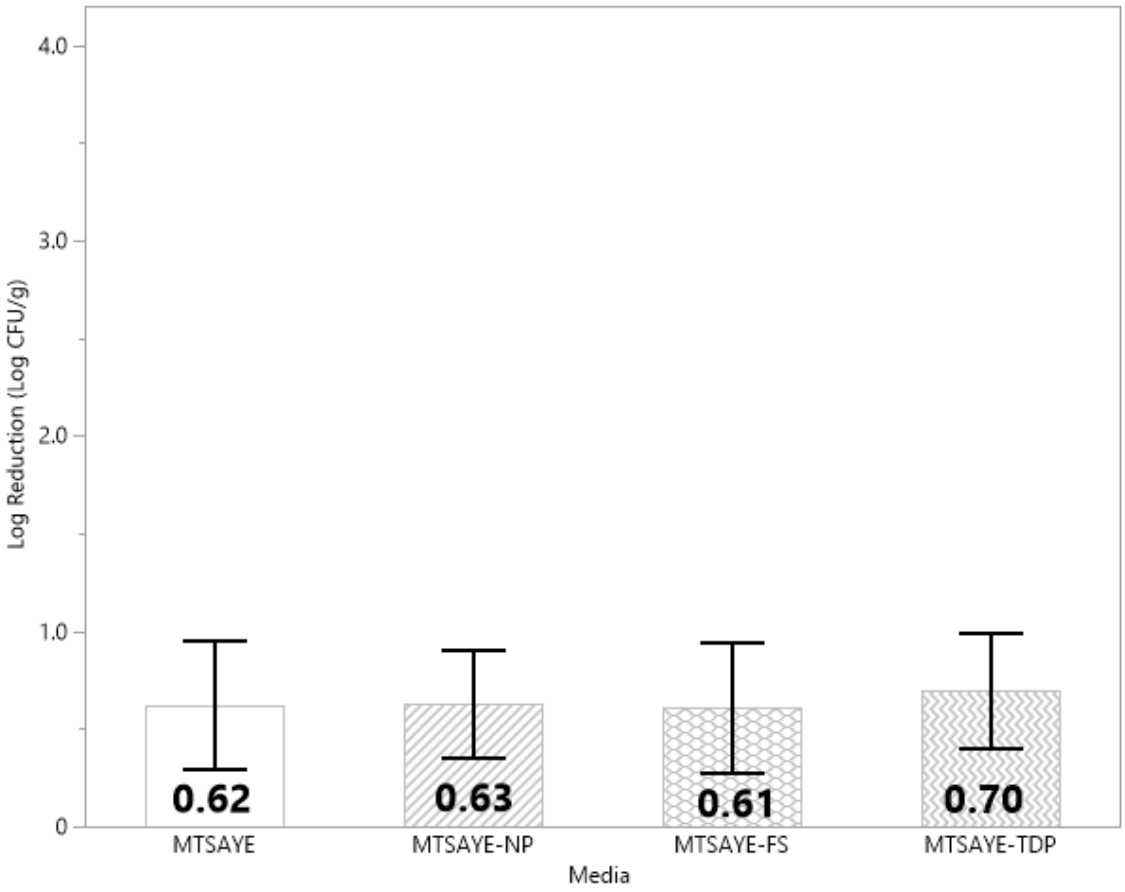

Figure 8. Comparison of the mean log reduction of *E. faecium* (CFU/g) plated on different media formulations recovered from basil leaves treated with H_2O_2 fumigation (n=3). Each error bar represents one standard deviation from the mean. Figure abbreviations: Modified TSA with Yeast extract (MTSAYE), MTSAYE with added Sodium Pyruvate (MTSAYE-NP), MTSAYE with added ferrous sulfate (MTSAYE-FS), MTSAYE with added 3'3'-thiodipropionate (MTSAYE-TDP)

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