SLOW-RELEASE CHLORINE DIOXIDE GAS TREATMENT AS A MEANS TO REDUCE *SALMONELLA* COMTAMINATION ON SPICES

by

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(Under the Direction of Mark A. Harrison)

ABSTRACT

Salmonella enterica is a major food safety issue for the spice industry. Slow-releasing chlorine dioxide (ClO_2) gas from self-contained sachets, which could be employed in a small-scale operation, was evaluated for its effectiveness in reducing *Salmonella* contamination on black pepper, cumin, and sesame seed. Three levels of chlorine dioxide gas (100, 200, or 500 mg ClO_2/kg spice) were applied to *Salmonella* inoculated spices. Spices were sampled immediately after treatment, and at 1, 10, and 30 days post-treatment. The combined effect of ClO_2 gas treatment and storage time on *Salmonella* numbers on spices was evaluated. *Salmonella* numbers on black pepper, cumin, and sesame seed samples were significantly reduced for each treatment level when compared to the control. Generally, *Salmonella* population numbers decreased over storage time.

INDEX WORDS: spices, *Salmonella*, chlorine dioxide, gaseous antimicrobial

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DEDICATION

I would like to dedicate my thesis to my mother and father. Without their constant love and support, there is no way I could have gotten to this point. There aren't two better people for me to model my life after. I want to especially thank my mom for her sharing and passing her love and passion for food down to me and being there for me at all times. My father for burdening me with his sense of humor that helps me get through good and bad times. There is nothing I enjoy more than making the two of you proud. Thank you for everything that you do for me.

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CHAPTER 1

INTRODUCTION

Salmonella enterica is a major food safety issue for the spice industry. From 1973-2010, there were 14 spice-related foodborne illness outbreaks worldwide *(109)*. *Salmonella* was the causative organism in 10 of the outbreaks and caused 87% of illnesses *(109)*. *Salmonella* is also the main organism responsible for spice-associated recalls *(113)*. Spices can become contaminated at many different points of the supply chain. *Salmonella* has shown ability to survive in low water activity environments *(3)*. Effective antimicrobial spice treatments are needed to mitigate the risk of foodborne illness *(53)*. Current spice industry processing techniques include: steam treatment, irradiation, and fumigation with ethylene oxide. While these processes can be effective at reducing numbers of *Salmonella* on spices, they can also adversely affect spice quality or, in the case of ethylene oxide, leave harmful residues on the spice after treatment *(57, 100, 106)*. These processes can also be unavailable or too costly for small-scale spice processors. A safe, inexpensive, and simple alternative would be useful for many small-scale spice processors.

Chlorine dioxide (ClO_2) has been used as an antimicrobial since the mid-1900s *(9)*. In the food and beverage industry, it has primarily been used in the disinfection of drinking water. Gaseous ClO_2 has been shown to be effective in reducing microbial loads on many different food products, including fruits and vegetables *(46)*. Research on the reduction of microbial populations in low water activity environments due to the effect of ClO_2 is still fairly limited. The U.S. Food and Drug Administration (FDA) has approved the use of aqueous ClO_2 in wash

waters for fruits and vegetables and water in poultry products, but the gaseous form has not yet been approved for use in food processing *(95)*. A major reason for lack of approval is inadequate characterization of ClO_2 residues on food products after gas treatment with gaseous ClO_2 *(95)*. Despite this lack of characterization, major ClO_2 residues, chlorite and chlorate, are not considered to be toxic *(81)*.

The current study was performed to evaluate efficacy of a slow-release ClO_2 gas system in reducing *Salmonella* numbers in spices. Black pepper, cumin, and sesame seed were chosen as representative spices for the study. ClO_2 gas was generated in self-containing sachets by the reaction of a granular porous solid impregnated with sodium chlorite and a ferric chloride activator. Three levels of ClO_2 (100, 200, and 500 mg ClO_2/kg spice) were applied to inoculated spices for 12 h and compared to a control treatment (0 mg ClO_2/kg spice). The effect of storage on *Salmonella* numbers of treated spices was also evaluated by evaluating the spices immediately after treatment and on day 1, 10, and 30 post-treatment.

CHAPTER 2

LITERATURE REVIEW

Salmonella spp.

Salmonella spp. are gram-negative, rod-shaped, motile, facultative anaerobic bacteria that are part of the *Enterobacteriaceae* family. The *Salmonella* genus contains two species, *Salmonella enterica* and *Salmonella bongori*. Each species contains many different serotypes that are differentiated by surface and flagellar antigens *(47, 62)*. In 2007, it was determined that there were 2,579 different serotypes in the *Salmonella* genus *(47)*. *S. enterica* is one of the most common causes of foodborne illness worldwide. In 2010, it was estimated that there are approximately 93.8 million cases of gastroenteritis and approximately 155,000 deaths due to *Salmonella* spp. infection worldwide annually, with approximately 80.3 million of the cases being foodborne *(65)*. About 95% of *Salmonella* infections are foodborne, resulting from non-typhoidal *S. enterica* (NTS) contamination *(68)*.

S. enterica serotypes Typhi, Sendai, and Paratyphi utilize the human body as their host and cause enteric fever, while NTS can often utilize many different types of hosts *(92)*. *Salmonella* serotypes that cause enteric fever are known as typhoidal serotypes. These serotypes are not commonly associated with foodborne illness outbreaks. Symptoms of enteric fever don't usually manifest until 14 days after infection from typhoidal *Salmonella* serotypes, while NTS infection symptoms usually take from 6-72 h to manifest *(1, 44)*. Enteric fever symptoms often last up to 3 weeks and are characterized by sustained fever, nausea, hepatosplenomegaly, and gastroenteritis symptoms *(99)*. The symptoms caused by NTS are often self-limiting, and can last

up to 10 days *(42)*. Symptoms from NTS infection include watery diarrhea, fever, and acute gastroenteritis. Bacteremia occurs when *Salmonella* cells enter the bloodstream after penetration of the intestinal epithelium *(31)*. Approximately 5% of NTS infections result in bacteremia, which can potentially be fatal *(1)*. This is most common in elderly and immunocompromised individuals. While typhoidal *Salmonella* and NTS infection symptoms differ greatly, the explanation for why they differ is still not well understood *(42)*.

Infection via NTS occurs when NTS cells are consumed orally, mainly through contaminated food products and water, as stated above. It can take less than 10^3 organisms to result in salmonellosis within a human *(15)*. *Salmonella* cells promote illness by penetrating the epithelial cells of the small intestine.

Most *Salmonella* pathogenicity genes are clustered together within *Salmonella* pathogenicity islands (SPIs) *(92)*. SPIs are large gene cassettes that are located within the organism's chromosome that are responsible for encoding proteins responsible for host interaction *(66)*. There have been 21 identified pathogenicity islands in *Salmonella,* and they are thought to have been acquired via horizontal gene transfer from unknown plasmids and phages, and are highly conserved across different *Salmonella* serotypes *(16, 66)*. SPI1 and SPI2 are the SPIs that are responsible for encoding the *Salmonella* type III secretion system. The type III secretion system encoded by SPI1 is mainly responsible for *Salmonella* penetration of human intestinal cells, while the SPI2 encoded type III secretion system plays a major role in translocating effector proteins into the cytoplasm of intestinal cells and intracellular survival of the organism *(26, 66)*. The injected effector proteins initiate a cell response that changes the structure of epithelial cell membrane and cause engulfment of the bacterial cell *(31)*. Once engulfed, the remaining SPIs are important in *Salmonella* cells adapting and surviving within

host cells *(66)*. Inside the epithelium, bacterial cells become trapped in a vacuole and release a thermolabile enterotoxin into the cytoplasm of the epithelial cell that activates a release of adenyl cyclase localized in the cell membrane *(62)*. This release of adenyl cyclase disturbs Na^+ and Ca^- levels which causes a release of fluid into the intestinal lumen, resulting in diarrhea *(40)*. *Salmonella* possess other virulence factors, including a thermolabile cytotoxin and membrane-associated antigens *(62)*.

Injured *Salmonella*

Contamination of food products by injured microorganisms has long been recognized as a problem *(18)*. After exposure to different types of processing techniques, some microbes might remain in an injured or stressed state and will not have the ability to grow in minimal media *(82, 83)*. The inability for cells to grow in minimal media is thought to be due to structural injury rather than metabolic injury, although metabolic injury can still be present *(12, 84)*. Injured cells can still possess limited metabolic capabilities, but are unable to reproduce *(119)*. Additionally, injured cells can rapidly repair cellular damage from the different stresses it encountered when given an environment that satisfies its nutritional needs *(19)*. This is important because injured cells that are present on a food product that satisfy its nutritional needs could potentially repair cell damage and promote foodborne illness if the contaminated food product is consumed *(18)*.

Microorganisms can be injured through sublethal processes such as freezing, heat, irradiation, and oxidation *(18, 90)*.These sublethal processes often cause damage to the cellular membrane of gram-negative bacteria *(12)*. Injury has been shown to cause leakage of intracellular materials, increased sensitivity to sodium chloride (NaCl), an alteration of the glucose transport system, and decrease of citric acid cycle enzyme activity as a result of increased sensitivity to 2,4-dinitrophenol in *Salmonella* Typhimurium *(20, 60, 77, 105)*.

Salmonella spp. have evolved many different types of stress responses and repair mechanisms to adapt to different host and non-host environmental stressors that cause injury to the cells. These stress responses are vital to the organism's ability to survive and promote its virulence once within a host *(90)*. *Salmonella* possess ways to detoxify destructive oxygen radical species once they are inside its host using the type III secretion system promoted by SPI2 *(24)*. The OxyR regulon transcribes genes encoding proteins which reduce hydrogen peroxide and other oxidizing agents *(98)*. Sigma factors RpoE and RpoH are very important in regulation of heat shock genes that encode heat shock proteins that allow *Salmonella* to endure thermal stress *(98)*. RpoH regulates the transcription of over 30 heat shock proteins, including chaperones and proteases that help to repair proteins damaged by elevated temperatures *(38)*. RpoE regulates genes encoding proteins that assist in cell membrane repair processes such as phospholipid and lipopolysaccharide biosynthesis and membrane-bound protein assembly *(88)*. These examples are included to demonstrate a fraction of the advanced processes that *Salmonella* cells undergo in response to injury or stress.

Methods have been developed to detect and enumerate injured cells to differentiate the cells from healthy unstressed cells. Methods to detect injured or stressed microorganisms are important, since the cells might not grow on typical selective plating. For example, enriching, using a nonselective liquid broth such as tryptic soy broth (TSB), containing the sample of interest and subsequent plating on selective media can allow for isolation of microorganisms or enumeration via the most probable number (MPN) method *(83)*. To allow for direct enumeration, the sample can be pour plated on a nonselective medium such as tryptic soy agar (TSA), incubated, then overlaid with a selective medium *(83)*. The overlay allows the selective agents to

diffuse through the nonselective medium, slowly creating a selective environment, allowing injured cells to repair before exposure to selective agents *(83)*.

Spices

The United States Food and Drug Administration (FDA) regulates the use of spices in the food industry in the United States. FDA defines a spice as an aromatic vegetable substance whose significant function in food is seasoning rather than nutrition (21 CFR 101.22). Spices are mainly used in the food industry to help impart flavor, color, and aroma, but can also be used as antimicrobials, antioxidants, and pharmaceutical substances. The spice definition covers all dried parts of a plant except for leaves, as herbs are considered the dried leaves of aromatic plants *(75)*. Plant parts commonly used as spices include berries, fruits, seeds, bulbs, and roots *(75)*. In 2012, the American Spice Trade Association (ASTA) published a list of 57 spices that can be listed as "spice" on food labeling in compliance to FDA food labeling requirements (21 CFR 101.22). India is the largest producer and exporter of spices, accounting for approximately 86% of worldwide spice production *(75)*. The United States is the largest importer of spices produced in India, and spices are very popular in United States cuisine *(75)*. In 2009, a survey showed that 86% of American households use spices, herbs, or spice blends *(69)*. Spices have also been gaining popularity in the U.S. in the past 4 decades. Annual per capita spice intake has increased from approximately 1.25 lbs (567 g) in 1970 to 3.64 lbs (1,575 g) in 2010 *(108)*.

Black pepper

Black pepper is one of the most imported spices into the United States. In 2010, 155.4 million pounds of black and white pepper were imported into the U.S., accounting for 12.8% of total spice import into the country *(108)*. In 2014, Vietnam, Indonesia, and India were the top 3 exporters of pepper worldwide, respectively *(35)*.

Black peppercorns are harvested from the tropical, climbing plant known as *Piper nigrum*, which is part of the Piperaceae family. Black peppercorns commonly used in modern cuisine are dried fruits, or berries, of the *P. nigrum* plant. The fruits of the plants are green when mature, but the drying process of the berries causes oxidation of phenolic compounds in the peppercorns which causes a blackening of color *(118)*.

The two main components of black pepper are its volatile oils and its pungent compounds, such as piperine. Piperine is the chemical that gives black pepper its spicy, pungent qualities. The aroma of black pepper is mainly due to the volatile oils active in the spice and account for about 2.0-2.6% of the spice *(118)*. Cellulose and fiber in the hull of the peppercorns also add bulk to the spice, which is an important factor industrially *(117)*.

On the farm, black pepper berries are normally harvested manually by detaching spikes that contain the berries from the parent vine of the *P. nigrum* plant *(117)*. Berries are then detached from the spikes by threshing and washed, either manually or mechanically. Different cultivars of *P. nigrum* are graded based on the size berry that it produces: large (> 4.25 mm), medium (3.25-4.25 mm), and small (<3.25 mm) *(117)*. After washing and grading, berries are blanched to activate the phenolase enzyme, which causes the oxidation of phenolic compounds mentioned above *(117)*. Drying the berries then reduces moisture content from about 65% to about 8-10% *(117)*. This low moisture content allows for the spice's lengthy shelf life.

Cumin

Cumin spice comes from the dried fruit of the annual plant, *Cuminum cyminum* that is part of the Umbelliferae family. Fruits, more commonly referred to as seeds, are harvested about 4 months after planting and sun dried before being stored for further use *(4)*. The seeds are often roasted to enhance the flavor of the spice. Cumin is of particular use in Indian, Mexican, and

Middle Eastern cuisine. India is the largest producer of cumin worldwide. Cumin is also indigenous to Iran and the Mediterranean region *(4, 55)*. Different regions produce different varieties of cumin that differ based on seed color, flavor, and oil content *(4)*. Cumin is one of the most imported spices into the U.S. In 2010, 22.7 million pounds of cumin seed was imported into the country, accounting for 1.9% of total domestic spice import *(108)*.

Characteristic odor and flavor are due mainly to the aldehydes present in the essential oil of the seeds *(4)*. Essential oils make up 2.3-5.0% of the seeds *(5)*. Cuminaldehyde is the most prevalent essential oil in cumin oil (40-65% of the oil), and is one of the main compounds responsible for the bitterness of cumin *(5)*. Moisture content in the seeds can range from 7-22% after drying *(94)*.

Sesame seed

Sesame seeds come from mature fruits of the *Sesamum indicum*, or sesame, plant. *Sesamum indicum* is a member of the Pedaliaceae family. Sesame seeds are often used as an oil source and in confections and baked goods. African and Asian countries are the top producers of sesame seed. From 2012-2014, United Republic of Tanzania, India, and Nigeria were, respectively, the top three producers of sesame seed in the world *(36)*. Sesame seed is also a major import for the U.S., accounting for 6.7% of total spice import in 2010 *(108)*.

Sesame seed oil extraction is very popular due to its high oil content (about 48-55%) *(52)*. About 80% of sesame seed oil content comes from unsaturated fatty acids, which is different than most other sources of vegetable oils, making it an important source of essential fatty acids *(59)*. Sesame oil also shows pronounced antioxidant activity, making it resistant to oxidative rancidity *(17)*. Sesame seeds contain about 25% protein, making them more desirable than other types of oil seeds if protein content of the product is an important consideration *(6)*.

When fruits of the sesame plant are mature, they are dried in the field, pre-harvest, to allow seed ripening, which helps seed quality *(52)*. Once harvested, sesame plants are cut and threshed to disperse the seeds. Seeds are then cleaned and allowed to dry in the sun to lower the moisture content to about 5% *(52)*. Sesame seeds are often dehulled to remove the bitterness that is associated with the outer hull of the seed. Dehulled sesame seeds are the type of sesame seed commonly used in food products. The dehulling process significantly alters the nutritional content of the seed. Dehulled seeds are higher in fat content than whole seeds, but contain less fiber and nutrients such as calcium and iron *(114)*.

Salmonella enterica contamination of spices

Salmonella exists naturally in the environment and can contaminate spices during production. Spices can be contaminated at the farm level, during transportation and storage, and in processing facilities, pre- and post-microbial intervention. From 1985-2012, *Salmonella* spp., *Bacillus* spp., *Clostridium perfringens, Cronobacter* spp., *Shigella,* and *Staphylococcus aureus* were detected in spices *(7, 8, 109)*. *Salmonella* and *Bacillus* spp. are the only pathogens that have been directly responsible for foodborne outbreaks definitively linked to contaminated spices *(109)*. *Salmonella* caused 87% of illnesses in these outbreaks, and remains the major reason for spice-associated recalls, making it the main pathogen of concern in the spice industry *(109, 113)*. Numbers of *Salmonella* as low as 0.04 MPN/g in food products linked to foodborne illness outbreaks has been demonstrated, highlighting the importance of proper handling and treatment of spices *(61)*.

Salmonella contamination can occur at many points along the processing chain for spices. Attachment of microorganisms to food surfaces is dependent on substrate properties, surface characteristics, and motility factors of the microorganism (e.g. flagella and fimbriae) *(72)*.

Surface irregularities of food products and food contact surfaces, such as roughness, crevices, and pits, have been shown to increase bacterial cell attachment to food products *(25)*. Many spices have irregular surfaces, suggesting increased probability of *Salmonella* attachment if exposed to the bacterium.

From 1973-2010, there were 14 documented spice-related foodborne outbreaks worldwide *(112)*. As indicated above, *Salmonella enterica* and *Bacillus* spp. were the causative pathogens of these outbreaks. Out of the 14 outbreaks, 10 (71.4%) were caused by *Salmonella* contamination and 4 (28.6%) were caused by *Bacillus* spp. contamination *(109)*. Spice-related *Salmonella* outbreaks that occurred in the United States within this time frame included seasoning mix and broccoli powder (*S.* Wandsworth and *S.* Typhimurium; 87 cases; 0 deaths), white pepper (*S.* Rissen; 87 cases; 1 death), and black and red pepper (*S.* Montevideo and *S.* Senftenberg; 283 cases; 0 deaths) *(21, 23, 97)*. Other spices that have been involved in foodborne illness outbreaks include paprika, turmeric, curry powder, anise seed, and fennel seed *(109)*. There have also been many spice-related food recalls over the past 50 years. From 1969-2003, there were 20 recalls of spices or foods containing contaminated spices *(113)*. In 2008-2009, there were 8 recalls of spices or foods containing contaminated spices *(109)*. These recalls were attributed to lack of supplier control, as well as inadequate sanitation and poor environmental monitoring *(109)*.

Many serotypes of *Salmonella* have been isolated from spices and herbs grown domestically or imported from various countries. Of the serotypes used in the current study, from 2009-2010, *S.* Agona was isolated from anise, black pepper, capsicum, cumin, curry powder, garam masala, mint, nutmeg, oregano, and sesame seed *(109)*. *Salmonella* Anatum was isolated from capsicum, coriander, cumin, fenugreek, and sesame seed *(109)*. *Salmonella* Montevideo

was isolated from arnica, black pepper, capsicum, coriander, cumin, mint, oregano, nutmeg, sesame seed, and thyme *(109)*. *Salmonella* Senftenberg was isolated from black pepper, capsicum, celery seed, coriander, cumin, curry powder, garam masala, nutmeg, sesame seed, and thyme *(109)*. *Salmonella* Tennessee was isolated from capsicum, celery, sesame seed, and was the causative organism of an outbreak of foodborne illnesses associated with peanut butter, another low water activity food product *(22, 109)*.

Spices are harvested from different parts of the parent plant's anatomy. The source of the spice can impact exposure to microorganisms, including *Salmonella*. Because of this, and different conditions in which spices grow, *Salmonella* prevalence in spices varies immensely. From FY2007-FY2009, the 5 spices with the highest prevalence of *Salmonella*-contaminated imported shipments were coriander (15%), oregano (12%), sesame seed (11%), curry powder (8.7%), and cumin (8%) *(109)*. Only 4.5% of imported black pepper shipments were found to be contaminated *(109)*. However, because of its wide use and role in many of the spice-related outbreaks, it is an important spice to understand.

Reducing the available water to *Salmonella* has long been used to inhibit its growth on food products. The lower limit of water activity to support the growth of *Salmonella* is 0.94 *(86)*. Although spices are almost always stored at a water activity below 0.75, *Salmonella* has shown capability to survive at low water activity over long times of storage *(3, 53)*. *Salmonella* can survive for up to 8 months in paprika samples *(61)*. Still, relatively little is known about the stress response of *Salmonella* to desiccated environments *(98)*. Studies have shown that cells can form an intracellular filamentous network to help in survival *(67)*. O antigen and extracellular polysaccharides have also been shown to help form a hydrated layer around cells and slow the rate of dehydration in the cell, which increases survival capabilities of the cells at low water

activities *(43)*. Desiccated *Salmonella* cells have also been shown to have increased resistance to heat, irradiation, bile salts, sodium chloride, and various other types of disinfectants relative to non-desiccated cells *(27, 48)*.

Antimicrobial spice processing techniques

Many processes exist within the food industry to rid spices of microbial contamination. As discussed above, spices are often cultivated in under-developed areas where standard food safety practices are not as developed as in first world regions. Therefore, the need for effective processing techniques to reduce microbial numbers on spices is very important. The following discusses some common methods of antimicrobial spice processing.

Irradiation

Ionizing radiation, or irradiation, involves the use of gamma rays, X-rays, or electron beams to transmit energy to microbial cells *(93)*. The bacterial chromosome is the major target of irradiation *(70)*. The use of irradiation can have direct and indirect actions on cells. Direct action includes the absorption of electrons by target molecules due to the ejection of energy into the cells by the radiation *(70)*. This usually occurs when cells are dry. Because cells are mostly comprised of water, ionization caused by irradiation usually takes place in water, causing an indirect mode of action *(70)*. Indirect action is characterized by formation of hydroxyl radicals (OH^-) due to decomposition of water reacting with DNA of irradiated cells *(70)*. Hydroxyl radicals are highly reactive compounds and can interact with the sugar-phosphate backbone of microbial DNA, causing breakage of the double helix.

Irradiation of food products, including spices, has been used for the past 50 years. In 1981, the World Health Organization (WHO) reported that irradiation of a food product with an average dose of up to 10 kGy does not produce any sort of toxicological, nutritional, or

microbiological hazard to consumers *(37)*. A level of 3.0-10.0 kGy of irradiation is required to reduce or eliminate microbial population in low water activity foods *(34)*. A maximum dosage of 30 kGy is currently permitted by FDA to treat spices (21 CFR 179.26). Irradiation has been shown to have potential for slight effect on the antioxidant activity and sensory quality of spices *(100, 106)*.

In 2002, it was estimated that 90,000 tons (81,646 tonnes) of spices, herbs, and dry vegetables seasonings were treated with irradiation worldwide *(89)*. Despite its relative effectiveness in reducing microbial numbers on foods, low consumer acceptance still inhibits irradiation from becoming a universal processing technique. Many factors contribute to low consumer acceptance. FDA requires that irradiated foods labeling be marked with a statement and logo indicating that the food has been irradiated *(74)*. This includes foods that are available for consumer purchase. Multi-ingredient foods that contain single ingredients that have been irradiated are not required to include the logo on its label. Consumers are often not aware of what irradiation is, confusing the term with radioactivity, and choose not to purchase irradiated food products *(33, 104)*.

Steam treatment

Steam treatment is another commonly used industry practice to reduce microbial contamination of spices *(3)*. The efficacy of steam treatment is dependent on the time and temperature the microorganisms are exposed to. Effective treatment processes are successful in eliminating problematic vegetative cells, including *Salmonella (109)*. Like other forms of heat treatments, steam inactivates microorganisms by denaturing proteins and nucleic acids in the cells. Various forms of steam including: dry, saturated, and superheated, are used based on available technology and food product *(3)*. If saturated steam is being used, a drying step post-

treatment might be necessary to return the product to its original moisture level *(3)*. It has also been shown that steam treatment when combined with storage can induce negative quality changes in spices *(57)*. Steam treatment can be applied to whole or ground spices, but it is mostly applied to whole spices *(103)*.

Various forms of steam treatments (e.g., various time, temperature, and pressure combinations) have been shown to be quite effective in reducing total microbial population numbers in spices. Aerobic plate count (APC) reductions have ranged from 0.8 to 7.9 log CFU/g in black pepper and paprika *(91, 116)*. There have been no published studies that examine the effect of steam treatment on *Salmonella* numbers on spices directly. Multiple steam technologies have been validated by the United States Department of Agriculture (USDA) for the almond industry, a similar low water activity food, and reduce *Salmonella* numbers by 4-5 log CFU/g.

Fumigation

Fumigation of spices involves the treatment of spices with an antimicrobial gas under various conditions (e.g., pressure, temperature, humidity) to inactivate microorganisms, including *Salmonella*, on the surface of spices. The most commonly used gas to treat spices is ethylene oxide (EtO). It is estimated that between 40 and 85% of spices are treated each year with EtO *(3)*. Propylene oxide (PO) is also used, but much less frequently than EtO *(3)*. The bactericidal effect of EtO results from the gas causing alkylation of nucleic acids within bacterial cells, leading to inactivation of the cells *(73)*. Like many other antimicrobials, EtO is more effective against vegetative cells than spores, with spores being 5-10 times more resistant to EtO *(76)*. Multiple studies have shown varying effectiveness of EtO in reducing APC in spices, with reductions ranging from 1.3 log CFU/g to 6 log CFU/g *(39, 111)*. EtO is also commonly used in the medical industry as a sterilizer for medical equipment to reduce microbial load *(109)*.

EtO is generally considered to be a carcinogen and mutagen, especially when inhaled, and has been shown to cause tumors in laboratory animals after intragastric exposure *(29, 32)*. To account for this risk, ASTA has developed a protocol for consistent EtO treatment of spices in accordance of the United States Environmental Protection Agency (EPA) Food Quality Protection Act Tolerance Reassessment Decision Document *(3)*. This document states that EtO residues should not exceed 7 ppm on spices and dried vegetable products and ethylene chlorohydrin, a residue that forms when EtO reacts with free chloride ions, should not exceed 940 ppm *(110)*. Following the protocol put forth by ASTA regarding EtO usage, residue levels should meet the standards stated above, and not cause any sort of toxicological risk for humans.

Chlorine dioxide

Chlorine dioxide (ClO_2) has been recognized as an effective disinfectant since the mid-1900s *(9)*. ClO_2 is similar to chlorine (Cl_2) in that it is a very powerful oxidizing agent, but ClO_2 has 2.5 times the oxidizing power as Cl_2 *(54)*. It is also versatile due to the fact that it is unaffected by pH *(13)*. It has commonly been used for drinking water disinfection, wastewater treatment, and in cooling tower water due to its ability to break down phenolic compounds and remove phenolic sensory qualities in water and for its antimicrobial activity *(30)*.

Chlorine dioxide can be applied in an aqueous or gaseous form. The gaseous form is desirable for most food products due to the fact that the aqueous form can increase the moisture level of a food product and potentially promote a favorable environment for growth of microorganisms *(109)*. The gaseous form also has greater penetrability potential than the aqueous form *(50)*. This allows the gas better access to surface irregularities on food products than the aqueous form *(45)*. Despite this fact, chlorine dioxide gas has been shown to be less

effective on injured produce and produce with many surface irregularities, like iceberg lettuce *(51, 102)*.

Chlorine dioxide can be formed through different types of chemical reactions. Chlorine dioxide gas in the current study was generated by the mixing of two dry solids, granular porous sand impregnated with sodium chlorite and a ferric chloride activator. The following reaction illustrates the chemistry taking place to release chlorine dioxide gas for the current study:

$$3NaClO_2 + 3H_2O + FeCl_3 \rightarrow 3ClO_2 + Fe(OH)_3 + 3NaCl$$

Due to its high reactivity and explosive nature, chlorine dioxide cannot be compressed and shipped, so it needs to be generated at the site of use *(46)*.

Much research has gone into determining the method of biocidal inactivation for chlorine dioxide. Chlorine dioxide causes nonspecific oxidative damage to the outer membrane of microorganisms, making it effective against a broad range of microorganisms *(46, 58)*. In *Escherichia coli*, the main mode of action of chlorine dioxide is thought to be due to a rapid efflux of potassium ion *(11)*. This causes a loss of permeability and disruption of the outer membrane of bacterial cells. Cell respiration is also inhibited by high doses of chlorine dioxide *(11)*. Additionally, chlorine dioxide abruptly inhibits the synthesis of proteins in bacterial cells *(10)*. The mechanism by which chlorine dioxide promotes protein synthesis inhibition is unknown. In *E. coli*, chlorine dioxide has demonstrated the ability to completely inhibit dehydrogenase enzymes, though this is not thought to be the main cause of death of bacterial cells *(87)*. Chlorine dioxide has been shown to readily react with cysteine, tryptophan, and tyrosine in viral cells *(71)*. Chlorine dioxide has greater sporicidal activity than chlorine, due to greater utilization of its oxidation potential by accepting up to 5 electrons in spore cells *(85)*.

Gaseous chlorine dioxide has been shown to be very effective in reducing pathogen numbers on produce. A ClO_2 concentration of 8.0 mg/L applied to blueberries, strawberries, and raspberries for 120 min reduced *Salmonella* numbers by 2.44-3.67, 3.76-4.41, and 1.54 log CFU/g, respectively *(101)*. *Salmonella* numbers on cabbage, carrots, and iceberg lettuce exposed to 4.1 mg/L ClO_2 for 30.8 min were reduced by 4.42, 5.15, and 1.58 log CFU/g, respectively *(102)*. Another study on iceberg lettuce showed a reduction of *Salmonella* by 2.8 log CFU/g when exposed to 5 mg/L ClO_2 for 10 min *(63)*. Cantaloupe that was treated with 5 mg/L ClO_2 for 6 min had reduced *S.* Poona numbers by 5 log CFU/g *(64)*. *Salmonella* numbers on apples and tomatoes treated with 4.1 mg/L ClO_2 for 25 min were reduced by 4.21 and 4.33 log CFU/g, respectively *(102)*. Vidalia onions and peaches exposed to 4.1 mg/L ClO_2 for 20 min had reduced *Salmonella* numbers by 1.94 and 3.23 log CFU/g, respectively *(102)*. Mungbean sprouts tumbled and treated with 0.5 mg/L ClO_2 for 60 min had reduced *Salmonella* numbers by 5.5 log CFU/g *(80)*. Similar 3-5 log CFU/g reductions have been demonstrated for other pathogens on produce such as *Listeria monocytogenes* and *E. coli* O157:H7 and spoilage organisms like *Alicyclobacillus acidoterrestris (46)*.

Research on reduction of microorganism numbers on low water activity foods is still rather limited. Refrigerated almonds exposed to 10 mg/L ClO_2 gas for 30 min had reduced *Salmonella* numbers by 5.29 log CFU/g *(115)*. A 200 µg/mL aqueous ClO_2 wash reduced *E. coli* O157:H7 by 3.8 log CFU/g on radish seeds *(56)*. ClO_2 gas treatment significantly reduced *S.* Poona numbers on tomato seeds when treated 10 mg/L for 3 min *(107)*.

Use of gaseous ClO_2 is not currently permitted by FDA to treat food products. Aqueous ClO_2 can be used in washing water for fruits and vegetables that are not considered raw agricultural commodities at a level of no greater than 3 ppm (21 CFR 173.300). These fruits and

vegetables must then by rinsed with potable water, blanched, cooked, or canned. FDA also permits the use of chlorine dioxide to treat water. A maximum level of 3 ppm ClO_2 can be used in water used in poultry processing (21 CFR 173.300). FDA has approved the use of chlorine dioxide as an equipment sanitizing agent at a maximum concentration of 200 ppm (21 CFR 178.1010) *(13)*.

Despite its proven efficacy as an antimicrobial, a major reason why chlorine dioxide gas has not yet been approved for treatment of produce in the United States is due to the fact that chemical residues of the gas in food products after fumigation have not been well characterized. After participation in oxidation reactions, chlorine dioxide gas rapidly breaks down to chlorite (ClO_2^-), chlorate (ClO_3^-), and perchlorate (ClO_4^-) ions *(46)*. Additionally, chlorine dioxide can be further reduced to chloride. Fumigation of tomatoes and cantaloupes with chlorine dioxide gas showed that greater than 80% of the main detectable residues in tomato homogenate and on cantaloupe rind were chloride *(95)*. Chlorite residues were not found at all on fumigated tomatoes and cantaloupes. Formation of chlorate and perchlorate residues can be prevented by eliminating light from the area being fumigated *(95)*. This shows that the rate of decomposition of ClO_2 gas can be slowed by preventing interaction with light. A subchronic toxicity test showed that a 553 mg/L solution of chlorine dioxide, chlorite, and chlorate administered orally to rats was not toxic *(81)*.

Because of its heavy use in the paper industry as a bleaching agent, chlorine dioxide's bleaching effect on vegetables has received much attention *(41)*. Bleaching of fruits and vegetables has been shown in many different studies. Strawberries, blueberries, tomatoes, iceberg lettuce, green bell peppers, and carrots are examples of fresh produce that have shown a bleaching effect after exposure to chlorine dioxide gas *(28, 63, 79, 101, 102)*. Browning of

produce has also been reported after exposure to chlorine dioxide gas; browning is likely due to oxidation of phenols by ClO_2 gas *(46)*.

CHAPTER 3

MATERIALS AND METHODS

Bacterial strains used

Salmonella enterica cultures were obtained from the Department of Food Science and Technology, University of Georgia, Athens, GA, and stored on cryogenic beads (Hardy Diagnostics, Santa Maria, CA) at -80°C. Five serotypes of *S. enterica* that have been isolated from foodborne salmonellosis outbreaks were used in the study: *S.* Agona and *S.* Anatum, isolated from alfalfa sprout-associated outbreaks; *S.* Montevideo, isolated from a tomato-associated outbreak; *S.* Senftenberg (ATCC 8400; ATCC, Manassas, VA); and *S.* Tennessee, isolated from a peanut butter-associated outbreak. To activate the frozen cultures, one bead was placed into 10 mL of tryptic soy broth (TSB; BD Biosciences, Franklin Lakes, NJ) and incubated at 37°C for 24 h. Broth cultures were then streaked via loop inoculum (~10 μL) onto tryptic soy agar (TSA; BD Biosciences, Franklin Lakes, NJ) slants and incubated at 37°C for 24 h. Slant cultures were stored at 4°C.

Antibiotic adaptation

A loopful of each slant culture was transferred into 10 mL tubes of TSB supplemented with nalidixic acid (Sigma-Aldrich, St. Louis, MO) at a concertation of 50 μg/mL (TSBN) and incubated at 37°C for 24 h. Cultures were streak plated for isolation onto xylose lysine desoxycholate agar (XLD; BD Biosciences, Franklin Lakes, NJ) plates supplemented with 50 μg nalidixic acid/mL (XLDN) and incubated at 37°C for 24 h. Isolated colonies were picked and streaked onto TSA slants supplemented with 50 μg nalidixic acid/mL (TSAN) and incubated at

37°C for 24 h. Slant cultures were stored at 4°C and transferred onto new TSAN slants every 2-4 weeks to ensure viable *Salmonella* cells.

Salmonella growth behavior

Cross-streak plates were prepared to test for cross-serotype growth inhibition. For each adapted serotype of *Salmonella*, a loop transfer was made from its TSAN slant to a 10 mL tube of TSBN and incubated at 37°C for 24 h. Cultures were then cross streaked onto a TSAN plate, with each serotype receiving a horizontal and vertical streak, so that each vertical streak crossed all five horizontal streaks and vice versa, and incubated at 37°C for 24 h. Each cross-streak junction was examined for growth inhibition.

Growth curves of each wild-type *Salmonella* culture and each adapted culture were obtained to ensure similar growth characteristics between the wild-type and adapted strains. For each wild-type strain, two successive transfers were performed into 10 mL of TSB and incubated at 37°C for 16 h. The same was done for the adapted cultures except TSBN was used as the growth medium. A transfer of 0.25 mL for each wild-type and adapted culture was made into 25 mL of TSB and TSBN, respectively, and incubated at 37°C. At 0, 8, 9, 10, 12, and 14 h incubation time, 1.0 mL of the 25 mL cultures were transferred to a 1.5 mL disposable cuvette (Thermo Fisher Scientific, Waltham, MA), and optical density values at 600 nm were measured using a spectrophotometer (Beckman Coulter, Inc., Atlanta, GA).

Spice procurement and storage

Organic black peppercorns (*Piper nigrum*; Frontier Co-op, Norway, IA), cumin seeds (*Cuminum cyminum*; Frontier Co-op, Norway, IA), and sesame seeds (*Sesamum indicum*; Frontier Co-op, Norway, IA) were purchased from a local supermarket in Athens, GA. Spices were stored at room temperature (20-21°C) in a dark storage area.

Chlorine dioxide consumption curves

Chlorine dioxide precursor media was obtained from ICA TriNova, LLC, Newnan, GA. The media consisted of two parts, a sodium chlorite precursor and a ferric chloride acid activator. When combined and agitated, the two parts emitted ClO_2 gas. The concentration of gas was dependent on the amount of chlorine precursor used and treatment time. The media was mixed in gas-permeable sachets (ICA TriNova, LLC, Newnan, GA).

Gas consumption curves were obtained for each spice to determine proper ClO_2 treatment time. To obtain the curves, 50 g of spice were placed in the chamber of a ClOClave machine (ICA TriNova, LLC, Newnan, GA) and a sachet formulated to release 100 mg ClO_2/kg spice was shaken and inserted into the chamber. Measurements of gas concentration in the headspace of the chamber were taken at over times until the concentration reached approximately 0 parts per million by volume (ppmv). When the concentration reached 0 ppmv, it was assumed that the spice had consumed all the emitted gas. Curves were plotted as headspace gas concentration (ppmv) against time (min). Two curves were made for each spice tested.

Inoculum preparation

To prepare the inoculation cocktail, a loopful of each adapted serotype was used to inoculate separate 10 mL tubes of TSBN and incubated at 37°C for 24 h. Fifty µL from each tube was subsequently transferred into 50 mL conical centrifuge tubes (ThermoScientific, Rochester, NY) containing 50 mL TSBN and incubated at 37°C for 24 h. Each centrifuge tube was centrifuged (Eppendorf Centrifuge 4810, Hauppauge, NY) at 2,500 x g for 10 min. The supernatant was discarded and the pellets were resuspended in 50 mL of 10 mM sterile phosphate buffered saline (PBS; 8 g sodium chloride; 0.2 g potassium chloride; 1.44 sodium phosphate, dibasic, anhydrous; 0.24 g potassium phosphate, monobasic, crystal per 1 L deionized

(DI) water). Tubes were centrifuged again at 2,500 x g for 5 min. The supernatant was discarded and the pellets were resuspended in 50 mL of PBS. All suspensions were combined in a 2,000 mL Pyrex bottle and swirled to mix the cocktail. Prior to inoculation, the cocktail was serially diluted in 0.1% peptone water (PW; BD Biosciences, Franklin Lakes, NJ) and spiral plated (Autoplate 4000, Spiral Biotech, Norwood, MA) on XLDN and brain heart infusion agar (BHI; BD Biosciences, Franklin Lakes, NJ) plates supplemented with 50 μg nalidixic acid/mL (BHIN) to enumerate *Salmonella* in the cocktail. The final inoculum cocktail contained approximately 10^8 CFU *Salmonella*/mL.

Inoculation procedure

Black peppercorns (800 g), sesame seeds (800 g), and cumin seeds (800 g) were weighed and divided equally into two 1,000 mL Pyrex bottles. After weighing, 400, 425, and 625 mL of the *Salmonella* suspension was added to each bottle for black peppercorns, sesame seeds, and cumin seeds, respectively. Enough suspension was used to cover the top of the spices in the bottle. Bottles were shaken in an environmental incubator shaker (New Brunswick Scientific Co., Inc., Edison, NJ) for 30 min at approximately 160 revolutions per minute (RPM). Spices were drained into sterile cheese cloth (Thermo Fisher Scientific, Waltham, MA) in approximately 100 g batches and left to dry on the cheese cloth for 24 h in a biosafety cabinet.

Chlorine dioxide treatments

Spices received four different gaseous ClO_2 treatments: 0 (control), 100, 200, and 500 mg ClO_2/kg spice (0, 2.57, 5.14, and 12.86 mg ClO_2/L). Four 175 g samples of inoculated spice were weighed and placed into the drums (drum volume=6.8047 L) of four different vacuum tumble marinators (Chard International, Two River, WI). Each drum was randomly assigned a treatment group using a random number generator. An empty sachet containing no ClO_2 media was placed

into the control drum. Sachets with media formulated to release 100, 200, and 500 mg ClO_2/kg spice over 12 h were shaken to activate the media and placed into their respective drums. Drum tops were placed onto the drums after insertion of the sachets and were further sealed using Parafilm (Heathrow Scientific, Vernon Hills, IL). All four drums were placed onto their respective bases, and the spices were tumbled at approximately 2.4 RPM for 12 h to allow mixing. Treatments took place in a ventilated area. Relative humidity of the ventilated are was measured using a temperature, humidity and dew point data logger (Omega Engineering, Inc., Stamford, CT). The average relative humidity and temperature in the ventilated area during treatment time were 41.5% and 23.7°C, respectively.

Microbiological analyses

Following 12 h treatment with ClO_2, four 30 g samples of each spice from each treatment group were measured into 120 mL sterile specimen containers (Cen-Med Enterprise, Inc., New Brunswick, NJ). One container from each treatment group was immediately used for analysis, while the other three were stored in a dark storage area at room temperature (20-21°C).

Triplicate 10 g sub-samples from each treatment group's first container were measured into stomacher bags (VWR International, Radnor, PA). Ninety mL of TSB containing 0.54 g of sodium thiosulfate (Sigma-Aldrich, St. Louis, MO) was added to each stomacher bag and the bag was hand-shaken for 1 min. Contents from the stomacher bag were serially diluted in 0.1% PW and spiral plated for enumeration on XLDN and BHIN plates that were incubated at 37°C for 24 h. Brain heart infusion agar supplemented with 50 µg nalidixic acid/mL was used to attempt to enumerate sublethally injured *Salmonella* cells that might have been damaged by the oxidation capability of ClO_2 gas that a selective medium, like XLDN, could not enumerate *(18)*. Colony forming units (CFU) were manually counted using the segment pair counting method.

Immediately after plating, each stomacher bag was incubated at 37°C for 24 h for enrichment. If the presence of *Salmonella* was below the detection limit of the spiral plater (800 CFU/g), a loopful of enrichment broth was streaked onto XLDN and BHIN and incubated at 37°C before being checked for presumptive *Salmonella* colonies. Presumptive positive *Salmonella* colonies from the enumeration and enrichment procedures were periodically confirmed using lysine iron agar (LIA; BD Biosciences, Franklin Lakes, NJ), triple sugar iron agar (TSI; BD Biosciences, Franklin Lakes, NJ), and Microgen *Salmonella* latex agglutination kits (Microgen Bioproducts, Camberley, UK).

On days 1, 10, and 30 post-treatment, spices were analyzed using the same plating/confirmation scheme described above.

Statistical analyses

All statistical analyses were performed using SAS (Cary, NC) software. In the case that counts were below the detection limit of 800 CFU/g and *Salmonella* was still detectable by enrichment, 800 CFU/g was used as the count. *Salmonella* population numbers were transformed to log CFU/g numbers before calculating mean values.

Chlorine dioxide treatment analyses were replicated three times for each spice. Each replication consisted of triplicate samples from each chlorine dioxide treatment and storage day combination. Each sample was duplicate plated and the mean colony count of the two plates was calculated. *Salmonella* colony numbers were averaged across the nine samples for each combination and the standard deviations were calculated for each subset of nine samples. For each type of spice and enumeration method, average *Salmonella* population numbers across the 4 treatment levels of ClO_2 at each storage day were compared using the Tukey-Kramer multiple comparisons method with an alpha level of 0.05. This analysis was performed to analyze ClO_2

treatment effect on *Salmonella* numbers in spices. For each type of spice and enumeration method, average *Salmonella* population numbers across each storage day for each ClO_2 treatment were analyzed using the Tukey-Kramer multiple comparisons method with an alpha level of 0.05. This analysis was done to evaluate storage effect on each ClO_2 treatment used in the experiment.

CHAPTER 4

RESULTS

For *Salmonella* enumerated from black pepper on BHIN and XLDN, all three chlorine dioxide treatments (100, 200, and 500 mg ClO_2/kg pepper) resulted in significantly different numbers ($p<0.05$) than the control treatment (0 mg ClO_2/kg pepper) at each storage day (Table 1). At storage day 0 and 1, treatment with 500 mg ClO_2/kg pepper resulted in significantly lower ($p<0.05$) numbers of *Salmonella* than 100 and 200 mg ClO_2/kg pepper treatments. *Salmonella* numbers were at or near the enumeration detection limit (2.90 log CFU/g) on days 10 and 30 for all three ClO_2 treatment groups; no significant difference ($p>0.05$) was seen between treatment groups. Reduction of *Salmonella* numbers across storage days for each ClO_2 treatment varied. In general, *Salmonella* numbers reduced from day 0 to day 30. For *Salmonella* enumerated on both BHIN and XLDN numbers were significantly reduced ($p<0.05$) from storage day 0 to 30 for 0, 100, and 200 mg ClO_2/kg pepper treatments. There was no significant difference ($p>0.05$) in *Salmonella* numbers on black pepper treated with 500 mg ClO_2/kg pepper from day 0 to day 30. There may have been further decrease had the detection limit been lower.

Each chlorine dioxide gas treatment resulted in significantly different *Salmonella* numbers ($p<0.05$) than the control treatment for cumin samples (Table 2). For *Salmonella* numbers enumerated on BHIN, all four treatments were significantly different ($p<0.05$) on storage day 0, 1, and 10. As chlorine dioxide concentration increased, *Salmonella* numbers decreased. This trend was similar for day 30, though there was no significant difference ($p>0.05$) between 200 mg ClO_2/kg cumin and 500 mg ClO_2/kg cumin. For cumin samples plated on

XLDN, *Salmonella* numbers generally decreased with increased chlorine dioxide gas concentration. Storage time also tended to influence *Salmonella* numbers in both media. While there was no significant difference in *Salmonella* numbers ($p>0.05$) between storage day 0 and day 1 for each treatment, numbers were significantly lower ($p<0.05$) on day 30 than on day 0.

For *Salmonella* enumerated from sesame seed on XLDN and BHIN, all three chlorine dioxide treatments resulted in significantly lower numbers than the control treatment (Table 3). For *Salmonella* enumerated on BHIN on days 0, 1, and 10, treatment with 100, 200, and 500 mg ClO_2/kg seed were significantly different ($p<0.05$) from one another, with increasing reduction with increasing gas concentration. Samples on day 30 showed this same trend, although 100 and 200 mg ClO_2/kg seed treatments were not significantly different ($p>0.05$). For *Salmonella* enumerated on XLDN on days 0 and 1, treatments with 100, 200, and 500 mg ClO_2/kg seed were significantly different ($p<0.05$) from one another, with increasing reduction with increasing gas concentration. Samples on days 10 and 30 showed this same trend, although 100 and 200 mg ClO_2/kg seed treatments were not significantly different ($p>0.05$). Storage time had an effect on *Salmonella* numbers across different treatment levels. For all treatments, *Salmonella* numbers significantly decreased ($p<0.05$) from storage day 0 to 30. Population numbers dropped the most from day 0 to day 30 for the 500 mg ClO_2/kg seed treatment group.

Two different types of media were used as part of the enumeration method to attempt to measure the amount of injury caused to *Salmonella* cells by the chlorine dioxide gas treatment. Subtracting the *Salmonella* numbers enumerated on XLDN from the numbers enumerated on BHIN gives an estimate of the number of injured *Salmonella* cells from the process. No statistical analysis was performed on the difference between *Salmonella* numbers on XLDN and BHIN, but numbers were generally lower on XLDN than on BHIN. For *Salmonella* enumerated

from each spice tested, differences between *Salmonella* numbers on BHIN and XLDN ranged from approximately 0.25-1.00 log CFU/g.

CHAPTER 5

DISCUSSION

Due to high prevalence of *Salmonella enterica* on imported and domestically grown spices, proper processing techniques are essential to reducing the risk of spice-related foodborne illness *(109)*. Although various processing methods to reduce *Salmonella* contamination on spices are currently utilized in the food industry, each method carries disadvantages to its use. Steam treatment, one of the most common methods used by large spice processors, is a heat treatment that can negatively impact spice quality *(57)*. It also introduces moisture to the spices, which forces a drying step to be included in the processing line *(3)*. Irradiation is not widely accepted by consumers and has also been shown to impact sensory quality and antioxidant activity of spices *(100, 104, 106)*. Ethylene oxide is widely considered a carcinogen and mutagen and has been banned as a spice treatment in the European Union and Australia *(29, 93)*. An antimicrobial processing method that does not have such drawbacks could be of potential use for the spice industry. The current study evaluated different concentrations of chlorine dioxide (ClO_2) gas as a means to counter *Salmonella* contamination of spices.

Starting inoculation levels differed greatly between black pepper and the other two spices tested, cumin and sesame seed. Inoculated black pepper contained approximately 5-6 *Salmonella* log CFU/g, while inoculated cumin and sesame seed contained approximately 7-8 *Salmonella* log CFU/g. The differences were likely due to the amount of cocktail used to inoculate the spices or structural properties of the spices. The low starting inoculation level for black pepper introduced a problem for the 500 mg ClO_2/kg pepper treatment and some of the longer storage

days. *Salmonella* levels were reduced to below the detection limit (<2.90 log CFU/g) for the enumeration method used.

The results of the current study do not show as great of a reduction in bacterial numbers on spices as has been shown in other types of low water activity food products treated with chlorine dioxide gas. In the current study, ClO_2 gas reduced *Salmonella* numbers on black pepper, cumin, and sesame seed by approximately 0.81 to 2.74 log CFU/g. Refrigerated almonds treated with 10 mg/L ClO_2 gas for 30 min had reduced *Salmonella* levels by 5.29 log CFU/g *(115)*. Tomato seeds that were treated with 10 mg/L of chlorine dioxide gas for 3 min reduced *Salmonella* levels by 5.30 log CFU/g *(107)*. A possible significant factor in the differing results could be relative humidity in the compartment where the food products were treated with the gas. Studies have shown that chlorine dioxide efficacy increases with an increase in relative humidity *(49)*. For the study on tomato seeds, seeds were treated with the gas at a relative humidity of 75% as controlled by a humidifier, while in the current study, relative humidity was not directly controlled, and ranged from approximately 40-49%. Another reason for lower reduction levels in the current experiment could have been due to ClO_2 degradation over treatment time. Chlorine dioxide gas that is quickly degraded will decrease the level of gas that is available to act on microorganisms present on the surface of food products *(46)*. An increase in temperature has also been shown to increase inactivation of *E. coli* O157:H7 on produce, so increasing the temperature during treatment of spices with ClO_2 could result in increased *Salmonella* reduction *(49)*. A combination of increased ClO_2 gas concentration, relative humidity, and temperature may be the most effective way of using chlorine dioxide gas as an antimicrobial spice process.

Research on reduction of *Salmonella* contamination on spices due to currently used spice processing methods is still quite limited. Much data has been collected on reduction of aerobic

plate count (APC), coliforms, yeasts and molds, and *Enterobacteriaceae* on treated spices. Log CFU/g reductions of APC due to irradiation treatments of 2 to 20 kGy on various spices have ranged from 1.6 to >6.9 log CFU/g *(109)*. Doses of 4 and 8 kGy have been observed to reduce *Enterobacteriaceae* levels by 3.0 and >4.7 log CFU/g, respectively, on ground black pepper *(96)*. Aerobic plate count reductions in steam treated spices have ranged from 0.8 to 7.9 log CFU/g *(91, 116)*. Reductions in *Enterobacteriacae* levels have ranged from 3.8 to 3.9 log CFU/g on steam treated paprika *(2)*. Various studies have shown APC reductions ranging from 1.3 to 6.0 log CFU/g on ethylene oxide fumigated spices *(109)*. Data on reduction of *Salmonella* or a surrogate on spices due to the above processes is lacking in the literature, as is aerobic plate count reduction data on spices due to ClO_2 gas. The present study suggests that chlorine dioxide may be less effective at reducing microbial levels on spices than some currently used industry processes.

Storage day also influenced *Salmonella* numbers on spices across the different treatment groups. Even though *Salmonella* levels dropped in the untreated spices across the 30-day storage period, chlorine dioxide treatment combined with subsequent storage could be a possible mechanism to further reduce *Salmonella* numbers on treated spices by up to 2.5 log CFU/g. Based on literature data, longer storage could lead to even further reduction of *Salmonella* numbers on spices. After 32 weeks of storage of inoculated inshell pecans, a similar low water activity food product, *S.* Anatum and *S.* Senftenberg numbers were reduced from approximately 10^5 CFU/g to undetectable levels *(14)*.

Contamination of spices with *Salmonella* can occur at many points during its supply chain. While physical cleaning of spices post-harvest can potentially reduce sources of pathogenic microorganisms, most microbial interventions are done once a spice enters a

processing facility *(109)*. During primary production, contamination can be caused by wildlife, contaminated irrigation water, and improper drying. Spices can also be contaminated during transportation and storage. In the manufacturing and processing environment, contamination of spices and other low water activity food products is often due to poor sanitation practices, poor facility and equipment design, lack of good manufacturing practices (GMPs), poor ingredient control and handling, and poor pest control *(78)*. Because of its ease of use and ability to be quickly generated onsite, chlorine dioxide gas can be used at any point along the spice supply chain, including at farm level during primary production or during transportation of the product. While it might not achieve as great of a reduction of *Salmonella* as other spice processing techniques, chlorine dioxide has potential to be used in conjunction with other antimicrobial processing methods.

Although spices did not appear to the naked eye to be affected by chlorine dioxide gas, effects on sensory qualities were not specifically studied in the current experiment. A bleaching effect has been observed in produce that has been exposed to chlorine dioxide gas *(28, 63, 79, 101, 102)*. Further research to measure the effect of chlorine dioxide gas on sensory qualities in spices, such as color and volatile chemistry, may be needed.

In conclusion, chlorine dioxide gas is effective to reduce *Salmonella* numbers on black pepper, cumin, and sesame seed. Increasing ClO_2 gas concentration generally leads to greater reduction in *Salmonella* on treated spice. *Salmonella* numbers also decrease over a 30-day storage period. Chlorine dioxide gas generated on site may be a cost-effective microbial intervention for small-scale processors that do not want to utilize one of the commonly used antimicrobial spice processes. Chlorine dioxide gas may have utility as an extra microbial intervention step earlier in the production, transport, or processing chain to ensure greater spice

safety. Additional data on the combined effect of chlorine dioxide and other factors such as relative humidity and temperature would be useful to determine if the efficacy of the gas treatment could be increased by manipulating these factors.

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Table 1. Mean $\log_{10}$ *Salmonella* CFU/g of ClO_2 treated black pepper.

		Salmonella population[a] ($\log_{10}$ CFU/g)			
		Storage day			
Media type	Treatment[b] (mg ClO_2/kg pepper)	0	1	10	30
BHIN[c]	0	$5.58^{Ax} \pm 0.13$	$5.37^{Ax} \pm 0.18$	$4.47^{Ay} \pm 0.30$	$3.50^{Az} \pm 0.28$
	100	$3.83^{Bx} \pm 0.13$	$3.83^{Bx} \pm 0.16$	$2.95^{By} \pm 0.14$	$2.90^{By} \pm 0.00$
	200	$3.81^{Bx} \pm 0.17$	$3.62^{By} \pm 0.12$	$2.91^{By} \pm 0.03$	$2.90^{By} \pm 0.00$
	500	$3.40^{Cx} \pm 0.15$	$3.23^{Cx} \pm 0.24$	$2.96^{Bx} \pm 0.11$	$2.90^{Bx} \pm 0.00$
XLDN[d]	0	$5.07^{Ax} \pm 0.08$	$4.91^{Ax} \pm 0.11$	$3.94^{Ay} \pm 0.39$	$3.05^{Az} \pm 0.16$
	100	$3.31^{Bx} \pm 0.14$	$3.11^{By} \pm 0.14$	$2.90^{Bz} \pm 0.00$	$2.90^{Bz} \pm 0.00$
	200	$3.18^{Bx} \pm 0.23$	$2.96^{By} \pm 0.09$	$2.90^{By} \pm 0.00$	$2.90^{By} \pm 0.00$
	500	$2.90^{Cx} \pm 0.00$	$2.91^{Cx} \pm 0.01$	$2.90^{Bx} \pm 0.00$	$2.90^{Bx} \pm 0.00$

$^{A\text{-}D}$Mean values (n=9) with unlike superscripts within a column and media type are significantly different by Tukey-Kramer test ($p<0.05$).
$^{x\text{-}z}$Mean values (n=9) with unlike superscripts within a row are significantly different by Tukey-Kramer test ($p<0.05$).
[a]The limit of detection for the enumeration method was 2.90 log *Salmonella* CFU/g (800 CFU/g).
[b]Spices were treated with gaseous ClO_2 for 12 h.
[c]Brain heart infusion agar supplemented with 50 µg nalidixic acid/mL (BHIN) was used to enumerate healthy and sublethally injured *Salmonella* cells.
[d]Xylose lysine desoxycholate agar supplemented with 50 µg nalidixic acid/mL (XLDN) was used to enumerate healthy *Salmonella* cells.

Table 2. Mean $\log_{10}$ *Salmonella* CFU/g of ClO_2 treated cumin.

		Salmonella population[a] ($\log_{10}$ CFU/g)			
		Storage day			
Media type	Treatment[b] (mg ClO_2/kg cumin)	0	1	10	30
BHIN[c]	0	$7.54^{Ax} \pm 0.27$	$7.31^{Axy} \pm 0.31$	$7.05^{Ay} \pm 0.21$	$6.02^{Az} \pm 0.55$
	100	$6.73^{Bx} \pm 0.20$	$6.46^{Bxy} \pm 0.32$	$6.05^{By} \pm 0.17$	$4.39^{Bz} \pm 0.69$
	200	$5.68^{Cx} \pm 0.68$	$5.66^{Cxy} \pm 0.67$	$4.95^{Cy} \pm 0.51$	$3.11^{Cz} \pm 0.31$
	500	$5.00^{Dx} \pm 0.58$	$4.82^{Dx} \pm 0.49$	$3.88^{Dy} \pm 0.40$	$2.90^{Cz} \pm 0.00$
XLDN[d]	0	$7.13^{Ax} \pm 0.28$	$7.05^{Ax} \pm 0.34$	$6.52^{Ay} \pm 0.42$	$5.28^{Az} \pm 0.46$
	100	$5.86^{Bx} \pm 0.18$	$5.80^{Bx} \pm 0.31$	$5.36^{By} \pm 0.31$	$3.49^{Bz} \pm 0.46$
	200	$5.42^{Bx} \pm 0.57$	$5.39^{Bx} \pm 0.66$	$4.56^{Cy} \pm 0.48$	$2.90^{Cz} \pm 0.00$
	500	$4.51^{Cx} \pm 0.50$	$4.31^{Cx} \pm 0.53$	$3.40^{Dy} \pm 0.38$	$2.90^{Cy} \pm 0.00$

$^{A\text{-}D}$Mean values (n=9) with unlike superscripts within a column and media type are significantly different by Tukey-Kramer test ($p<0.05$).

$^{x\text{-}z}$Mean values (n=9) with unlike superscripts within a row are significantly different by Tukey-Kramer test ($p<0.05$).

[a]The limit of detection for the enumeration method was 2.90 log *Salmonella* CFU/g (800 CFU/g).

[b]Spices were treated with gaseous ClO_2 for 12 h.

[c]Brain heart infusion agar supplemented with 50 µg nalidixic acid/mL (BHIN) was used to enumerate healthy and sublethally injured *Salmonella* cells.

[d]Xylose lysine desoxycholate agar supplemented with 50 µg nalidixic acid/mL (XLDN) was used to enumerate healthy *Salmonella* cells.

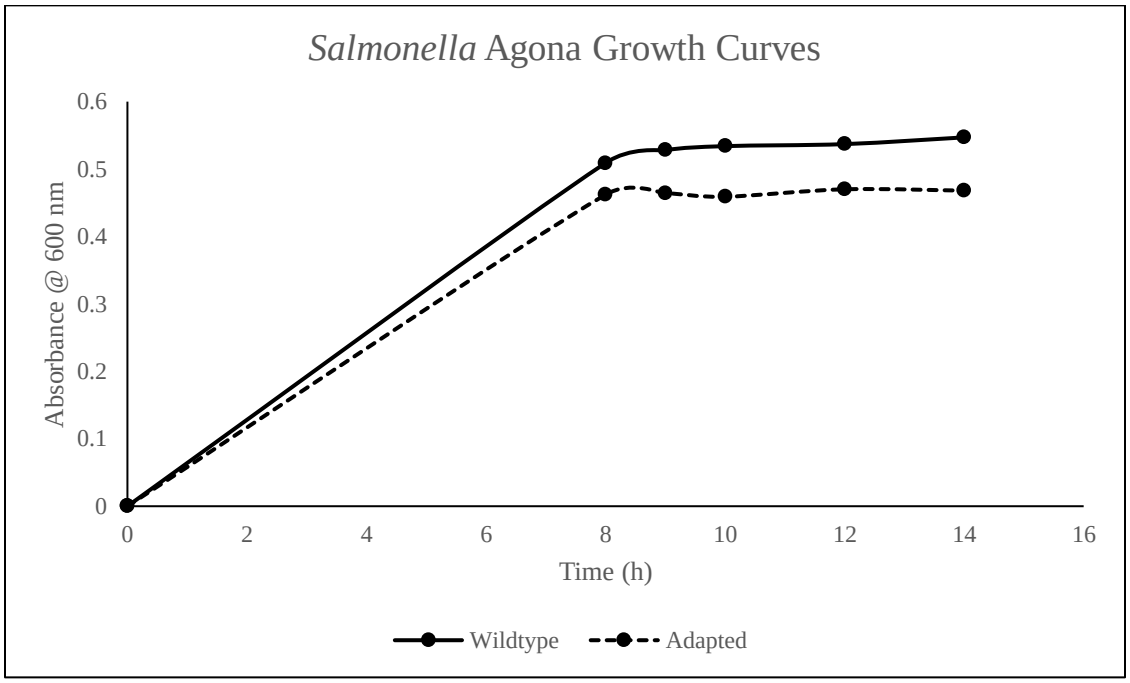
Table 3. Mean $\log_{10}$ *Salmonella* CFU/g of ClO_2 treated sesame seed.

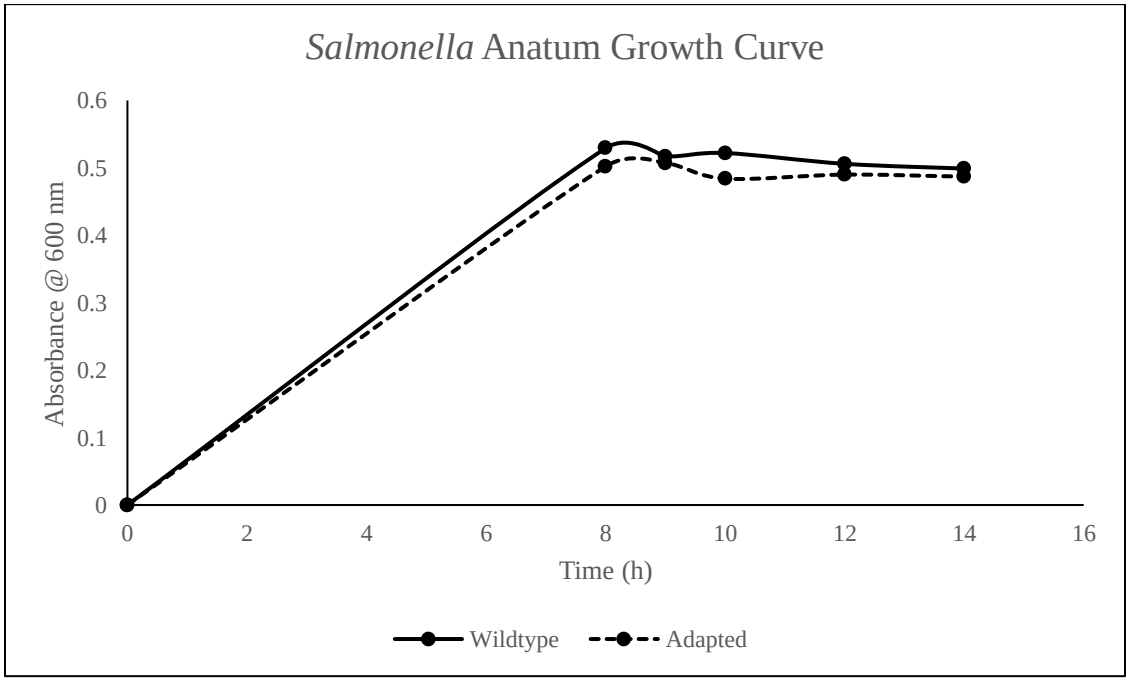
		Salmonella population[a] ($\log_{10}$ CFU/g)			
		Storage day			
Media type	Treatment[b] (mg ClO_2/kg seed)	0	1	10	30
BHIN[c]	0	$7.77^{Ax} \pm 0.17$	$7.74^{Ax} \pm 0.17$	$7.45^{Ay} \pm 0.12$	$6.93^{Az} \pm 0.27$
	100	$6.20^{Bx} \pm 0.14$	$6.20^{Bx} \pm 0.07$	$6.09^{Bx} \pm 0.08$	$5.47^{By} \pm 0.62$
	200	$5.69^{Cx} \pm 0.32$	$5.80^{Cx} \pm 0.20$	$5.69^{Cx} \pm 0.20$	$5.31^{By} \pm 0.33$
	500	$5.17^{Dx} \pm 0.57$	$5.31^{Dx} \pm 0.25$	$4.97^{Dx} \pm 0.11$	$3.32^{Cy} \pm 0.40$
XLDN[d]	0	$7.61^{Ax} \pm 0.05$	$7.57^{Ax} \pm 0.18$	$7.30^{Ay} \pm 0.17$	$6.62^{Az} \pm 0.29$
	100	$5.96^{Bx} \pm 0.05$	$5.95^{Bx} \pm 0.03$	$5.86^{Bx} \pm 0.06$	$5.01^{By} \pm 0.75$
	200	$5.49^{Cx} \pm 0.46$	$5.60^{Cxy} \pm 0.24$	$5.48^{Bxy} \pm 0.31$	$5.01^{By} \pm 0.53$
	500	$4.87^{Dx} \pm 0.45$	$4.95^{Dx} \pm 0.26$	$4.69^{Cx} \pm 0.56$	$2.92^{Cy} \pm 0.06$

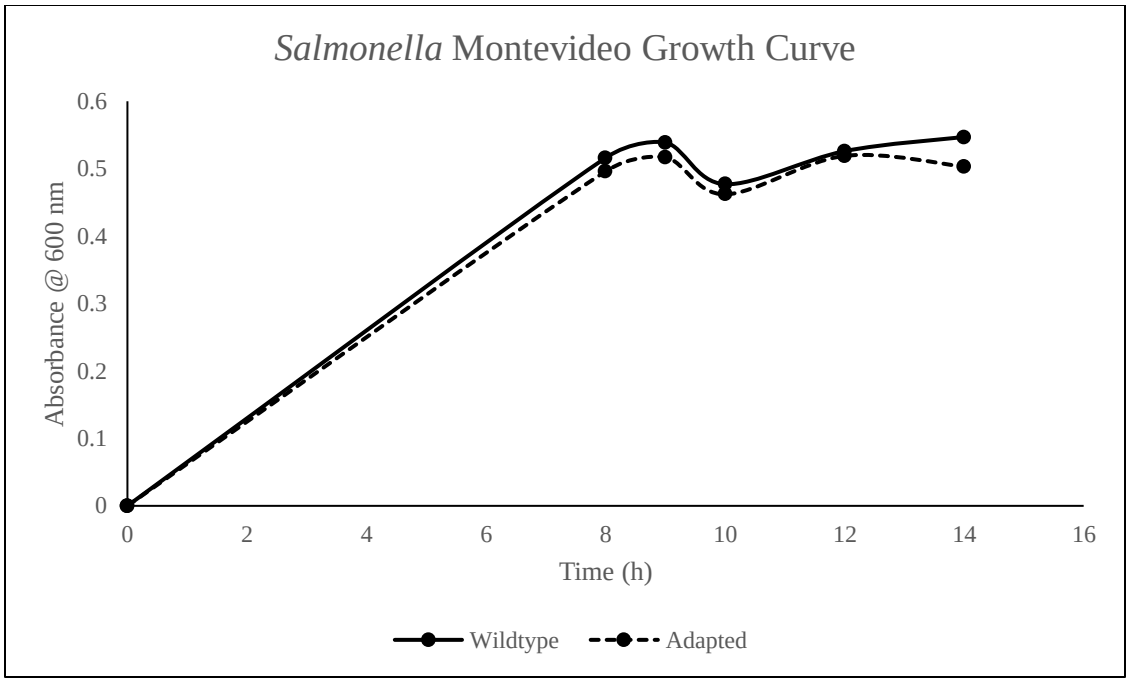
[A-D]Mean values (n=9) with unlike superscripts within a column and media type are significantly different by Tukey-Kramer test ($p<0.05$).
[x-z]Mean values (n=9) with unlike superscripts within a row are significantly different by Tukey-Kramer test ($p<0.05$).
[a]The limit of detection for the enumeration method was 2.90 log *Salmonella* CFU/g (800 CFU/g).
[b]Spices were treated with gaseous ClO_2 for 12 h.
[c]Brain heart infusion agar supplemented with 50 µg nalidixic acid/mL (BHIN) was used to enumerate healthy and sublethally injured *Salmonella* cells.
[d]Xylose lysine desoxycholate agar supplemented with 50 µg nalidixic acid/mL (XLDN) was used to enumerate healthy *Salmonella* cells.

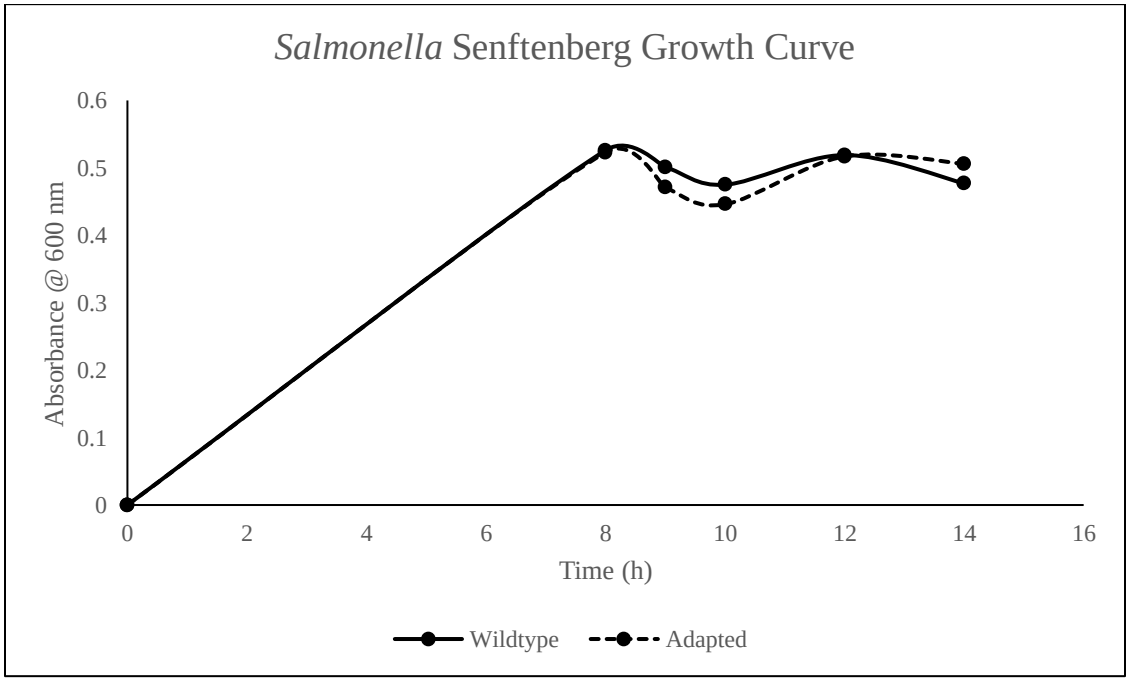
APPENDIX A

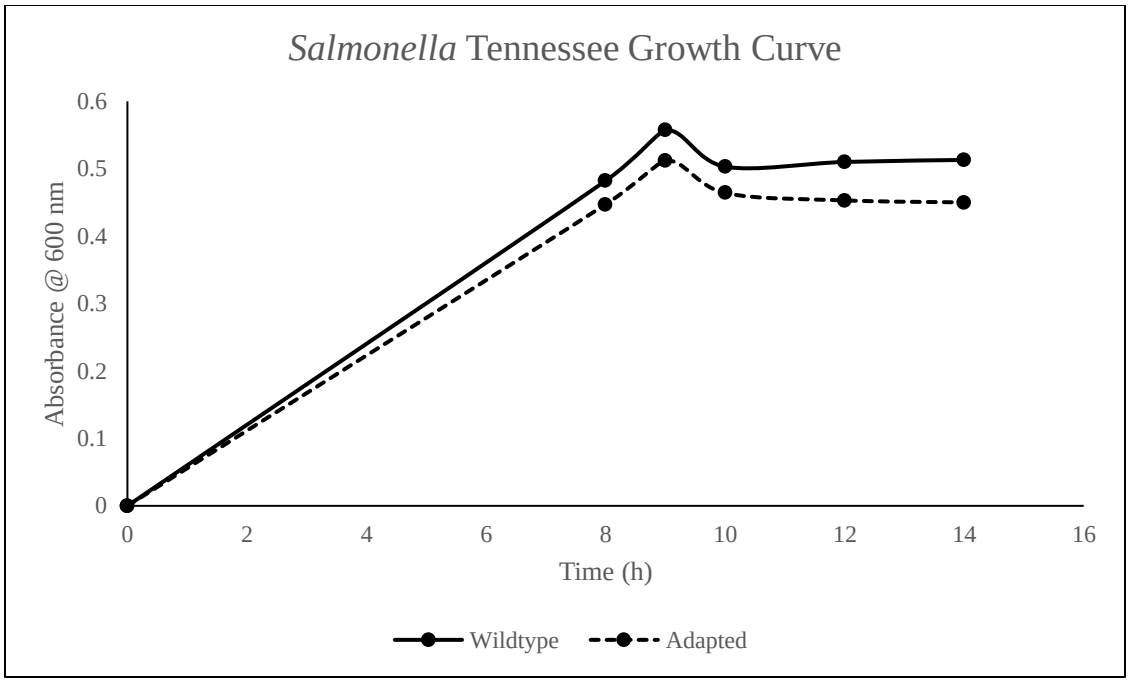
SALMONELLA GROWTH CURVES





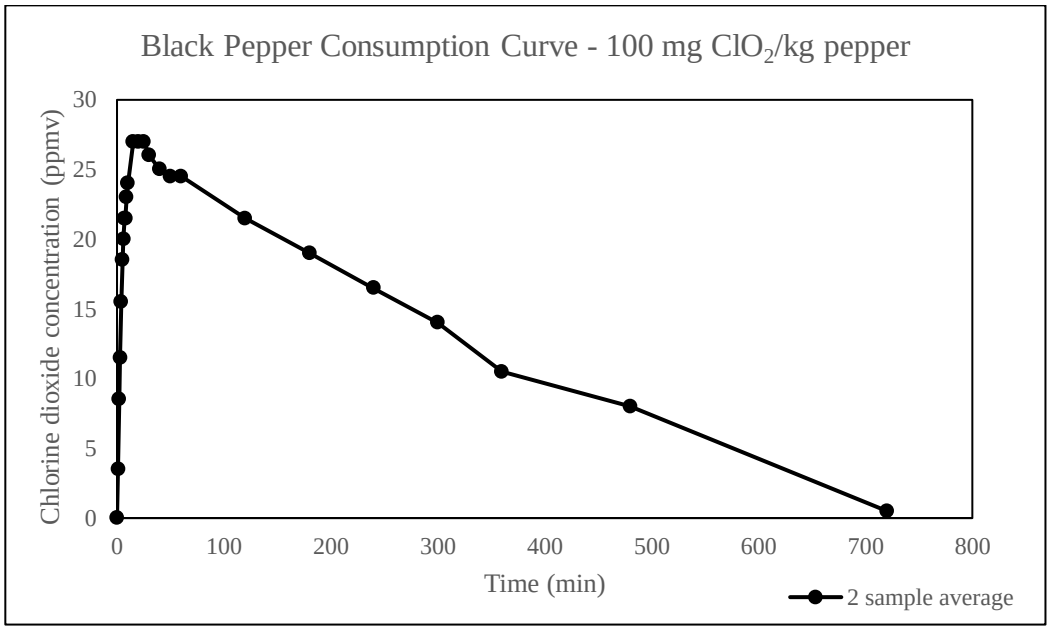

Salmonella Montevideo Growth Curve
Absorbance @ 600 nm
0.6
0.5
0.4
0.3
0.2
0.1
0
0
2
4
6
8
10
12
14
16
Time (h)
Wildtype
Adapted

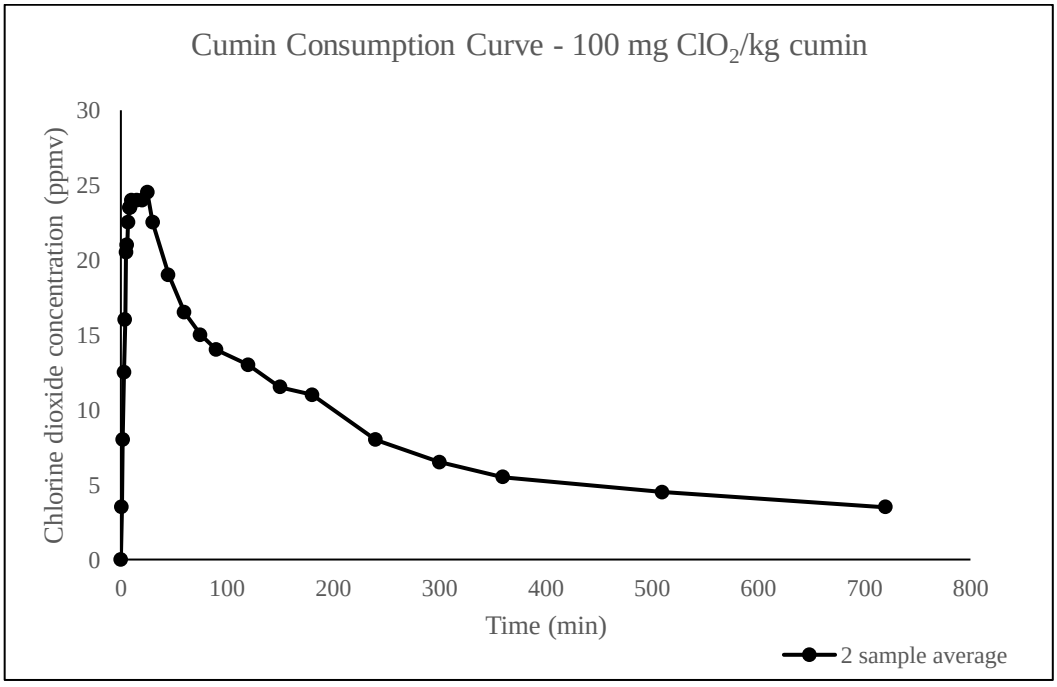

Salmonella Senftenberg Growth Curve
Absorbance @ 600 nm
0.6
0.5
0.4
0.3
0.2
0.1
0
0
2
4
6
8
10
12
14
16
Time (h)
Wildtype
Adapted

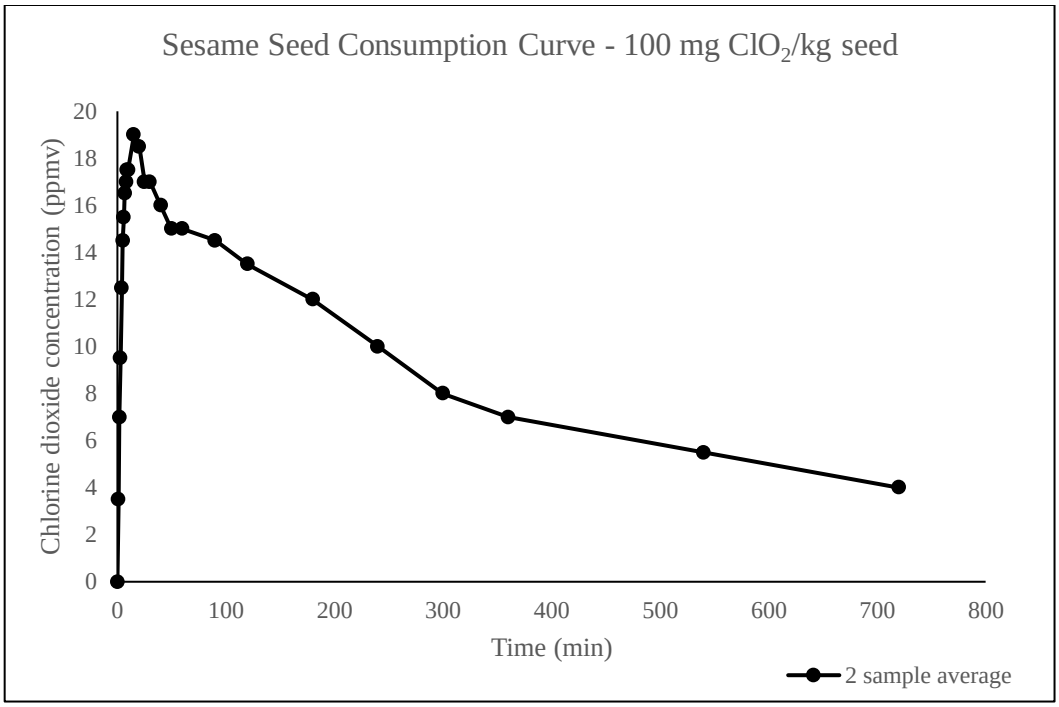

Salmonella Tennessee Growth Curve
Absorbance @ 600 nm
0.6
0.5
0.4
0.3
0.2
0.1
0
0
2
4
6
8
10
12
14
16
Time (h)
Wildtype
Adapted

APPENDIX B

CHLORINE DIOXIDE CONSUMPTION CURVES






Sesame Seed Consumption Curve - 100 mg ClO_2/kg seed
Chlorine dioxide concentration (ppmv)
0
2
4
6
8
10
12
14
16
18
20
0
100
200
300
400
500
600
700
800
Time (min)
2 sample average