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Radiofrequency and Gaseous Technologies for Enhancing the Microbiological Safety of Low Moisture Food Ingredients

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Radiofrequency and Gaseous Technologies for Enhancing the Microbiological Safety of Low Moisture Food Ingredients

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by

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Abstract

High heat resistance and long survival of *Salmonella* in low moisture food ingredients (LMFIs) such as spices and seeds are concerning as they are typically consumed without cooking. Therefore, it is challenging to effectively inactivate pathogenic bacteria without negatively impacting the quality of the treated product. This dissertation aimed to develop and evaluate novel intervention technologies: in-package radiofrequency steaming and non-thermal gaseous technologies to improve the microbial safety of LMFIs. The dissertation can be divided into three parts.

The first part of this dissertation on the thermal inactivation kinetics of *Salmonella* and a surrogate, *Enterococcus faecium* NRRL B-2354on black pepper powder indicated that microbial inactivation increased with increasing treatment temperature and water activity. Inoculation protocol also influenced the heat resistance of *Salmonella* where inoculation of black peppercorns pre-grinding had higher D-values compared to those inoculated post-grinding.

The second part of this dissertation aimed at developing an in-package pasteurization process to inactivate *Salmonella enterica* in spices (black peppercorn) and herbs (dried basil leaves). During RF heating, the one-way steam vent enabled the accumulation of steam inside the package improving the heating uniformity before venting off excess steam. In-package radiofrequency steaming reduced *Salmonella* below detection levels on dried basil leaves within 35 s in a bottle sealed with a steam vent and 40 s in polymer packages with steam-vent and on black peppercorns within 155 s in a polymer package.

A single intervention technology is not fit for all LMF matrices. Thermal processing would not be feasible for chia seeds due to the potential oxidation of fats and gelling in the presence of moisture. Therefore, the third part of the study explored non-thermal antimicrobial

gaseous technologies, such as chlorine dioxide (ClO_2), and ethylene oxide (EtO) gas on the decontamination of chia seeds. The developed response surface model suggested that an increase in gas concentration, relative humidity, and treatment time enhanced the microbial reduction on chia seeds. At gas concentration of 10 mg/L and 80% RH over a 5 h exposure period; *Salmonella* and *E. faecium* populations were reduced by 3.7 ± 0.2 and 3.2 ± 0.3 log CFU/g, respectively. Mild heating at 60 °C after ClO_2 (90 %RH, 3 mg/L for 2 h) followed by ambient storage for seven days enhanced the inactivation to achieve 5-log reduction. The quality of treated products was not significantly impacted except for an increase in peroxide value after ClO_2 treatment. EtO inactivation was faster than ClO_2 treatment on chia seeds providing more than 5 log reduction of *Salmonella* within 10 minutes at 50% RH and 60 °C without significantly affecting its quality. *E. faecium* was a suitable surrogate for *Salmonella* in all intervention technologies investigated in this study. The developed predictive models would benefit food industries in identifying the process parameters for improving LMFIs safety without altering the nutritional and sensorial qualities of food.

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Dedication

I wholeheartedly dedicate this dissertation to my cherished family, friends, and the divine presence of God who have played an integral role in shaping my academic journey.

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List of Published Papers

Chapter 2. in this dissertation has been published in Comprehensive Reviews in Food Science and Food Safety

Wason, S., Verma, T., & Subbiah, J. (2021). Validation of process technologies for enhancing the safety of low-moisture foods: A review. *Comprehensive Reviews in Food Science and Food Safety*, 20(5), 4950-4992.

Chapter 3. in this dissertation has been published in Food Research International

Wason, S., Verma, T., Wei, X., Mauromoustakos, A., & Subbiah, J. (2022). Thermal inactivation kinetics of *Salmonella enterica* and *Enterococcus faecium* NRRL B-2354 as a function of temperature and water activity in fine ground black pepper. *Food Research International*, *157*, 111393.

Chapter 4. in this dissertation has been submitted to Journal of Food Safety

Wason, S., Fuerte, Y.K.N., Villa-Rojas, R., & Subbiah, J. Effect of Inoculation (pre- versus post-grinding) of black pepper on decimal reduction time of *Salmonella* spp. and *Enterococcus faecium* NRRL B-2354.

Chapter 5. in this dissertation has been published in Food Control

Wason, S., Verma, T., Irmak, S., & Subbiah, J. (2022). In-package pasteurization of dried basil leaves using radiofrequency heating. *Food Control*, *141*, 109179.

Chapter 6. in this dissertation has been submitted to the Journal of Food Protection

Wason, S., Villa-Rojas, R., Subbiah, J. Radiofrequency pasteurization of black peppercorns and dried basil leaves using in-package steaming.

Chapter 7. in this dissertation has been published in Food Control

Wason, S., & Subbiah, J. (2023). Gaseous chlorine dioxide for inactivating *Salmonella enterica* and *Enterococcus faecium* NRRL B-2354 on chia seeds. *Food Control*, 109736.

Chapter 8. will be submitted to Innovative Food Science and Emerging Technologies.

Wason, S., Eskridge, K., Acuff, J., & Subbiah, J. Mild heating and ambient storage following gaseous chlorine dioxide treatment of chia seeds enhanced inactivation of *Salmonella* spp.

Chapter 9. in this dissertation will be submitted to Journal of Food Microbiology

Wason, S., Irmak, S., & Subbiah, J. Effect of temperature and relative humidity on ethylene oxide inactivation of *Salmonella* and *Enterococcus faecium* NRRL B-2354 on chia seeds

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Chapter 1. Introduction

1.1 Background

Low moisture food ingredients (LMFIs) are those that have water activity lower than 0.85 (FAO/WHO, 2014). Low moisture foods are becoming a major concern in terms of foodborne illness outbreaks and recalls due to *Salmonella* contamination. Low moisture food products including flour, spices, nuts, and herbs have been considered low-risk commodities for microbial contamination as they do not offer a favorable environment for the pathogenic bacteria to reproduce. However, in the past decade, the association of pathogenic bacteria with low moisture foods has gained the attention of the regulatory and scientific community. While growth no longer happens, the pathogenic bacteria that may have been present already in food survive in the desiccated environment for a long period of time. Therefore, low moisture foods can no longer be considered microbiologically safe simply because they do not support the growth of pathogenic bacteria like *Salmonella* (Podolak et al., 2010). To mitigate and control such hazards, food industries are required to implement and validate the processing interventions under the U.S. Food and Drug Administration (FDA), Food Safety Modernization Act (FMSA) that was passed in 2011 (Bracket et al., 2014).

The number of foodborne illness outbreaks associated with LMFIs during 2011-2015 was 21 and it increased by 67% to 35 during 2016-2020 (Acuff et al., 2023). It is evident from these statistics that the low moisture food safety concern is on the rise. Nuts, nut products, and seeds contributed to most outbreaks and recalls. These accounted for over half (51.9%) of outbreaks reported from 2012 to 2020. Furthermore, spices were linked to only one foodborne illness outbreak, but led to 82 recalls. The major cause of this foodborne illness outbreak is due to *Salmonella* contamination in food (Acuff et al., 2023; Wason et al., 2021). It is also important to

consider the fact that many of the outbreaks across the world would remain untracked or unreported. The variation in susceptibility due to immune responses to pathogenic infection between the population of developed, developing, and under-developed countries can also exist. The outbreak incidents and recalls necessitate the development and validation of process controls to ensure the microbial safety of the finished product. Even though the number of recalls is less for low moisture foods when compared to the meat and fresh produce industry, the impact can still be significant. Low moisture foods such as spices and nuts are consumed as ready to eat food or seasoning without prior cooking, increasing the risk of foodborne illnesses. Further, the infectious dose of *Salmonella* is as low as 13 cells, indicating a greater risk from the consumption of ready-to-eat foods such as cereals, spices, chocolate, etc (Wason et al., 2021). Low moisture foods have a longer shelf life and therefore recalling food has always been difficult following an outbreak. Because low moisture foods are used as ingredients in many food products, a particular foodborne illness outbreak may lead to recall of multiple food products produced since the last cleaning of the food facility. These outbreaks and associated food recalls pose a huge economic burden on food industries.

Several thermal and non-thermal technologies such as the use of heat, steam, radiofrequency, cold plasma, high pressure, and gaseous treatments have been studied to improve the safety of low moisture foods. Thermal inactivation using dry heat and steam could lead to quality deterioration in low moisture foods because of the long treatment times required to achieve desired log reduction. Traditional pasteurization of low moisture food is not sufficient to ensure safety as a result of increasing heat resistance among most foodborne pathogens specifically in dry environments. Radiofrequency is a volumetric heating method which is rapid and could provide desired log reduction at lower treatment times without significantly affecting

the quality of food. Non-thermal techniques such as irradiation, pulsed electric field, and high pressure have been evaluated as alternative treatments but have limitations to be used on different physical forms of food. Some non-thermal techniques such as CO_2 and high-pressure processing require the presence of water for microbial inactivation and are not suitable for low moisture foods. In the present research, it was intended to explore the potential of gaseous technologies such as ozone, ethylene oxide, and chlorine dioxide as intervention technologies against microbial pathogens in low moisture foods. In addition, gaseous technologies could be more effective due to their high diffusion and penetration abilities.

With the Food Safety Modernization Act (FSMA) regulations for process validation, a suitable non-pathogenic surrogate needs to be evaluated for the food industry to adopt. Surrogate microorganisms are those that show the following characteristics: non-pathogenic (Biosafety level 1); show similar inactivation kinetics as the pathogen of concern; easy to propagate; and easy to differentiate from other microorganisms. There is a need for identification of surrogate microorganisms and develop standard protocols to help the food industry in the validation of processes that are necessary for the control of pathogenic microorganisms.

1.2 Current Research Gap

Low moisture food ingredients (LMFIs) are increasingly reported for foodborne illness outbreaks and recalls due to *Salmonella* contamination. *Salmonella* exhibits enhanced heat resistance at desiccated conditions. Therefore, it is challenging to effectively inactivate pathogenic bacteria without negatively impacting the quality of the treated product. Additionally, the effectiveness of these treatments can be influenced by factors such as water activity, pH, and particle size, which can vary significantly between different low moisture foods. However, comparing different published microbial models can be often difficult due to variations in the

thermal inactivation protocols, serovars of *Salmonella* used in the study, and the difference in modeling approach. For instance, Gautam et al., (2020) developed two secondary models to predict the effect of either temperature or water activity (but not both simultaneously) on the inactivation of *Salmonella* in black pepper powder. For easier application of the developed models to determine the process parameters for thermal pasteurization, our study aimed to develop a combined model for predicting the effect of both temperature and water activity on thermal inactivation simultaneously. Earlier studies with black pepper (Gautam et al., 2020; Wei et al., 2021), did not report the effect of particle size on the thermal inactivation of *Salmonella*, even though the particle size was reported to impact the heating rate and inactivation kinetics of *Escherichia coli* ATCC 25922 in red pepper powder (Zhang et al., 2020). Hence, **a comprehensive inactivation kinetics of *Salmonella* in black pepper at various water activities is required to evaluate existing processes or identify optimal process conditions to achieve a desirable food safety.**

LMFIs such as spices and nuts are consumed as a whole and in ground form. They are prone to contamination if hygienic conditions are not maintained during production, processing, grinding, packaging, etc. Black pepper may get contaminated in the farms during production. When black peppercorns are ground to powder, any *Salmonella* contamination could transfer to the ground black pepper. Moreover, ground black pepper can get cross-contaminated post processing if proper sanitary conditions are not maintained in the manufacturing plant. Limcharoenchat et al. (2018) reported *Salmonella* to be more heat resistant in almond meal and date paste when almonds and dates were inoculated before fabrication as compared to inoculation of products post-fabrication. However, the opposite was true in the case of wheat flour, where inoculation pre-fabrication made *Salmonella* more sensitive to heat as compared to

post-fabrication. It is evident that inoculation protocol might affect the thermal sensitivity of *Salmonella* necessitating to identify appropriate thermal treatment to ensure the safety of processed product. Therefore, spice industries must consider the **impact of inoculation method (pre-grinding and post grinding) on heat resistance of *Salmonella* for thermal processing of black pepper**.

Radiofrequency (RF) heating is one promising technology that can be used to revolutionize low moisture foods manufacturing. With extensive research in the past few years, RF heating has been proven to be effective in pasteurizing various agricultural food products, including spices, nuts, powders, grains, and others (Altemimi et al., 2019; Huang et al., 2018; Jiang et al., 2020; Ling et al., 2020; Zhang et al., 2021; Zhou & Wang, 2019). However, current RF treatment still mainly pasteurizes the bulk products first and then packaged separately, which does not eliminate the potential cross-contamination issue. Non-uniform heating also has been identified as another big challenge in RF heating that needs improvement (Huang et al., 2018). Therefore, there is a critical need to improve current RF pasteurization technology that addresses the cross-contamination and non-uniform heating issues for manufacturing low moisture foods and ingredients. Steam vent packages have been used in microwavable food products to improve the heating uniformity and safety of the meals. The packaging is designed to build steam in a sealed plastic pouch or container that has a self-venting release valve. Microwaving and simultaneously generated steam offer even distribution of heat and decreased cooking time improving food safety. For instance, microwave-assisted steam generation could inactivate *Vibrio vulnificus* and *Vibrio parahaemolyticus* in oyster meat also retaining the sensory quality of the cooked meat (Espinoza, 2013). However, the steam vent packages that are typically used for microwavable food products do not seal after it is ruptured. This type of steam vent package is

suitable for foods that are designed to be consumed immediately after heating. Recent technological advances in packaging allow for the integration of one-way steam vent in the package that can reliably seal after steam release. In-package pasteurization of spices (dried basil leaves and black peppercorns) using steam vent package must be evaluated **for its effect on RF heating profiles, microbial inactivation, and the quality of treated products** to guide the spice industries in application of this novel technology.

A single technology could not fit all LMFI processing. Due to fat oxidation in high-fat foods such as chia seeds and potential gelling in the presence of moisture, RF processing would not be feasible for processing chia seeds. Moreover, the enhanced thermal resistance of *Salmonella* in low moisture foods prevents effective decontamination. Therefore, alternative non-thermal antimicrobial gaseous technologies must be explored. Gases generally have higher diffusion coefficients than liquids allowing them to diffuse deeper and quicker into pores inside bulk low moisture foods. Ethylene oxide (EtO) sterilization is commonly used by the food industry to inactivate pathogens in spices, but its efficacy on edible seeds, a high-risk food commodity, remains unexplored. The Environment Protection Agency (EPA) has emphasized the need to investigate alternative decontamination strategies for commodities that typically have high pathogen loads and currently lack an effective decontamination strategy (EPA, 2023b). Because there is currently no standard processing method available for chia seeds, studies on ethylene oxide inactivation would provide useful information on the processing parameters for effective decontamination of chia seeds using EtO gas. Gaseous chlorine dioxide (ClO_2) is an antimicrobial gas with high oxidizing potential offering bactericidal properties. ClO_2 gas has been used to improve the microbial safety of drinking water for the past two decades (Benarde et al., 1965; Jonnalagadda & Nadupalli, 2014). Research on ClO_2 gas technologies is extensively

studied for decontamination of high moisture foods; while studies exploring its inactivation efficacy on low moisture foods are limited. ClO_2 oxidizes bacterial cell constituents while EtO acts on the sulfhydryl, hydroxyl, amino, and carboxyl groups of bacterial cells by adding alkyl groups to affect the cellular metabolism ultimately leading to cell lysis (Wason et al., 2023). The difference in microbial inactivation mechanisms between the two gaseous technologies could contribute to variation in bacterial inactivation. Therefore, the use of gaseous technology as a single intervention strategy and as a combination treatment such as mild heating needs further exploration for bacterial inactivation in low moisture foods Process conditions such as temperature and RH influence the efficacy of gaseous technologies. By optimizing process conditions, it is possible to reduce the severity (concentration or treatment time) of gaseous technologies to achieve a desired microbial inactivation. Therefore, a comprehensive study to investigate **the antimicrobial efficacy of chlorine dioxide and ethylene oxide gas, the impact of process parameters such as relative humidity, temperature, gas concentration, and its impact on the quality of treated chia seeds** is necessary.

Studies on gaseous treatments and in-package RF steaming of low moisture foods are very limited and food processors have no reference point for selecting process parameters and critical limits to comply with the Food Safety Modernization Act (FSMA) Preventive Controls rule, thus hampering trials/adoption of the technology. Hence, there is a need for validation of decontamination methods using surrogates that are more thermally resistant than *Salmonella.* In general, *Enterococcus faecium* B2354 has shown higher heat resistance than *Salmonella* and thus has been confirmed as a suitable surrogate for *Salmonella* in various low moisture foods, including ground black pepper (Wei et al., 2019; Wei et al., 2021a), milk powders (Wei et al., 2021b), paprika and white pepper (Ozturk et al., 2020), dried basil leaves (Verma et al., 2021),

etc. Although some studies have confirmed the use of *Enterococcus faecium* NRRL B-2354 as a surrogate for certain processes, there is little information available for its use as a surrogate for other technologies such as the gaseous treatments, since the selection of surrogate is product and process specific. Therefore, it is critical to **validate *E. faecium* as a surrogate against *Salmonella* for gaseous treatment of various low-moisture foods to help the food industry with in-plant validations.**

1.3 Goals & Objectives

Objective I: Investigate the parameters impacting the heat resistance of *Salmonella.*

1. Thermal inactivation kinetics of *Salmonella enterica* and *Enterococcus faecium* NRRL B- 2354 as a function of temperature and water activity in fine ground black pepper.
2. Effect of Inoculation (pre- versus post-grinding) of black pepper on decimal reduction time of *Salmonella spp.* and *Enterococcus faecium* NRRL B-2354.

Objective II: Radiofrequency processing for enhancing the microbial safety of pre-packaged spices.

3. In-package pasteurization of dried basil leaves using radiofrequency heating in bottle.
4. Radiofrequency pasteurization of black peppercorns and dried basil leaves using in-package steaming.

Objective III: Antimicrobial gaseous technologies for improving the microbial safety of chia seeds.

5. Gaseous chlorine dioxide for inactivating *Salmonella enterica* and *Enterococcus faecium* NRRL B-2354 on chia seeds.

6. Mild heating and ambient storage following gaseous chlorine dioxide treatment of chia seeds enhanced inactivation of *Salmonella* spp.
7. Effect of temperature and relative humidity on ethylene oxide inactivation of Salmonella and Enterococcus faecium NRRL B-2354 on chia seeds

1.3 Dissertation Organization

This dissertation provides insight into the challenges and potential solutions for ensuring the safety of low moisture food ingredients. It consists of 10 chapters. Chapter 1. Provides the background/introduction to low moisture food safety, microbial interventions, current research gap, and significance of the study. Chapter 2. provides a comprehensive literature review on process technologies and their effectiveness in ensuring the safety of low-moisture foods.

Chapters 3 and 4 are part of Objective I. In chapter 3, the thermal inactivation kinetics of *Salmonella enterica* and *Enterococcus faecium* NRRL B- 2354 were determined as a function of temperature and water activity in fine ground black pepper. A modified Bigelow model was developed which could be beneficial for the industries to assess the impact of process parameters on microbial inactivation and identify suitable processing conditions. In Chapter 4, the effect of inoculation protocol on the decimal reduction time (D-value) thermal resistance of *Salmonella cocktail* and *E. faecium* in black pepper. Pre-grinding and post-grinding inoculation was compared based on D-value of *Salmonella enterica. E. faecium* was evaluated as a surrogate for *Salmonella* during thermal treatment.

Objective II includes Chapters 5 and 6, on radiofrequency processing for enhancing the microbial safety of spices and herbs packaged in two different types of packaging material. Chapter 5 studied the efficacy of in-package pasteurization of dried basil leaves in a polypropylene bottle using radiofrequency heating. Chapter 6 examined the radiofrequency

pasteurization of black peppercorns and dried basil leaves by in-package steaming using a steam vent film package. The effect of in-package steaming on the quality of spices and herbs was analyzed in terms of color, a_w, moisture content (MC, % wb), antioxidant activity, total phenolic content, and volatile composition.

Objective III consists of Chapters 7 – 9 on antimicrobial gaseous technologies for improving the microbial safety of chia seeds. In Chapter 7, gaseous chlorine dioxide was investigated for inactivation of *Salmonella enterica* and *Enterococcus faecium* NRRL B-2354 on chia seeds. Chapter 8 assessed the effect of mild heating and ambient storage post ClO_2 treatment to improve the inactivation of *Salmonella* spp. on chia seeds. Chapter 9 evaluated the ethylene oxide inactivation of *Salmonella* and *Enterococcus faecium* on chia seeds as a function of temperature and relative humidity. Following the gaseous treatment, the quality of chia seeds was analyzed in terms of color, a_w, MC, fatty acid composition, peroxide value, germination ability, and gas residues or byproduct formation.

As per the FSMA and the need for validation of decontamination methods using surrogates, *E. faecium* was evaluated for its suitability as a surrogate for the above-mentioned technologies. The last chapter (Chapter 10) summarizes the whole dissertation and provides some suggestions for future research.

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Chapter 2. Literature Review

2.1 Abstract

The outbreaks linked to food borne illnesses in low moisture foods are frequently reported due to the occurrence of pathogenic microorganisms such as *Salmonella* Spp. *Bacillus cereus, Clostridium* spp., *Cronobacter sakazakii, Escherichia coli, and Staphylococcus aureus*. The ability of the pathogens to withstand dry conditions and to develop resistance to heat are regarded as the major concerns for the food industry dealing with low moisture foods. In this regard, the present review is aimed to discuss the importance and the use of novel thermal and non-thermal technologies such as RF, steam pasteurization, plasma, and gaseous technologies for decontamination of food borne pathogens in low moisture foods and their microbial inactivation mechanisms. The review also summarizes the various sources of contamination and the factors influencing the survival and thermal resistance of pathogenic microorganisms in low moisture foods. The literature survey indicated that the non-thermal techniques such as CO_2, high-pressure processing etc., may not offer effective microbial inactivation in low moisture foods due to their insufficient moisture content. On the other hand, gases can penetrate deep inside the commodities and pores due to their higher diffusion properties and are regarded to have an advantage over thermal and other non-thermal processes. Further research is required to evaluate newer intervention strategies and combination treatments to enhance the microbial inactivation in low-moisture foods without significantly altering their organoleptic and nutritional quality.

Keywords: microbial inactivation, ozone, hydrogen peroxide, chlorine dioxide, low moisture foods

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2.2 Introduction

According to the Food and Agriculture Organization, low moisture foods are those that have water activity lower than 0.85 (Batz et al., 2014). They are either low in moisture or those with initial high moisture that undergoes a drying or dehydration process (Grocery Manufacturers Association, 2009). Low moisture food products including flour, spices, nuts, and herbs have been considered as low-risk commodities for microbial contamination as they do not offer a favorable environment for the pathogenic bacteria to reproduce. However, in the past decade, the association of pathogenic bacteria with low moisture foods has gained the attention of the regulatory and scientific community. While the growth no longer happens, the pathogenic bacteria that may have been present already in food survives in the desiccated environment for long period of time. Therefore, low moisture foods can no longer be considered microbiologically safe simply because it does not support the growth of pathogenic bacteria like *Salmonella* (Podolak, Enache, Stone, Black, & Elliott, 2010).

Table 1 summarizes the outbreaks pertinent to low moisture foods from 1973-2019. It is evident from the Table that the number of outbreaks reported in low moisture foods have seen reasonable increase over the decades. In the last decade alone about 18 incidences have been recorded infecting more than 1300 individuals. About 80% of the foodborne illnesses was due to *Salmonella* contamination, followed by *E. coli* (16%) and *Listeria* (4%). Among the various foods associated with *Salmonella* contamination, butter products such as peanut butter, nut butter, and soynut butter were more frequently reported probably due to its higher fat content. Infants were found to be the most susceptible group to foodborne illnesses in low moisture foods. For instance, 3000 infections were reported for *Salmonella* contamination in infant milk formula in Trinidad in 1973. Interestingly, all the outbreaks reported have been in western countries or

developed countries, except for an outbreak in Trinidad. However, it is important to note that many of the outbreaks across the world are under-reported. Variation in susceptibility due to immune response to pathogenic infection among the population of developed, developing, and under-developed countries may exist. The outbreak incidents and recalls necessitate the development and validation of process controls to ensure microbial safety of low-moisture foods and ingredients.

To mitigate and control such hazards, the food industries are required to implement and validate the processing interventions under the U.S. Food and Drug Administration (FDA), Food Safety Modernization Act (FMSA) that was passed in 2011 (Brackett, Ocasio, Waters, Barach, & Wan, 2014). FSMA requires processors to validate all intervention technologies. There is a strong need to evaluate and validate both the legacy and the novel technologies as a kill step for pasteurization purposes. In this context, this review article attempts to summarize the literature on the use of thermal and non-thermal pasteurization technologies for improving the microbial safety of low moisture foods.

2.3 Sources of Contamination

In general, commodities such as spices and nuts that grow under open fields and orchards are prone to multiple contaminants leading to foodborne illness outbreaks (Table 1). The variations in the prevailing conditions in the cultivation areas can pose a severe threat for increased outbreaks of foodborne illness and intoxications (Rendlen, 2004). Because the collection and operation of spices do not always take place under extreme hygienic conditions, it can increase the microbial load and related damage to produce (McKee, 1995). Spices are dried under the natural solar energy by placing them on the floor, thereby increasing the risk of

contamination by birds, filth, insects, etc. In addition, the fate of the pathogen which enters the food system is not certain and can elevate at various stages right from food production, processing, and until consumption. Despite having low water activity, these commodities are also prone to pathogenic contamination at various stages of processing. The products may get contaminated at various unit operations such as drying, dehulling, milling, packaging, and storage. For instance, the outbreak related to *Salmonella* Enteritidis contamination in raw almonds was reported in Canada and the United States in 2000 and 2001. It was anticipated that the field colonies of *Salmonella which* had colonized the plant environment and the processing equipment could have attributed to the contamination of almonds during processing (Elliot, 2005; Isaacs et al., 2005). Frelka and Harris (2015) reported that aerobic plate count and *Escherichia coli* counts in inshell walnuts increased by 1 log during harvest of walnuts from the tree. Walnut shells could break during harvesting and processing which exposes the kernels to the outside environment leading to contamination. In another case, *Salmonella* Tennessee found in environmental samples within the plant was associated with the contamination of peanut butter in an international outbreak in 2007. The cross contamination between the two processing lines was found to be the cause for illness due to *Salmonella* contamination in infant milk powder (Funk, 2007). Cross contamination of a cereal batch with the cleaning remains from the milling machine was reported to be the cause of an outbreak related to the contamination of infant food with *Salmonella* Senftenberg in 1995 (Rushdy et al., 1998). Carrasco, Morales-Rueda, & García-Gimeno (2012) stated low plant standards such as poor sanitation, improper structural and equipment design, and low maintenance are the probable causes for contamination of *Salmonella* in low moisture foods. For instance, the outbreak related to *Salmonella* Agona contamination in toasted oat cereal necessitated the investigation for source of contamination

within the facility. It was observed that there was a high level of contamination at multiple points within the plant including in floor, production equipment, and the exhaust system in the plant (Breuer, 1999). In a 2008-2009 nationwide outbreak associated with *Salmonella* Typhimurium contamination in peanut, the open gaps located at the air conditioner in the roof of the plant was found to be the source for draining in rainwater into the packaging room, roasted product, and packaging line of the facility (FDA, 2009). The cross-contamination between the product lots, tainted equipment, packing operations, spillage, and the pathogenic survivors on cardboard and other packaging materials are also the sources of contamination.

Foods such as bakery products get contaminated with mold spores as well as endospores of *Bacillus subtilis* when allowed to cool for a longer time in contaminated and tainted food contact surfaces before packaging (Erickson, 2019). Apart from food contact surfaces, worker's hygiene is another critical source of contamination. For example, Morita, Kitazawa, Lida, & Kamata (2006) suggested restricting the movement of operators to avoid *Salmonella* contamination in oil meal factory, as the employee's shoes and gloves were found to be contaminated with *Salmonella* after 1 day of disinfection.

In a low moisture food plant, wet cleaning is not common due to food safety issues. Introduction of water to a low- moisture food production area could adulterate the produce, develop mold growth and affect the shelf life of the product. Hence, it was suggested that reducing water in these environments would increase the safety of low moisture foods as a whole (Kornacki, 2012; Lupo, 2013). On the other hand, in high moisture foods like meat, the facility is cleaned every day and if there is an outbreak, the product produced during that particular day is recalled. However, in case of low moisture foods, the complete cleaning of the facility may occur only once a year. Any associated foodborne outbreak would require the recall of all the products

since the last cleaning of the facility. Moreover, low moisture foods are often used as ingredients in other food products resulting in recall of several other food products produced with those low moisture ingredients. This makes it a huge task for the industry and some brands may even go out of business. For instance, paprika and sesame used as ingredients in spice mixture, snacks and tahini-based foods were reported to be the source of *Salmonella* contamination (Lehmacher, Bockemühl & Aleksic, 1995; Unicomb et al., 2005). In another scenario, cocoa powder used as an ingredient in the confectionery products was found to be the source of contamination of *Salmonella* Durham in an outbreak infecting 110 people in Sweden (Gastrin et al., 1972).

Spices and sesame seeds are prone to *Salmonella* contamination during growth, storage, and processing and can contaminate other finished products if good manufacturing practices (GMP) are not followed. In an outbreak involving *Salmonella* Typhimurium DT 104 contamination in sesame candy, the source of contamination was investigated at various points of processing. It was found that, because the commercial manufacturing of sesame candy involves high-temperature processing, there may not be any potential contamination during the production process. Hence, the cross-contamination of sesame products following the heat treatment step was reported to be the point of contamination in the manufacturing plants (Brockmann, Piechotowski, & Kimmig, 2004).

Even though the recalls are lower for low moisture food when compared to meat and produce industries, the impact can still be significant. Moreover, low moisture foods have a longer shelf life and therefore recalling food has always been difficult following an outbreak. The consumers may not be aware of the food safety issues and may be exposed to huge risk of foodborne illness after consumption of the recalled produce.

Pathogenic bacteria such as *Salmonella* sustain its viability even on dry stainless-steel surfaces and have been reported to contaminate the foods for a minimum of 4 days from highly contaminated surfaces (Kusumaningrum, Riboldi, Hazeleger, & Beumer, 2003). In addition, the pathogens were able to transfer from these test surfaces with higher transfer rates of 20 to 100% depending on the initial contamination levels. Due to the increased tolerance of *Salmonella* even in stainless steel, which has no nutrients for the pathogens, the incidence of foodborne illness in low moisture foods is not surprising.

2.4 Foodborne Pathogens in Dry Environments - Persistence and Thermal Resistance

Biological agents require nutrients, water, optimum pH, and temperature for its growth. Microorganisms can grow at a water activity (a_w) of as low as 0.60. The majority of the bacteria can grow at water activity greater than 0.87. Among bacteria, *Salmonella* is the most common foodborne pathogen associated with repeated outbreaks in low moisture foods such as chocolate, milk powder, peanuts, nuts, spices, among others (Table 1). It is universal and may spread through cross-contamination in the environment from animals, soil, and food (Winfield & Groisman, 2003).

2.4.1 Factors affecting survival of foodborne pathogens in low moisture foods

a. Water activity (a_w)

Water activity (a_w) is a crucial factor affecting the survival rate of foodborne pathogens in low moisture foods. Although lower water activity may inhibit the growth of pathogenic microorganisms, vegetative cells and spores can persist for a longer period (Podolak et al., 2010). The minimum levels of water activity required for growth depends on the temperature, pH, and

type of solute used to reduce a_w level (Mattick et al., 2001). According to the International Commission on Microbiological Specification for Foods (ICMSF), under optimal conditions *S. aureus* can grow at a_w as low as 0.83; but the minimum a_w is 0.85 in most foods. (ICMSF, 1996). Some species of xerophilic spoilage molds and osmophilic yeasts can grow at a_w between 0.60 to 0.70, but the minimum a_w for mycotoxin production by molds is 0.80; majority of them do not produce mycotoxins at a_w of 0.85 (Cousin, Riley, & Pestka, 2005). In a storage study, Keller, VanDoren, Grasso, & Halik (2013) observed that black pepper inoculated at 7.0-8.0 log CFU/g and stored at higher RH (97%) showed longer *Salmonella* survival at 25°C than at 35°C with its population reaching below detection limit (1.69 log CFU/g) after 100 and 45 days, respectively. In contrast, storage at lower ambient RH levels (47% RH), *Salmonella* decay was observed initially, but as a_w reached 0.13-0.23, the bacterial population remained stable throughout the year. It was further reported that *Salmonella* could readily grow to maximum population densities (~9.0 log CFU/g) at permissive growth conditions (>0.96 a_w and 30°C) within 24 hours which might occur in a food facility when small quantities of water come in contact with the ground pepper due to condensation. In general, *Salmonella* survival is longer under lower RH conditions than at high RH, a higher ambient temperature at the same RH results in higher population decay rates (Keller et al., 2013).

Similarly, Farakos, Pouillot, & Keller (2017) indicated the significant effect of a_w on the survival characteristics of pathogenic microorganisms in tree nuts by observing decreased survival at a higher a_w of 0.54 as compared to a lower a_w of 0.37. Heat resistance may also depend on the type of dry matrix in which the microorganism exists (Fong & Wang, 2016). Further, *Salmonella* survives longer in low moisture food at adverse environments such as very low water activity, when compared to slightly higher water activity. For example, Rachon,

Peñaloza & Gibbs (2016) reported that during heating, *Salmonella* was better able to survive in the inoculated chicken meat flour ($a_w = 0.38$) followed by confectionery ($a_w = 0.57$) and culinary seasoning ($a_w = 0.66$) or pet food ($a_w = 0.65$).

b. Temperature

Salmonella can grow at temperatures from 2-54°C, optimal growth is observed at temperatures between 35-37°C (Beuchat, 2009). Hiramatsu, Matsumoto, Sakae & Miyazaki (2005) investigated the survival resistance of 18 strains of *Salmonella*, 35 Shiga-toxin *E. coli* (STEC), and 5 *shigella* under desiccated conditions in a paper disk. The results from their study showed that all the *Salmonella* strains and majority of STEC strains survived for 24 months at 4°C without a significant log reduction demonstrating strong resistance to dryness during refrigerated storage following drying for 24 hours. However, these strains failed to survive at higher temperatures of 25°C and 35°C. Farakos et al. (2017) observed that the persistent survival of *Salmonella* increased when the temperature was decreased from 25°C to 4°C. Gruzdev, Pinto, & Sela (2011) observed higher heat resistance of *Salmonella* at dry environments. Desiccated *Salmonella* demonstrated tolerance to 1 hour exposure to dry heat showing no significant log reduction at 60°C and 1.5 and 3.1 log reductions at 80°C and 100°C, respectively. However, non-desiccated and rehydrated *Salmonella* could completely be inactivated within 10 and 20 min, respectively. Tapia, Alzamora & Chirife, 2020 suggested that less vibration of water molecules during heating due to less amount of water present at low a_w conditions resulted in increased heat tolerance of pathogens in low moisture foods. Mattick et al. (2001) reported higher tolerance of *Salmonella* cells at lower a_w to temperatures greater than 70°C. The response mechanism in bacteria leads to change in morphology when exposed to temperature stresses. For instance, some bacterial cells shrink in size, while other cells tend to increase in size under unfavorable

conditions. At refrigeration temperatures, *E. coli* and *Salmonella enterica* subsp. *enterica* serovars Enteritidis and Typhimurium developed filaments which continue to grow, multiply and eventually lyses into normal cells when the temperature was increased to 30-37°C. Similarly, cells of *Clostridium acidiurici* and *E. coli* elongated when grown at elevated temperatures (Hoffman & Frank, 1963; Terry, Gaffar, & Sagers, 1966; Mattick et al., 2003).

c. Presence of solutes

The presence of sucrose significantly enhanced the survival (log_{10} (dry food/dried paper disk)) of the *Salmonella* and STEC strains in chocolate containing 30 – 40% sucrose by more than 100 times as compared to the survival in dried paper disks without sucrose. In contrast, presence of salt in dry foods could reduce the survival rates of *Salmonella* and STEC strains (Hiramatsu et al., 2005). Interestingly, it was observed that corn syrup and salt enhanced the survival of *S.* Typhimurium on egg powders (a_w = 0.29 – 0.37) at 37°C. However, it did not have protective ability at higher a_w levels of 0.51 – 0.61 at 37°C (Jung & Beuchat, 1999). At temperatures greater than 100°C, the time taken by the bacteria to reduce by 5.0 log was higher in confectionery than in chicken flour; mostly due to the protective effect of sugars present in the confectionary (Rachon et al., 2016). Under desiccated conditions, added sugars such as sucrose and trehalose inhibit structural damage by replacing water in the bacterial cell membrane and by preserving the structure of protein (O'Byrne and Booth, 2002). In addition, the bacteria might possess thermal resistance by accumulating intracellular sucrose/trehalose by de novo synthesis and/or translocation (O'Byrne & Booth, 2002; Young et al., 2015). In contrast, glycerol did not result in increased heat resistance when *Salmonella* serovars were adapted to lower a_w (Peña-Meléndez, Perry & Yousef, 2014). Gruzdev et al. (2011) found that the survival of *Salmonella* exposed to NaCl, bile salts, ethanol, hydrogen peroxide enhanced upon desiccation. While

Salmonella inoculated in acidic LMFs such as dry apples and dry pickled sour plums with pH less than 4 was found to be susceptible to acidity and could not survive for 24 hours at 25°C.

d. Food composition

Along with temperature and a_w, proximate composition of food such as fat, protein, sugar content also plays a significant role in desiccation resistance of pathogenic microorganisms. Exposures to increased fat content and decreased a_w both were associated with a protective effect on the survival of *S.* Tennessee in the simulated gastric fluid compared with control cells. Peanut butter inoculated with *S*. Tennessee after a simulated intestinal phase treatment for 5 hours resulted in an increase in the populations of *S.* Tennessee inoculated in peanut butter by 2.0 log CFU/g as compared to non-digested control cells. This study demonstrates that cross-protection from low-a_w stress and the presence of high fat result in improved survival in the low pH of the stomach (Aviles, Klotz, Smith, Williams, & Ponder, 2013). In the case of treenuts, Farakos et al. (2017) observed no effect on survival with respect to variation in fat content. In addition, He, Guo, Yang, Tortorello, & Zhang (2011) reported that higher carbohydrate content in peanut butter and incubation at lower temperature (25°C) for 30 days enhanced survival during storage but reduced heat resistance of *S. enterica* and *E. coli* O157:H7. Further, *S. enterica* population survived better than *E. coli* O157:H7 in peanut butter. The potential for interaction of food matrix and stress adaptations could influence the virulence of *Salmonella* and should be considered for risk analysis (Aviles et al., 2013).

e. Type of foodborne pathogenic bacteria

Salmonella is ubiquitous (Podolak et al., 2010) and has been reported in a variety of low moisture foods such as infant milk powder and peanut butter (Table. 1). At favorable conditions

such as temperature, humidity, and pH, they can survive for weeks in water and for years in soil by adapting to different hosts and medium such as soil and water. For instance, *Salmonella enterica* serovar Typhimurium was reported to survive in artificially contaminated water for about 8 weeks (Moore, Martinez, Gay & Rice, 2003) and about 231 days in contaminated compost-amended soil (Islam et al., 2004). In addition, *Salmonella* is reported to survive for a longer period of time in dry foods and feeds (Hiramatsu et al., 2005). Janning, Veld, Notermans, & Krämer (1994) studied the effect of dry conditions (a_w = 0.20) at 22 °C on the survival of 18 bacterial strains. They observed that though there was an initial decrease in cell numbers up to 2.7 log (CFP/ml), the strains of *Salmonella* were stable for 112 days, requiring 248 to 1,351 days to observe about 1.0 log reduction**.** *Salmonella* was more desiccation resistant than *Enterobacter cloacae* and *E. coli* (Podolak et al., 2010). Further, strain-specific survival of pathogens such as *Salmonella* have been reported by fewer studies. For instance, Ng, Bayne, & Garibaldi (1969) observed strain specific response in *Salmonella,* where *S.* Senftenberg 775W was found to be the most heat resistant in aqueous solution among the 300 different strains studied. While Goepfert & Biggie (1968) observed *S.* Senftenberg strain to be less heat resistant than *S*. Typhimurium in chocolate.

Cronobacter species is another important opportunistic pathogen which can grow both in high as well as in low a_w. It was linked to foodborne illness in infants due to the consumption of reconstituted powdered infant formula produced from dried milk powder (Norberg et al., 2012). Different strains of *C. sakazakii* are reported to exhibit varying survival characteristics attributed to its physical composition. Beuchat (2009) reported that the *C. sakazakii* environmental strain, which was able to survive longer, formed crinkled, matte colonies with tough, rubbery texture as compared to mucoidal colonies formed by clinical strain on violet, red bile glucose agar. *C.*

sakazakii (strains 2 and 25), *C. vulneris*, and *Klebsiella oxytoca* were able to survive for up to 2.5 years (Barron & Forsythe, 2007). The most resilient species was *K. oxytoca*, which had a 3.9 log reduction in viability over 2.5 years of observation; whereas other microorganisms had a 7.0 log decline or were not recoverable. These findings also revealed that capsulated strains had higher survival and greater recovery even after 2 years of storage. The ability of *C. sakazakii* strains to survive under stress conditions could be attributed to their ability to form extracellular polysaccharide (Lehner et al., 2005). Erickson (2019) reported that toxigenic and pathogenic fungal species were the main concerns related to food safety in low moisture foods.

2.4.2 Mechanism of survival

The exposure of *Salmonella* to a single stress increases the chances of developing cross-tolerances to multiple stresses leading to more virulent cells (Abee and Wouters, 1999; Hecker & Volker, 2001; Kultz, 2005; Vorob'eva, 2003). There are a variety of regulators that control the stress response in *Salmonella*. These regulators such as sigma factors, transcriptional regulators or phospho-relay-based two component systems are responsible for up or down regulation of genes specific to the stress response. Two-component system comprises a response mechanism to the external stimuli through a variety of target gene expression. This contributes to the protective ability for pathogens to survive and persist in dry environments (Spector & Kenyon, 2012). Under low nutrient, osmotic and other stress conditions, the sigma factor RpoS regulate the gene expression required for virulence in *Salmonella* spp. and pathogenic *E. coli* strains (Dineen et al., 1998; Dong, Joyce, & Schellhorn, 2008). The action of RpoS-regulated stress response results in a sequence of physiological and morphological changes (Hengge-Aronis, 2002). RpoS directs the transcription of proP, aiding in transportation of the compatible solute glycine betaine and otsBA gene involved in trehalose biosynthesis (Dong & Schellhorn, 2009; Ibanez-Ruiz, Robbe-

Saule, Hermant, Labrude, & Norel, 2000; Mellies, Wise, & Villarejo, 1995; Vijayakumar, Kirchhof, Patten, & Schellhorn, 2004).

Even though *Salmonella* has been implicated in several foodborne illness outbreaks, the mechanism by which *Salmonella* survives in dry environments is not clear. However, Mandal & Kwon (2017) elucidated the role of genes to overcome desiccation stress. *S. typhimurium* was selected and its library of more than 350000 mutants were exposed to desiccation. The desiccated Tn5 mutant enzymes were revived, DNA extract were prepared for genome sequencing. Gene encoding for proteins attributing to the survival characteristics during desiccation were identified through Con-ARTIST pipeline (Conditionally essential loci - Analysis of high-Resolution Transposon Insertion Sequences Technique). This technique is used for classifying specific regions critical for growth and survival under optimal and stressed conditions, respectively. These genes were associated in transport of ions, repair to overcome DNA damage, energy utilization for maintenance of metabolism, formation of extracellular polysaccharide, translation, ribosomal structure, and transcription, post translational modification, protein turnover, and chaperones, etc. Another important characteristic of pathogenic microorganisms is structural features that allow them to persist for longer times under desiccation. For instance, thin aggregative fimbriae or curli and extracellular cellulose in *S.* Typhimurium is attributed for its long-term survival under desiccation (Barnhart & Chapman, 2006; Riedel & Lehner, 2007; White, Gibson, Kim, Kay, & Surette, 2006). Biofilms play an important role in imparting desiccation resistance to a variety of foodborne pathogens. Biosynthesis of extracellular cellulose and curli protein is regulated by CsgD protein and it is also required for formation of *Salmonella* biofilms (Gerstel & Römling, 2003; Jain & Chen, 2007; Römling, 2005). The majority of *Salmonella* serotypes exhibit the patterned, aggregative colonies on the growth surface (Anriany,

Weiner, Johnson, De Rezende, & Joseph, 2001; Romling et al., 2003). This characteristic is known as rdar morphotype (it is red, dry, and rough and produces both curli and cellulose). White, Gibson, Kim, Kay, & Surette, 2006 suggested that this morphotype among *S. enterica* along with its survival advantages could contribute to its transmission between hosts. This provides an understanding of desiccation resistance in *Salmonella*.

Another crucial foodborne pathogen is *L. monocytogenes* that imparts varying degrees of desiccation resistance depending on its serotypes. Serotype 1/2b shows higher resistance to dryness than other serotype strains based on evaluation of 31 known desiccation-associated genes (Zoz et al., 2017). Proteomics could potentially demonstrate adaptation mechanism of osmotically stressed cells. During osmotic stress, the response of bacterial cells includes release of water leading to a decrease in turgor pressure and increase in the concentration of intracellular metabolites and ions. This water was restored by an active potassium influx and glutamate synthesis which was then replaced by osmoprotectants.

Riedel & Lehner (2007) investigated the changes in the protein profiles of *C. sakazakii* to two types of dry stress (physical desiccation and growth in hyperosmotic media). The upregulation of outer membrane porins (OmpC gene) in both stressed cells exhibited broad substrate specificity by gaining benefits from the osmoprotectants contained within the growth medium. It was also proposed that the presence of osmoprotectants in the growth media decreases the transcription of the osmotically induced genes. In addition, the supercoiling state of the cell promotes transcriptional activation of osmotic stress response genes. Elevated levels of osmolarity enables the rapid negative DNA supercoiling followed by a set of events linked to restore intracellular water and transcription of osmoregulated genes for synthesis of osmoprotectants (Cheung, Badarinarayana, Selinger, Janse, & Church, 2003; Jordi & Higgins,

2000; Potts, 2010). Osmoprotectants balance the osmolarity of the cell with that of external environment by concentrating to high levels in order to prevent the water loss.

Many studies reported that pathogenic microorganisms exposed to adverse conditions such as low water activity is more virulent than those suspended at higher water activities (Finn, Condell, McClure, Amézquita, & Fanning, 2013; Werber et al., 2005). A low infectious dose as low as 13.0 CFU/g has been reported in several foodborne illness outbreaks due to *Salmonella* contamination in low moisture foods (Finn et al., 2013). In case of *Escherichia coli* O157:H7, a low infectious dose of 5-50 CFU/g was reported for skimmed milk powder (Paswan and Park, 2020). While for the *Cronobacter*, WHO/FAO (2007) reported an infectious dose of 104 bacteria in infants by ingestion.

The high fat content of chocolate and peanut butter assists the ability of *Salmonella* to survive acidic environments in gastrointestinal phase and colonize (D'Aoust, 1977; Aviles et al., 2013). Along this line, Gruzdev et al. (2011) reported induced cross-tolerances to multiple stresses in *S. enterica.* A good understanding of the mechanism of bacterial survival will be helpful to develop new interventional technologies. Considering the limitations of current chemical and physical treatments, it is imperative to investigate intervention technologies to target the resistant pathogenic populations in low moisture foods.

2.5 Process Technologies for Microbial Inactivation in Low Moisture Foods

2.5.1 Thermal processes

Although several food preservation methods exist, the most widely used method is thermal processing. The use of heat to eliminate microbial hazards is usually more efficient in products

with a high-water activity ($a_w > 0.85$) (Bialka and Demirci, 2007; Nieto, Castro, and Alzamora, 2001). While thermal treatments will eliminate or reduce the microbial load, one needs to carefully evaluate the quality of final products in selecting the right processing method and parameters. Most of the technologies evaluated for microbial safety in low moisture foods are also commercially available. In this section, we briefly review different thermal methods and their efficacy to reduce microorganisms in low moisture foods. The mechanistic action of thermal inactivation of microorganisms is provided in Figure 1.

a. Extrusion

Extrusion cooking is a traditional process that has been widely used by the food industry for several decades. The extrusion process simultaneously combines various unit operations like cooking, mixing, kneading, shaping, and forming to create a variety of low-density, puffed cereals, and snack foods (Riaz, 2000). While the new designs and applications have been developed, the basic science behind extrusion has not undergone a drastic change in over 60 years. In an extrusion process, the raw materials are added to the extruder barrel, and the screws inside the barrel convey the food forward. During this time, the food material undergoes kneading with the help of screws and is exposed to high temperature and high pressure. Finally, the food material exits the barrel under pressure through a dye where the food material usually puffs due to moisture evaporation (Riaz, 2000). A variety of food products like breakfast cereals, corn puffs, pet foods, snacks can be produced using the extrusion process.

Extrusion process reduces the microbial load on food commodities due to the high temperature, pressure, and shear exerted on the product. Extrusion of products such as maize was reported to degrade the product DNA into many smaller fragments (Murray, Butler, Hardacre, &

Timmerman-Vaughan, 2007). Ukuku, Charles, & Sudarsan (2012) suggested that the shear stress created by the extrusion process on the bacterial cell is the primary factor responsible for their inactivation. Furthermore, the effect of shear stress on microbial inactivation was positively correlated with the temperature.

During the production of low moisture extruded food products, the moisture is added to the preconditioner either in the form of steam or water. The added moisture and the high temperature of the extruder barrel helps with the physicochemical transformation of the ingredients (Harper, 1994). The processing of ingredients at higher temperatures reduces the microbial population in the final food product. It is well known that pathogen such as *Salmonella* is easier to eliminate with the presence of excess moisture. Therefore, extrusion processing was assumed to eliminate the biological hazards from food due to the involvement of high temperature and high moisture, even though the final product has low moisture. However, with the implementation of the Food Safety Modernization Act (FSMA), the food industries are required to validate their process as a kill step such that it will effectively control the identified hazard.

According to the FSMA, validation is defined as the collection of scientific and technical evidence of the preventive control measure, if adequately implemented, can control the identified hazard. Table 2 summarizes the validation studies related to extrusion processing in different food products. Results from these studies showed that performing the extrusion process above 85˚ C is effective in reducing the microbial load during the extrusion process. A majority of these studies were conducted on a single-screw extruder with a limited number of parameters (temperature and moisture content) evaluated on the microbial reduction. The results from these studies suggested that temperature and moisture content have a positive effect on pathogen inactivation. Anderson et al. (2017) conducted a pilot-scale extrusion validation study where the

effect of temperature and water activity on *Salmonella* reduction was evaluated. The results showed that temperature above 82°C and a_w of 0.89 (20% moisture content) was sufficient to achieve 5.0 log reduction of *Salmonella* in oat flour. Although temperature and moisture content are critical parameters to monitor, it is equally important to evaluate other process variables such as screw speed, screw configuration, fat content, and their effect on microbial reduction.

Verma *et al.* (2018a) conducted a similar but more comprehensive study on a lab-scale single-screw extruder where a response surface model was developed to describe the effect of moisture content, fat content, screw speed, and temperature on the inactivation of *Salmonella* in oat flour. The results from their study suggested that >5.0 log reduction of *Salmonella* can be achieved when oat flour is extruded at a temperature above 85°C and screw speed of 150 rpm. Additionally, Verma et al. (2018a) reported that fat content had a protective effect on microbial reduction. For instance, as the fat content of the oat flour is increased from 5 to 15%, *Salmonella* reduction decreased from 4.5 to 2.0 log when extrusion was performed at 65°C and 150 rpm. Therefore, a higher extrusion temperature is required to achieve a desired reduction of *Salmonella* to compensate for the protective effect of higher fat content.

Even though the twin-screw extruder involves a higher capital and maintenance cost, the food industry still prefers to use a twin-screw extruder over a single screw because it offers many advantages. These included consistent product quality, easy processing of a wide range of raw materials, lower energy consumption, a higher level of process flexibility, better control of process parameters, and easy to use and clean (Riaz, 2000). Verma and Subbiah (2019) evaluated the effect of various product and process parameters on *Salmonella* inactivation during twin-screw extrusion of oat flour. The results showed that the *Salmonella* population was below the detection limit (<10.0 CFU/g) at a temperature ≥65°C. Overall, their study demonstrated that the

twin-screw extrusion was more effective in reducing the microbial load from the product than the single-screw extrusion.

The validation studies presented are specific to the process and product matrix. When a validation study is conducted on a different process, extrapolation of results using the response surface models should be avoided as the results may not be consistent with the new process being validated. Instead, the microbial inactivation trends seen in the literature can be utilized as a baseline for planning the extrusion validation experiment.

Due to the complexity of the extrusion process, the scale-up of the lab-scale results to the industrial scale extruder is difficult, which is one of the limitations associated with the extrusion validation study. Ainsworth, Ibanoglu, & Hayes (1997) suggested using residence time as a parameter for scaling-up the extrusion process and identifying the optimal conditions. However, several researchers found that parameters such as moisture content, screw speed, temperature, screw design, and die diameter affect the mean residence time (Harper, 1989; Kumar, Ganjyal, Jones, & Hanna, 2006; Nwabueze & Iwe, 2010; Yu, Meng, Ramaswamy, & Boye, 2014). Verma and Subbiah (2020) developed a response surface model where the effect of moisture content, fat content, screw speed, and temperature on the mean residence time was evaluated. The results showed that all the parameters except temperature had a significant effect on the mean residence time during the single-screw extrusion of oat flour. Additionally, Verma and Subbiah (2020) reported that replacing the screw speed with mean residence time did not significantly improve their previously developed inactivation models for *Salmonella* and *E. faecium* (Verma *et al.* 2018a, 2018b). Therefore, it was suggested to use screw speed instead of mean residence time for conducting the extrusion validation studies as it is easier and convenient for the food processors to control.

The other approach for scaling up the results would be to identify the critical parameters required to achieve the desired microbial reduction and conduct the industrial scale extrusion validation study using the non-pathogenic surrogate. Bianchini *et al.* (2012) reported *Enterococcus faecium* NRRL B-2354 as a suitable surrogate for *Salmonella* during extrusion of the carbohydrate-protein meal. The results of their study showed that *E. faecium* required a much higher temperature to inactivate than *Salmonella,* providing an appropriate margin of error for eliminating pathogens in the extrusion process. Verma, Wei, Lau, Bianchini, Eskridge, & Subbiah (2018b) used the same surrogate and compared its inactivation with *Salmonella* at different process parameters and product compositions during the extrusion of oat flour. They reported that *E. faecium* might be an acceptable surrogate for *Salmonella* during extrusion of low-moisture foods due to higher heat resistance; however, a surrogate with similar inactivation behavior may be preferred and needs identification.

Overall, the extrusion process is a promising thermal technology that has the capability to reduce or eliminate biological hazard from the food. Numerous combinations of extruder barrel screw and nozzle dies are used in the food industry to customize their products. Those factors will significantly impact the process conditions and therefore the microbial inactivation. The developed response surface models or optimal conditions for bacterial reduction are identified in the lab-scale extruder which may not work on an industrial-scale extruder. A multiphysics model can be developed to predict the process conditions (temperature, moisture content, shear) along the barrel screw. However, the validation of such a multiphysics model is challenging, as it is hard to measure those parameters at various points along the screw.

b. Radiofrequency

Radiofrequency (RF) heating is a novel thermal processing method that has been used by several researchers for the pasteurization of various food products. RF heating is a dielectric heating method operating in the range of 3 kHz – 300MHz. The heat is volumetrically generated due to the friction caused by ionic conduction and dipole rotation of water molecules (Piyasena, Dussault, Koutchma, Ramaswamy, & Awuah, 2003; Boreddy, Rose, & Subbiah, 2019; Wei, Lau, Stratton, Bianchini, & Subbiah, 2018; Lin, Subbiah, Chen, Verma, & Liu, 2020). RF heating offers many advantages such as faster heating rate, better heating uniformity, and a higher penetration depth, when compared to the conventional heating method (Jiao, Tang, & Wang, 2014).

The use of RF treatment for pasteurization of food products have been reported to exhibit thermal effect in inactivating microorganisms. Thermal effects are mostly regarded as the possible reason for the inactivation, as the heat generation attributes the cell death of microorganisms (Awuah, Ramaswamy, Economides, & Mallikarjunan, 2005; Shazman, Mizrahi, Cogan, & Shimoni, 2007; Hamoud-Agha, Curet, Simonin, & Boillerwaux, 2014). On the other hand, fewer studies have reported a high degree of microbial inactivation at lower product temperatures due to non-thermal effects (Saadi *et al.,* 2014). Non-thermal effects are highly unlikely, as low electric field intensity involved in RF heating cannot penetrate the cell membrane to achieve microbial inactivation.

According to the U.S. Federal Communications Commission, only three frequencies, i.e., 13.56, 27.12, and 40.68 MHz, have been authorized for industrial, scientific, and medical applications (Piyasena et al., 2003). These frequencies are selected to avoid any interference with

the communication system. RF heating has been commercially used for post-baking of cookies and thawing of meat (Dag, Singh, & Kong, 2020). Several studies have used the RF heating process for inactivating pathogenic bacteria in various low moisture foods. Table 3 summarizes the studies using RF heating for pasteurization of low moisture foods. Although RF heating proved to be a promising technique to inactivate pathogens, its industrial applications are limited due to the higher initial cost or is still under development for microbial decontamination.

In traditional thermal processing, the temperature gradient drives the heat transfer in the food product. In low moisture food, the heat transfer rate is low due to the lower thermal conductivity. This results in overheating of the edges due to which food products such as spices lose volatiles leading to quality deterioration (Boreddy, Thippareddi, Froning, & Subbbiah, 2016). However, with RF heating, the temperature gradient is not required as RF volumetrically heats up the food product. RF process allows to heat the food product rapidly to a high temperature within a short time which not only reduces the microbial load but also minimizes the quality deterioration. RF heating has also been reported to enhance the quality of low moisture foods compared to the traditional thermal processing. For example, Boreddy *et al.* (2016) reported that the foaming and gelling properties of egg white powder were significantly increased post RF-assisted thermal processing.

One of the disadvantages associated with the RF technique is the non-uniform heating of the food product. The non-uniform heating may lead to the overheating of some parts of the food product while the other part may still be cold, thereby causing the food safety issues in the product and deteriorate its quality (Piyasena et al., 2003; Tiwari, Wang, Tang, & Birla, 2011; Jiao et al., 2014). The heating non-uniformity in the food product could occur due to several factors such as container geometry, moisture content, thermal properties, and dielectric properties of

food. To reduce the non-uniform heating problem, Liu, Wang, Mao, Tang, & Tiwari (2013) suggested the use of hot air during the RF treatment of the sample. Similarly, Wang, Tang, Sun, Mitcham, Koral, & Birla (2006) reported that the mixing of the sample during RF treatment would improve its heating uniformity. However, it is a challenge to build a mixer that does not interact with the RF electric field and therefore, no successful mixing mechanisms have been developed or reported so far. The best method is to intermittently mix the samples without the presence of RF electric field. Therefore, it is critical to identify the cold spots and hot spots generated during non-uniform heating and should be used to evaluate the microbial inactivation in the food product.

During the RF heating, the multiple rays hit the food product from different directions which leads to the hot spots at the edges and corners. Liu et al. (2018) and Wei et al. (2018) reported that the cold spot was the top center in their food samples primarily due to the heat lost to the environment. Because of the low dielectric properties, the outside air is not heated up during the RF treatment causing the heat loss from the food product to the surrounding air. Due to this effect, the geometric center is usually hotter and the top center is the cold spot. Therefore, it is critical to identify the cold spot in the sample prior to conducting the validation studies. The cold spot and hot spots can be identified by inserting the fiber optic sensors in different layers of the sample and acquiring the temperature data during RF heating (Liu et al., 2018; Wei et al., 2018; Chen, Wei, Irmak, Chaves, & Subbiah, 2019; Lin et al., 2020). An inoculated pack method has been used by several researchers where the inoculated food product is packaged in a pouch and is placed in the cold spot (Liu et al. 2018; Wei et al. 2018; Chen et al. 2019; Lin et al. 2020). This method is followed in the RF validation studies.

During the RF heating, a considerable amount of moisture is lost from the food product due to steam generation. This not only affects the final food quality but also its shelf life. The American Spice Trade Association (ASTA) stipulates that moisture content of the black pepper during the storage should be less than 10.5% (American Spice Trade Association, 2011). The black pepper will be susceptible to fungal attack if the moisture content is >10.5%. To avoid the reduction in mass due to moisture mass, the spice industry tries to keep the moisture of black pepper close to the maximum safe moisture content, 10.5%. To achieve close to 10.5% moisture content after RF heating, several validation studies have suggested increasing the moisture content of the sample before the treatment. This not only contributes towards inactivating the microorganisms easily but also brings the final moisture content of the sample close to its native level post treatment. For instance, Wei, Lau, Stratton, Irmak, & Subbiah (2019) increased the moisture content of the ground black pepper to 12.8% before RF heating. The moisture content of the sample after RF heating reached 10.5%, meeting the ASTA storage guidelines. In addition, Wei et al. (2019) and Chen et al. (2019) reported that covering the sample container with a plastic film with the venting nut would help in releasing the excess steam from the treated sample and enhance the heating uniformity.

The use of RF heating for decontamination purposes for the food industry applications is still under development. With the FSMA regulations for process validation, a suitable non-pathogenic surrogate need to be evaluated for the food industry to adopt by validating RF heating at the industrial level. Several researchers have investigated the use *E. faecium* as a suitable surrogate for *Salmonella* in various low moisture foods (Table 3). These study results suggested *E. faecium* as an acceptable surrogate owing to its higher thermal resistance than *Salmonella*.

Overall, RF heating is a more promising thermal technique than the conventional methods to decontaminate food products. FSMA requires that the appropriate surrogate for target pathogen needs to be validated for different food matrices. Therefore, the identification of a surrogate for different food products is imperative to help the food industry conduct the in-plant validation studies. Modeling RF heating, adjusting the electrode gap, and configuring the package would help in improving the heating uniformity. If the food product is RF heated in a package, the moisture condensation may lead to the caking and lumping of the final food product. The use of steam vent packages will help in releasing the excess steam during the RF treatment and improve the final food quality.

c. Steam

Steam has been used for the decontamination of various food products due to its ability to effectively penetrate cavities and crevices that may provide protection to microorganisms (Morgan, Goldberg, Radewonuk, & Scullen*,* 1996).

The mechanism for microbial inactivation of steam pasteurization is similar to thermal process, as the steam process increases the temperatures of the food. These high temperatures are detrimental to the structure of proteins, nucleic acids, and lipids. It leads to the denaturation of proteins and nucleic acids, thus disrupting the cell metabolism. Lipids become too fluid within the cell membrane to continue to maintain the cellular content, thus, leading to cell lysis and inactivation of the microorganism.

Among different steam methods, wet steam is most commonly used to treat spices in the United States (ASTA, 2011). Although the wet steam method has proved to be effective in reducing the microbial load, the major disadvantage of using this method is the significant

increase in the moisture content of the treated sample, which can result in a reduction of shelf life of the final product. Lee, Oh, Chung, Reyes-De-Corcuera, Powers, & Kang (2006) used steam pasteurization method to achieve >4.0 log reduction of *Salmonella* Enteritidis from the surface of raw shelled almonds within 65 s of treatment. However, the wet treatment led to a significant quality loss as the almonds became puffy and skin peeled off very easily due to the high moisture content (Lee et al., 2006). Therefore, an additional drying step is necessary following the steam treatment to remove the excess moisture prior to storage.

Controlled condensation steam (CCS) is another strategy in steam processing which is employed to control the condensation on the products (Anderson, 2019). In a typical CSS process, the temperature of the system is usually raised to just above the saturation temperature to avoid condensation on the product (Gurtler, Doyle, & Kornacki, 2014). However, the efficacy of the CSS system for bacterial reduction in a food product depends on the temperature, exposure time, and pressure. Schweiggert, Reinhold, & Andreas (2007) reported that the use of high temperatures during CSS treatment had a negative impact on the quality of low moisture foods such as spices and herbs. Therefore, researchers investigated the use of CSS in combination with vacuum for reducing microbial load in various food products. Shah, Gladys, Julie, Kari, & Teresa (2017) evaluated the efficacy of vacuum steam pasteurization on the inactivation of foodborne pathogens in various low moisture foods. It was concluded that maintaining the temperature at 95°C for 120 s resulted in reduction of pathogens below the detection limit (<10.0 CFU/g). Further, lower processing temperature helped in retaining the quality of low moisture foods.

Super-heated steam (SHS) pasteurization is another dry steam method reported in the literature that has recently attracted a lot of attention for its various advantages such as efficient heat transfer, prevention of nutrient oxidation in food, and energy efficiency (Sook, Wahidu, &

Tajul, 2015). SHS is typically produced by providing additional heat to the steam to raise the steam temperature above the saturation temperature. A drop in temperature will not result in condensation unless the temperature is decreased to below the saturation temperature point (Cenkowski, Pronyk, Zmidzinska, & Muir, 2007). SHS has been long known as a safe, non-polluting technology with low energy consumption, if the steam is recycled (Chou & Chua, 2001). Ban and Kang (2016) reported that the application of SHS treatment at 200°C for 15 and 30 s resulted in >5.0 log reduction of foodborne pathogens in almonds and pistachio without altering their quality. Another study reported that the SHS treatment of black peppercorn, pecans, and almonds at 180°C for 13 s reduced the *Salmonella* spp. population below the detection limit (Ban et al., 2018).

2.5.2 Non-thermal processes

Of many processing interventions, thermal treatments are the most commonly used for the reduction of pathogenic bacteria in low moisture foods. However, the use of a thermal process may impact food quality; for instance, it might reduce the amount of heat-sensitive components in spices and herbs. Additionally, pathogens like *Salmonella* may develop higher heat resistance in the desiccated environment, which is a massive challenge while working with thermal treatments (Podolak et al., 2010). Because no single technology can provide a universal solution for a problem, it is crucial to search for non-thermal technologies as alternatives. Gaseous technologies are non-thermal methods that have been used to reduce the microbial load in high moisture foods. One of the main advantages of using gases is their ability to diffuse through the air spaces and pores. This allows the gaseous technologies to perform well with irregularly shaped food products. The following section discusses non-thermal methods such as antimicrobial gaseous technologies, high-pressure processing, and plasma technology for the

elimination of pathogenic bacteria in various foods. The mechanism of microbial inactivation of non-thermal processes and their modes of action is given in Figure 2.

a. Chlorine dioxide

Chlorine dioxide (ClO_2) is a strong oxidizing agent that has been used as a sanitizer both in the gaseous as well as in the aqueous form. The gaseous form has been widely used as an antimicrobial agent and is approved by Environmental Protection Agency (EPA) and the U.S. Food and Drug Administration (FDA) as a food additive for its antimicrobial action in food production (21 CFR 173.300) (Mahmoud, & Linton, 2010). Smaller size (0.124 nm) of the ClO_2 gas molecule (Chai, Hwang, Huang, Wu, & Sheen, 2020) provides a high diffusion and penetration capacity. ClO_2 gas is known to disrupt the cell membrane and protein synthesis in microorganisms (Vandekinderen et al., 2009). Due to the strong penetration ability, ClO_2 gas is regarded as an effective and promising non-thermal technology for reducing the microbial load in various food products (Park & Kang, 2015).

The mode of action of ClO_2 on pathogenic microorganisms includes the enzyme inhibition and disintegration of the complex protein targeting cysteine, tryptophan, histidine, and tyrosine amino acids (Benarde, Snow, Olivieri, & Davidson, 1967; Finnegan, Linley, Denver, McDonnell, Simons, & Maillard, 2010). The microbial action of ClO_2 is due to the inhibition of many cellular processes (Roller, Olivieri, & Kawata, 1980). In addition, ClO_2 is also known to interfere with the nucleic acid-amino acid complexes and inhibit the dehydrogenase activity (Benarde et al., 1967).

Gaseous ClO_2 slowly dissociates into chlorine and oxygen and becomes explosive under pressure when stored for an extended period of time (Demirci & Ngadi, 2012). Due to its

explosive and unstable nature, ClO_2 is usually produced on-site either by acidification of sodium chlorite ($NaClO_2$) or sodium chlorate ($NaClO_3$) or by oxidation of sodium chlorite with chlorine (Cl_2). Alternatively, ClO_2 can also be produced by the acidification of sodium chlorite and sodium hypochlorite (NaOCl) (White, 2010). The main reactions for the generation of ClO_2 is given below (Lee, Burgess, Rubino, & Auras, 2015):

$$5NaClO_2 + 4HCl \rightarrow 4ClO_2 + 5NaCl + 2H_2O$$

$$2NaClO_2 + Cl_2 \rightarrow 2ClO_2 + 2NaCl$$

$$2NaClO_2 + NaOCl + HCl \rightarrow 2ClO_2 + 2NaCl + NaOH$$

The literature survey revealed that the application of ClO_2 gas successfully eliminated foodborne pathogens in various high moisture foods (Table 5). It can be noted from the Table that gas concentration and treatment time were the primary factors whose effect on pathogen reduction was evaluated in most of the studies. Han, Floros, Linton, Nielsen, & Nelson (2001) conducted systematic research and developed a response surface model to assess the effect of gas concentration, temperature, relative humidity, and treatment time on the inactivation of *E. coli* O157:H7 in green peppers. Results from this study indicated that the highest reduction of *E. coli* (5.5 log CFU/5g) was achieved when green pepper was treated with 0.4 mg/L of ClO_2 for 65 min at 20°C and 85% relative humidity. Han, Guentert, Smith, Linton, & Nelson (1999) reported that the concentration, exposure time, and relative humidity play an essential role in the inactivation of microorganisms during the ClO_2 gas treatment.

Rane, Bridges, & Wu (2020) investigated the efficacy of ClO_2 gas to control the foodborne pathogens in whole black peppercorns and almonds. The moisture content of the almond kernels

was increased by 4% to increase the efficiency of ClO_2 gas. At 0.40 mg ClO_2/g almonds, 2.6 log reduction of *Salmonella* was achieved after 6 h of exposure time. The treated samples were then heat-treated 65°C to bring the final moisture content of almonds below 5%. The results showed that an additional 1.7 log reduction was achieved post heat treatment. However, for whole black peppercorns, 3.7 log reduction of *Salmonella* was achieved after 4 h of treatment at 80% relative humidity and 0.40 mg ClO_2/g peppercorn. No heat treatment was performed for whole peppercorns post gas treatment.

Overall, the ClO_2 gas treatment has shown positive results in terms of reducing bacterial load in food products. However, majority of the studies available on the use of ClO_2 for microbial inactivation are only limited to high moisture foods. Since relative humidity is one of the critical factors for microbial reduction, it is therefore imperative to conduct a systematic investigation on the efficacy of ClO_2 gas treatment in a variety of low moisture foods such as spices (cumin seeds), herbs (oregano, basil leaves), nuts (walnuts), etc. This will provide the food processors with a reliable starting point for the implementation of ClO_2 gas for improving the safety of low moisture foods.

b. Ozone

Ozone (O_3) has been used for the purification of bottled water, swimming pools, and wastewater since the 19th century (Guzel-Seydim, Greene, & Seydim, 2004). It is regarded as a potent antimicrobial gas due to its high oxidizing capacity. In the food industry, O_3 has been applied both in gaseous as well as aqueous forms for the decontamination of foods. The use of O_3 for the treatment of raw commodities was approved by the U.S. Food and Drug Administration in 2001 and is also registered with the U.S. Environmental Protection Agency as

a food contact sanitizer (Selma, Ana, Marita, & Trevor, 2008). O_3 is the strongest oxidant with an oxidation potential of 2.07 V, which is higher than that of hydrogen peroxide (1.80 V), chlorine (1.36 V) and chlorine dioxide (0.95 V) (Bialka & Demirci, 2007; Khadre & Yousef, 2001).

The production of O_3 involves the breakdown of diatomic oxygen into free-radical oxygen, which further reacts with another diatomic oxygen. The final reaction of free-radical oxygen and diatomic oxygen results in the formation of triatomic oxygen or O_3 (Rice, Robson, Miller, & Archibald, 1981). A high amount of energy is usually required to break the covalent bond in the diatomic oxygen, which is achieved either by using UV radiation or the corona discharge method (Rice et al., 1981). Since O_3 is very reactive and unstable, it cannot be stored for significant periods; therefore, it must be generated at the point of application as needed.

O_3 is regarded as a broad-spectrum antimicrobial agent due to its high reactivity and related oxidizing power of free radicals (Brodowska, Nowak, Kondratiuk-Janyska, Piątkowski, & Śmigielski, 2017). O_3 is highly unstable both in the aqueous and gaseous forms, which decomposes to form highly reactive free radicals such as hydroperoxyl, hydroxyl, and superoxide radicals when stored for long period of time (Manousaridis, Nerantzaki, Paleologos, Tsiotsias, Savvaidis, & Kontominas, 2005; Brodowska et al., 2017; Pirani, 2010). The mechanism of action of O_3 on microbial inactivation has been researched and studied on several occasions. For instance, Giese and Christenser (1954) indicated that the cell surface of the bacteria as the major site for O_3 activity. In addition, O_3 is reported to target the enzymes, proteins, cytoplasm, nucleic acids, spore coats and virus capsids of microorganisms for inactivation (Oizumi, Suzuki, Uchida, Furuya, & Okamoto 1998; Guzel-Seydim et al., 2004; Pirani, 2010; Greene, Guzel-Seydim, & Seydim, 2012; Brodowska, Nowak, & Śmigielski, 2018;). Furthermore, Scott (1975) reported the modification of pyrimidine bases in bacterial DNA of *E. coli*, upon ozonation, while thymine

being more sensitive than uracil and cytosine. The O_3 primarily disintegrates the cell walls, then the cytoplasmic membrane and finally the DNA, thereby inhibiting the resistance against O_3 treatments (Oizumi et al., 1998). On the other hand, a different mechanism has been proposed for the inactivation of viruses using O_3 treatments. A study by Kim, Gentile, & Sproul (1980) indicated the release of RNA materials from the Phage and reduced infectivity for spheroplasts after ozonation.

O_3 gas has been used to inactivate gram-positive and gram-negative bacteria in various food products. The inactivation of microorganisms post O_3 treatment is due to the oxidation of vital cellular components and damage of nucleic acids (Daş, Gürakan, & Bayındırlı, 2006). Table 6 summarizes the literature available on the use of gaseous O_3 for microbial inactivation in both high and low moisture foods. The efficacy of O_3 in low moisture foods was achieved when the relative humidity of the treatment chamber was above 70% (Akbas & Ozdemir, 2006; Akbas & Ozdemir, 2008). Zhao & Cranston (1995) reported that the effectiveness of O_3 on the microbial reduction was strongly influenced by the moisture content of black pepper, while higher moisture content led to a greater reduction of the microbial population. The O_3 treatment (6.7 mg/L) of black pepper for 60 min resulted in >3.0 log reduction of *Salmonella* and *E. coli* O157:H7; whereas in the case of dried oregano, 3.70 log reduction of *Salmonella* was achieved at a gas concentration of 5.3 mg/L treated for 120 min (Zhao & Cranston, 1995; Torlak, Durmuş, & Pelin, 2013). The effect of temperature on the efficacy of O_3 treatment in microbial reduction is currently unknown, as most of the studies have either not studied the effect of temperature or the experiments were conducted only at room temperatures (20-22°C). With an exception, Perry & Yousef (2013) ramped up the temperature to 55-58°C and obtained >6.0 log reduction of *Salmonella* in shell eggs when treated at 160 mg/L for 60 min.

Overall, the results from various studies given in Table 6 revealed that O_3 is an effective antimicrobial gas in low moisture foods such as dried oregano, flaked red pepper and black pepper (Table 6). However, there is a need to evaluate the efficacy of O_3 in different low moisture foods by conducting a systematic study investigating the effect of gas concentration and treatment time at different temperature and relative humidity on microbial reduction.

c. Hydrogen peroxide

Hydrogen peroxide (H_2O_2) is a known oxidizing agent that is toxic to pathogens (Alexandre, Brandão, & Silva, 2012). Juven & Pierson (1996) summarized the reports on the antibacterial effects of hydrogen peroxide and its application in the food industry. H_2O_2 possesses bactericidal and inhibitory properties due to its capacity to generate more reactive and cytotoxic oxygen species such as hydroxyl radical (HO^-). As the hydroxyl radical is a powerful oxidant, it can induce bacterial oxidation and cause damage to nucleic acids, proteins, and lipids.

The mode of action of H_2O_2 on pathogenic microorganisms follows similar mechanisms to other oxidizing agents. The microbial inactivation of H_2O_2 is mainly attributed by the oxidative mechanism of sulfhydryl groups and inhibition of osmosis within the cytoplasmic membrane owing to the changes in the cell wall (Kitis, 2004). Furthermore, H_2O_2 is also known to denature the protein structure by targeting their side chains (Finnegan, Linley, Denyer, McDonnell, Simons, & Maillard, 2010). The use of H_2O_2 for sanitizing different food products is classified as GRAS (generally recognized as safe) in the United States (Sapers & Simmons, 1998). Aqueous H_2O_2 is usually used at a concentration between 1-5% for sanitizing the food contact surfaces (Sapers & Simmons, 1998; Parish et al., 2003). However, at higher concentrations (4-5%), the efficiency of H_2O_2 was reported to be similar to chlorine treatment (Olmez & Kretzschmar,

2009). Beuchat (1997) reported 3.5 log reduction of *Salmonella* when alfalfa seeds were treated with 6% H_2O_2 for 10 min. Treating organic leafy greens with 3% H_2O_2 for 2 min was less effective in reducing the levels of *Salmonella* Newport (0.2-2.6 log CFU/g) (Moore, Patel, Jaroni, Friedman, & Ravishankar, 2011). Similarly, only 0.8 log reduction of *E. coli* O157:H7 was obtained when button mushrooms were treated for 30 s at 3% H_2O_2. These studies primarily discussed the effect of H_2O_2 concentration and exposure time on bacteria reduction.

Lin, Moon, Doyle, & McWatters (2002) and Huang & Chen (2011) evaluated the effect of temperature (50°C) in combination with a low concentration (2%) of H_2O_2 on microbial inactivation in lettuce and baby spinach, respectively. The results showed that, treating lettuce for 1 min and baby spinach for 2 min was effective in reducing the microbial load from fresh produce without affecting their quality. However, the studies pertaining to the use of H_2O_2 as a sanitizing method is only limited to high moisture foods. With the increasing concerns over the presence of pathogenic bacteria in low- moisture foods, it is imperative to test the efficacy of H_2O_2 as a non-thermal technology in various low moisture foods such as nuts, herbs, and spices.

d. Ethylene oxide

Ethylene oxide (EtO) fumigation is a dry sterilization process that has been widely used by the U.S. spice industry as an intervention to control the presence of pathogenic bacteria such as *Salmonella* and *E. coli* (Leistritz, 1997; Schweiggert et al., 2007; Gurtler et al., 2014). Historically, EtO fumigation has been used to decontaminate spices. According to the American Spice Trade Association (ASTA), the spice industry uses approximately 800,000 pounds of EtO for sterilization purposes in the United States (ASTA, 2017). The main advantage of using EtO is that it does not significantly affect the appearance or flavor of the spices.

EtO treatment first involves generation of vacuum inside the chamber before release of EtO gas to enhance diffusion capacity through the packaged product which is critical for the inactivation of microorganisms (Phillips & Miller, 1973). The alkylation reaction is considered to be the primary mechanism behind the inactivation of microorganisms (Mendes, Brandã, & Silva, 2007). The addition of alkyl groups to proteins, DNA, and RNA in microorganisms by binding to the sulfhydryl, hydroxyl, and carboxyl groups prevents the cellular metabolism and growth of microorganisms, thus makes the microbes nonviable (Mendes et al., 2007).

The Environmental Protection Agency (EPA) regulates the use of ethylene oxide under the U.S. Federal Insecticide, Pesticide, and Rodenticide Act (FIFRA) (EPA, 2012). The use of ethylene oxide for the treatment of spices is registered under 40 CFR 180.151. The U.S. EPA has established strict tolerances for EtO gas residues (7 ppm) and ethylene chlorohydrin (940 ppm) in spices and dried vegetables (EPA, 2012). ASTA recommends the chamber temperature to be at least 46°C throughout the processing to minimize the formation of byproducts (ASTA, 2009). Dried basil has been listed as an exception that cannot be treated with EtO, because the treatment may result in the formation of high levels of ethylene chlorohydrin due to the presence of naturally occurring chlorides (Gurtler et al., 2014). European Union has banned EtO as plant protection product effective from 1991 considering its harmful effects on human health and environment (Pan, 2009).

Because EtO is a toxic and carcinogenic gas, a vacuum cycle is usually integrated with the fumigation process, which helps in preventing the exposure of gas to the operator. However, the use of a low-pressure cycle and high temperature has been reported as the reason behind the loss of volatile compounds in spices (Farkas & Andrassy, 1988). Most of the EtO fumigation studies on food commodities were conducted prior to the 1970's; since then, this technology has been

commercialized for decontamination of spice products (Phillips & Kaye, 1949; Gilbert, Gambill, Spiner, Hoffman, & Phillips, 1964; Wesley, Rourke, & Darbishire 1965). The gas concentration, relative humidity, and temperature were reported to have a considerable impact on the microbial inactivation during EtO fumigation. To support, Wei, Chen, Chaves, Ponder, & Subbiah (2020) reported significant linear relationship of temperature, RH, and exposure time on microbial inactivation. They demonstrated that EtO gas treatment at 53°C and 50 % RH could achieve 4.92± 0.13 log reductions of *Salmonella* within 20 min of exposure time. Further, Chen, Wei, Chaves, Jones, Ponder, & Subbiah (2021) stated that RH greater than 40% is required during EtO treatment to achieve efficient microbial inactivation in cumin seeds. However, still there are no up-to-date standard protocols available to guide the spice industry on how to conduct an effective fumigation process, which can ensure the microbial safety of spices.

Newkirk (2016) tested the efficacy of a two commercial EtO system on the inactivation of *Salmonella* in whole black peppercorn and cumin seeds. The results from the study revealed that the EtO fumigation significantly reduced the microbial load on both the food products. However, there was a considerable variability associated with the commercial-scale EtO treatments. While on an average, EtO treatment achieved 6.62 ± 0.62 log reduction in black peppercorn and 4.9 log CFU/g in cumin seeds, there were replicates samples that received only 1.0 log reduction. Therefore, it is critical to systematically evaluate the effect of EtO fumigation parameters (gas concentration, relative humidity, temperature, exposure time) on microbial inactivation. The identification of the optimal conditions will serve as a guide for the spice industry to conduct in-plant validation studies.

e. Propylene oxide

Propylene oxide (PPO) fumigation has been used in the past to decontaminate various food products when applied under vacuum or pressure. It is a volatile liquid flammable compound having a wide range of activity against bacteria, molds, and yeast. Mrak and Stadtman (1946) studied the effects of PPO treatment in dried fruits and reported it to decrease the product spoilage effectively. They found that the mold and yeast require half as much PPO as required for the inactivation of bacteria.

The antimicrobial activity of the PPO is linked to its oxidation of enzymes and fatty acids. It is also reported that they target the components of the cell such as denaturation of proteins in the cell membranes and the cytoplasm thereby causing cell disruption and lysis (Sánchez-Maldonado, Alvin, & Jeffrey, 2018). Several factors like water activity, relative humidity, and exposure time, influence the pathogen inactivation during PPO treatment. Danyluk, Uesugi, & Harris (2005) found that PPO treatment at 20,833 ppm gas concentration for 4 h could achieve more than 5.0 log reduction of *S.* Enteritidis PT 30 in whole almonds (Table 8). Spoilage of dried fruits could be invariably reduced by introducing PPO into the package during sealing (Mrak, 1951). The effectiveness of PPO treatment can be achieved by adjusting the moisture within the food package. In addition, the gram-positive cocci were found to be highly resistant to PPO at drier conditions but exhibited reduced resistance at increasing relative humidity. A study by Bruch & Koesterer (1961) indicated that the PPO vapor treatment on flaked cereals inoculated with dry spores of *Bacillus subtilis* resulted in 95% bacterial reduction in 4 hours while extended holding times only resulted in additional bacterial reduction.

Danyluk et al. (2005) reported that alarming level of residues (>400 ppm) in almonds raised consumer concerns. PPO is suspected to have carcinogenic effect on humans at higher exposure levels than no-observed-adverse-effect levels (NOAELs) of 100 and 200 ppm (Sweeney et al., 2009). Hence, Environmental Protection Agency (EPA) requires the exposure time to PPO should not exceed 4 h and the residue on the product should be less than 300 ppm. Considering the need of longer processing time required for microbial inactivation, and related human health and environmental concerns, the industrial application of PPO is limited.

f. **High Pressure CO2**

Carbon dioxide (CO_2) is a non-toxic, inert and economically viable gas that does not leave any toxic residues on the treated commodities. Gaseous CO_2 has been effectively used in modified atmosphere packages to control the microbial growth in a variety of agricultural and food products (Jayas & Jeyamkondan, 2002). In low moisture foods such as grains, CO_2 gas has been used to induce hypoxia, lethal to insects and molds; but may not be enough to achieve pasteurization at shorter durations. At near atmospheric pressure, CO_2 treatment is reported to increase the lag phase and multiplication time of bacterial population (Tomkins, 1932). Under high pressure (up to 50 MPa), CO_2 exhibits better bactericidal effects in liquid foods (Damar & Balaban, 2006; Ermen, 2000). It is referred to as cold pasteurization as it does not involve high temperatures to inactivate the microbial load and enzymes and does not significantly impact the nutritional, physiochemical and sensorial quality of food (Spilimbergo, Matthews & Cinquemani, 2010).

The physical state and properties of CO_2 are greatly influenced by the temperature and pressure. Any change in the critical pressure (7.11 MPa) and/or critical temperature (31 °C) of

CO_2 changes its physical state either to solid, liquid, gas, dense phase or super critical phase. Dense phase is the fourth phase of CO_2, where the pressure of CO_2 is above the critical pressure, but below the critical temperature. It has been successfully used to inactivate pathogenic and spoilage microorganisms in liquid foods and preferably in carbonated beverages (Ferrentino & Balaban, 2011; Park, Lee & Park, 2002; Wei, Balaban, Fernando & Peplow, 1991). In the supercritical phase, the viscosity of the CO_2 is similar to that of the gaseous phase and a similar density to the liquid phase. It has attracted the attention of the researchers as a non-thermal pasteurization method to control food borne pathogens including *Salmonella typhimurium, Listeria, E. coli* (Choi et al., 2009, Jung et al., 2009, Kamihira et al., 1987, Kim et al., 2007b).

The main mechanism of inactivation is due to its solubility in water to produce carbonic acid exhibiting bacteriostatic effect in its undissociated form. Increased diffusivity of CO_2 at supercritical conditions facilitates intercellular carbonation, leading to cell death. Lin, Yang, & Chen (1992) suggested that high pressure super critical carbon dioxide can rupture the microbial cells as they are released, and improvement of the cell rupture can be achieved by continuous release of carbon dioxide pressure. In addition, Dixon & Kell (1989) reported that microbial inactivation was the result of changes in the cell membrane, cytoplasm, and enzyme properties.

Research on microbial inactivation of low moisture foods using high pressure CO_2 is limited, while fewer studies have been discussed here. Ballestra, Da Silva, & Cug (1996) reported that high pressure (1.2, 2.5 & 5 MPa) and temperature (25, 35 & 45 °C) enhanced the antimicrobial effects of carbon dioxide against food borne pathogens. However, Calvo and Torres (2010) indicated that the pressure did not significantly affect the action of carbon dioxide for microbial inactivation in paprika, while higher hydrostatic pressure (>10 MPa) resulted in oleoresin extraction. In addition, the initial water content (which possibly reduced the external pH) and

higher temperature (>80°C) enhanced the inactivation of spores but affecting the color of the spice. The application of supercritical carbon dioxide under pressures of 30 MPa at 65°C resulted in complete inactivation of aerobic bacterial load without affecting the polyphenolic and flavanol content in defatted cocoa product with initial addition of 10 % water to cocoa derivative (Calvo, Muguerza & Cienfuegos-Jovellanos, 2007). High pressure (30 MPa) resulted in 100 % extraction yield but was not effective in achieving desired microbial inactivation after the CO2 treatment at 65°C when only 5 % water was added initially to cocoa derivative. The application of high temperature (65-80°C) combined with addition of water (<10%) to cocoa powder helped to inactivate thermoresistant spores, pressure cycles alone did not have any significant effect on microbial inactivation. The effect of carbon dioxide treatments on the microbial inactivation in various low moisture foods is given in Table 8.

The microbial inactivation of CO_2 is affected by many parameters including, temperature, pressure, time, and the moisture content of the food product (Lin, Chan, Chen, & Chen, 1991; Isenschmid, Marison, & Von Stockar, 1995). Higher temperature reduces the density, thus increases the diffusivity of CO_2 and inactivation rate. The penetrating power of CO_2 is higher under supercritical conditions, and there is a rapid change in solubility and density of CO_2 with respect to temperature at the near-critical region. In addition, Kumagai, Hata, & Nakamura (1997) demonstrated the need for higher water content for effective microbial inactivation. As the moisture content in dry basis (g/g dry matter) increased from 0.358 to 2.286, the microbial kill increased from 1.0 to 8.0 log in the cells that are suspended in a medium. Hence, it is imperative to know that CO_2 treatment may not be effective and applicable for low moisture foods due to their lower water activity (Haas et al. 1989; Dillow, Dehghani, Hrkach, Foster & Langer, 1999; Nakamura, Enomoto et al. 1994). Furthermore, the addition of water to the low

moisture foods may not be viable option to enhance the CO_2 efficacy as it may affect the product quality. The effect of carbon dioxide treatments on the microbial inactivation in various low moisture foods is given in Table 8.

g. Plasma

Plasma is one of the emerging non-thermal techniques used for the microbial pasteurization of fresh fruits and vegetables, nuts, and other commodities (Niemira, 2012; Misra, Schlüter, & Cullen, 2016). Plasma is a partially ionized gas and contains high energy ions, electrons, atoms, and reactive neutral species (Pankaj, Misra, & Cullen, 2013). The increased amount of energy in plasma can break down the strong covalent bonds and induce chemical reactions (Moisan, Barbeau, Moreau, Pelletier, Tabrizian, & L'H, 2001). It can be categorized as thermal and non-thermal plasma, based on the energy levels of electrons and heavy species of plasma (Misra, Tiwari, Raghavarao, & Cullen, 2011). Thermal plasma is generated at a higher pressure and needs more power, while non-thermal plasma (NTP) can be generated at atmospheric pressure and lower power. Any kind of energy capable of ionizing the gases can be used for generation, which includes radioactive, electrical, thermal, electromagnetic radiation, or UV light. Among them, the electromagnetic energy and the electrical energies are preferred for generating cold plasma. Furthermore, the composition of plasma is related to the source, the working gas, and the application (Ehlbeck, Ohl, Maaß, Krohmann, & Neumann, 2003; Weltmann et al., 2008).

Principally, the commercial cold plasma devices were linked to their use in research and for biomedical applications. Hence, customization was needed in order to use the device for food applications. The dielectric discharge generated from two parallel electrodes is widely used for NTP. The application of cold plasma in the food industry had recently gained lot of interest

including its use in the packaging (Pankaj et al., 2014), decontamination (Niemira, 2012), and in altering the structure and properties of food (Bahrami, Bayliss, Chope, Penson, Perehinec, & Fisk, 2016). Cold plasma generation involves the deployment of a strong electromagnetic field to a neutral gas, which stimulates the ionization process (Banu, Sasikala, Dhanapal, Kavitha, Yazhini, & Rajamani, 2012).

The main advantage of plasma technology is the availability of the option to choose a gas or a gas mixture. The ionization process is convenient and is a rapid approach to ionize any gas present within the electric field inside the package using high voltage electrodes. For microbial decontamination of food at the industrial scale, the commodity is conveyed through the discharge (Misra et al., 2011). Furthermore, they offer uniform diffusion into complex food structures which are closer to the applicator within a short time without altering the quality of the seeds and leaving no toxic residues on foodstuff and is also safe for operators (Dhayal, Alexander, & Bradley, 2006). However, it might not be effective for the sample far from the applicator. Therefore, plasma may only be suitable for treating a single layer of granular materials, films and equipment surfaces. The effect of plasma has been reported on many pathogenic microorganisms including isolates of *Pseudomonas, Salmonella, Staphylococcus, Geobacillus, Aspergillus, Bacillus, Saccharomyces, Cryptosporidium,* and *Candida* species and bacteriophages and viruses (Moisan, Barbeau, Crevier, Pelletier, Philip, & Saoudi, 2002; Purevdorj, Noriyuki, Isao, & Osamu, 2002; Lee et al., 2005; Lee, Paek, Ju, & Lee, 2006a; Ohkawa, Akitsu, Tsuji, Kimura, Kogoma, & Fukushima, 2006; Alkawareek, Algwari, Gorman, Graham, O'Connell, & Gilmore, 2012; Maisch et al., 2012). Moreover, non-thermal plasma was also reported to inactivate the biofilms (Ermolaeva et al., 2011), and aflatoxins (Basaran, Basaran-Akgul, & Oksuz 2008) produced by pathogenic microorganisms.

Many studies have previously investigated the underlying mechanism responsible for microbial inactivation of cold plasma treatments in order to improve the efficiency of the process. However, the exact mode of action of plasma on microbial inactivation has not been fully understood or explained, and it is known to vary based on the biological material involved and the plasma system. Since plasma generation involves reactive species, electrostatic disruption, and charged particles, it is anticipated that they may be involved in the inactivation mechanism (Liao et al., 2017; Takamatsu et al., 2015). Cold atmospheric plasma, when operated in air is reported to produce enormous amounts of reactive species such as hydrogen peroxide, free radicals, ozone, superoxide, nitrous oxide, nitrites, and others (Shintani, Akikazu, Peter, & Gerald, 2010). These reactive species may damage the microbial cell by targeting their cellular membrane, proteins, lipids, DNA, and their components invariably disrupting the physiological pathways leading to cell death (Thirumdas, Chaitanya, & Uday, 2015).

The antimicrobial activity of plasma is attributed to the effect of heat and UV light on pathogenic microorganisms (Laroussi, 2002; Moisan et al., 2002). The inactivation efficacy of plasma treatment on microorganisms is dependent upon many factors including, plasma generator, process either gas or gas mixture, direct or indirect application, microorganism studied, and the treated product (Schlüter et al., 2013). The effect of plasma treatment on the bacteria is related to the influence of sterilization by UV light capable of exhibiting significant lethal damage to the bacterial cell (Laroussi, 2005). Besides, Surowsky, Antje, Nathalie, Oliver, & Dietrich (2014) reported the surface conditions, and the microbial load of the produce could also affect the antimicrobial characteristics of plasma treatments. Cold atmospheric pressure plasma can also be used to inactivate bacterial and fungal spores; the rate in inactivation is significantly slower at low pressure (Trompeter et al., 2002; Klämpfl et al., 2012). Since the

atmospheric pressure plasma involves low temperature, it is mostly preferred for the microbial inactivation of heat sensitive products (Moreau, Orange, & Feuilloley, 2008). The effect of plasma on the microbial inactivation in various low moisture foods is given in Table 8.

Sun, Nathan, & Susanne (2014) reported about 5.0 log reduction of *Salmonella enterica* in black peppercorns (10 g sample in about 6 layers) following cold atmospheric plasma treatment for 0-90 s. In contrast, Hertwig, Reineke, Ehlbeck, Knorr, & Schlüter (2015) reported a 4.0 log reduction of *Salmonella enterica* in black peppercorns upon treatment to cold atmospheric plasma for 30 min. The rapid inactivation in the former study could be due to the variation in the source of plasma generation and experimental conditions like relative humidity. The former study utilized arc discharge plasma while later study used microwave-driven plasma torch having a frequency of 2.45 GHz and a power consumption of 1.2 kW. Hertwig, Leslie, Meneses, Reineke, Rauh, & Schlüter (2017) suggested treatment of dried almonds with cold atmospheric plasma for 15 min was required to induce 5.0 log reduction of *S. enterica*. In another study, Niemira, Boyd, & Sites (2018) indicated 3.3 log reduction of *E. coli* O157:H7 biofilms on a model food contact surface treated with cold plasma jet for 15 s. Atmospheric pressure plasma is reported to reduce the aflatoxin contents in nuts significantly. For instance, Basaran et al. (2008) reported that 20 min of plasma treatment reduced aflatoxins in hazelnut by half of its initial level (950 ng/g). Similarly, Park et al. (2007) revealed that the mycotoxins and aflatoxins (AFB1, AFB2, AFG1, and AFG2) were degraded in nuts upon treatment to microwave-induced argon plasma. However, the physiochemical properties and structural changes of mycotoxins post treatment were not studied and needs further investigation. Although cold plasma system is not yet available on a commercial scale, it is gaining importance as a non-thermal treatment for low moisture foods. The equipment for large scale treatments are currently under development

using various sources and methods. Moreover, it might not be feasible to treat powders in large scale.

h. High-pressure processing

High-pressure processing or high hydrostatic pressure processing (HPP) has got considerable attention over the traditional food processing and preservation techniques of high-moisture foods. It is a non-thermal technique, where the food is subjected to 100–1000 MPa pressure utilizing water as a medium for pressure transmission at room or mild process temperatures (Yordanov & Angelova, 2010). The parameters such as water activity, nature of solute used to control water activity, temperature, time, and pressure influence the microbial inactivation by HPP. It has a wide array of applications in fruit and vegetable industry and use in meat products. However, the research on HPP application in low moisture foods is limited and may not be suitable for dry products where water content is too less to transmit the pressure within the food products.

The degree of susceptibility and resistance among the bacterial populations is known to vary for HPP treatments (Alpas, Kalchayanand, Bozoglu, Sikes, Dunne, & Ray, 1999; Pagan & Mackey, 2000). HPP mainly targets the non-covalent bonding in the protein, DNA, and lipids of microorganisms leading to decontamination (Rifna, Sushil, Snehasis, & Madhuresh, 2019). The microbial inactivation in low moisture foods can be critical, as lower activity can prevent cells for HPP treatment. Moreover, HPP for inactivation of microbial spores is little effective and often requires a combination of treatment (Wilson, Lukasz, Sandra, Roy, & Tim 2008; Daryaei & Balasubramaniam, 2012; Tao, Da-Wen, Eamonn, & Alan, 2014). A combination of high pressure with heat treatment is regarded more effective for pasteurization and sterilization of foods

(Patterson, Quinn, Simpson, & Gilmour, 1995). Park et al. (2019) reported that HPP treatment at 600 MPa for 5 minutes achieved 2.0 log reduction in aerobic bacterial count as well enhanced the antioxidant activity and total polyphenolic content in garlic powder. One of the significant advantages of using HPP is its ability to maintain the color and odor of treated foods. To support, Picouet, Sarraga, Cofán, Belletti, & Guardia, (2015) reported no significant difference in color between the unprocessed and high pressure processed samples. Different compositional factors are known to impact the efficacy of HPP towards microbial inactivation. Higher fat content in milk showed a baroprotective effect on the microorganism (Gervilla, Sendra, Ferragut, & Guamis, 1999). Goodridge, Willford, & Kalchayanand (2006) reported the effect of water activity in low moisture foods to be a significant factor impacting the ability of HPP to inactivate pathogenic microorganisms on raw almonds. However, low moisture foods suspended in water before HPP treatment and later drying, lead to a higher log reduction (3.8 log CFU/ml) as compared to direct pressurization (0.8 log CFU/ml) (Goodridge et al., 2006). Similar observations were made by Butz, Heinisch, & Tauscher (1994) on HPP processing of spice mixtures suspended in water. They further reported complete microbial inactivation after three cycles of pressure processing at 80 MPa, followed by 350 MPa for 30 min at 70°C. However, addition of water is not feasible for all low moisture foods such as spice powders and chocolate, furthermore, it might also have deleterious effect on the product quality. The effect of microbial inactivation in various low moisture foods is given in Table 8. In summary, HPP is not suitable for low moisture foods.

i. Irradiation

Irradiation is a non-thermal technique used for the preservation of food products and is approved US Department of Agriculture and US-FDA for the elimination of microorganisms,

insect pests and parasites, to extend shelf life of produce and to inhibit sprouting and ripening (FDA, 2016; Lima, Vieira, Santos, & de Souza, 2018). Ionizing radiation is a residue free treatment, causing no thermal damage to food products and affects the surface and inside of the foods (Ray and Bhunia, 2007). For food irradiation, three types of radiation consisting of variable energy levels are used such as, gamma rays (source from radioactive cobalt-60 or cesium 137), electron beam (by using electron beam accelerator) and X-ray generated by X-ray machine (Farkas and Moha´csi-Farkas, 2011). Gamma rays and the electron beam are the two radiation types extensively used by the food industry for microbial inactivation (FDA, 2020; Bouzarjomehri, Dad, Hajimohammadi, Shirmardi & Salimi, 2020).

The mechanism of action of radiation involves the radiolysis (breakdown of water molecules) resulting in generation of H^+ and OH^- ions and reactive oxygen species (HO- hydroxyl and H- hydrogen radicals) (Niemira, 2014). The hydrolytic process disrupts the structural and metabolic functions of the microbes including the fragmentation of the DNA molecule inhibiting the synthesis of DNA and cell division leading to the inactivation of the microorganisms (Clavero, Monk, Beuchat, Doyle, & Brackett, 1994; Moseley 1989).

Gamma rays is mostly preferred for food irradiation since it has better penetration power compared to electron beam which makes it applicable for treatment of bulk commodity (Miller, 2005). It is produced from two radioactive sources, Cobalt 60 (0.66 MeV) and Caesium 137 (1.33 MeV). Among them, Cobalt 60 radioisotope is used widely for food irradiation; gamma radiation is generated by exposing pure natural cobalt-59 pellets to neutrons inside a nuclear reactor. Numerous studies have reported the microbial inactivation of gamma radiation in low moisture foods. For instance, Ban & Kang (2014) reported that a radiation dose of 3.0 kGy combined with storage for 14 days at 25°C was capable of inactivating *S.* Typhimurium in peanut

to an undetectable level. They further suggested that the rate of inactivation was dependent on the a_w of the peanut butter, wherein lower a_w, reduced the radiolysis of water, affecting the microbial inactivation efficacy of irradiation.

Irradiation is widely used for the treatment of dried spices enhancing their shelf life (Suhaj, Rácová, Polovka, & Brezová, 2006) and is legally permitted for usage in about 51 countries (Farag, Aziz, & Attia, 1995). The FDA has approved 30 kGy dosage of radiation for microbial decontamination of spices, seeds and herbs. It was reported that gamma irradiation dose of 5-7.5 kGy was found to be effective in microbial inactivation of black pepper (Horvathova, Suhaj & Polovka, 2007). Similarly, Song et al (2014) suggested that gamma irradiation of black pepper and red pepper at a dosage of 5 kGy could reduce the population of *E. coli* O157:H7 and *S. Typhimurium* by 3.8 to >5 log CFU/g without affecting the product color. Furthermore, Calucci et al. (2003), reported changes in quality of products as a result of oxidation leading to reduction in ascorbate and carotenoid contents in black pepper and rosemary sample treated with irradiation at a dose of 10 kGy. The effect of irradiation on the aflatoxin content in inoculated white pepper was investigated and reported by Jalili, Jinap & Noranizan (2012). It was noticed that gamma irradiation even at a dose of 30 kGy with a product moisture of 18 % could not eliminate total aflatoxin content, instead resulted in about 39.2 – 55.2 % reduction in aflatoxin content in treated samples.

Electron beams consists of electrons of high energy which is driven out of an electron gun. They have a poor penetration capacity and reach up to a product depth of 3.80 cm only Hence, electron beams are converted to X-rays in order to increase its penetration capacity specifically for treatment of large items (FSNAZ, 2011). X-rays are yet another source of radiation treatment of low moisture food, possessing a high penetration capacity and can pass through thicker

commodities (FSNAZ, 2011). It is produced when the charged particle moving at a very high velocity is reflected by a heavy metal such as tungsten (FDA, 2020). Studies by Jeong, Shin, Chu, & Park (2012) evaluated the effect of a_w (0.23, 0.45, 0.64, and 0.84) on the survival of *S.* Enteritidis PT30 and *S.* Tennessee to X-ray irradiation treatments in almond and walnuts. They found that *S.* Enteritidis PT30 and *S.* Tennessee populations were less resistant to irradiation on surface-inoculated almonds and when the walnuts were at their dry state (i.e a_w = 0.24). *S.* Enteritidis PT30 and *S.* Tennessee showed higher sensitivity to X-ray irradiation on almonds (D_{10}-value = 0.226–0.431 kGy) than on walnuts (D_{10}-value = 0.474–0.930 kGy) at all water activities. Further, it was reported that the sensory properties of the irradiated almonds did not differ significantly between the control and irradiated samples. On the other hand, a significant difference in color change was reported in walnuts irradiated to doses leading to 5.0 log reduction. The use of X-rays to low moisture food commodity is limited as it is energy intensive and is extremely expensive. Hvizdzak, Beamer, Jaczynski, & Matak (2009) evaluated the efficacy of electron beam radiation on the inactivation of *S.* Tennessee and *S.* Typhimurium in peanut butter. They reported that e-beam dose of 3.0 kGy achieved 5.00 and 4.19 log reduction of *S.* Tennessee and *S.* Typhimurium respectively. *Salmonella* Typhimurium was found to be more resistant (D_{10}-value = 0.82 kGy) than *S.* Tennessee (0.72 kGy) to electron beam radiation. Based on this study, electron beam could be considered as an effective non-thermal pasteurization technique.

Byun, Cho, Park, Chun, & Ha (2019) compared the efficacy of the three sources of radiation on the inactivation of *A. flavus* spores in red pepper powder. They reported gamma irradiation at a dose of 2.0 kGy to have the faster rate of microbial inactivation of *A. flavus* spores than electron beam treatment at 3.0 kGy; both treatments reduced the spores count below the

detectable limit. X-ray exhibited the least efficacy with a 4 log reduction of *A. flavus* spores at a dosage of 3.5 kGy. In addition, an irradiation dose of 6 kGy (from all sources) was found to inhibit the population of aerobic pathogens efficiently without altering the product quality (Jung et al., 2015). They observed that neither of the radiation types affected the pungency (capsaicinoid content) or color of the irradiated red pepper samples in comparison to the control. However, the sensory analysis revealed development of off flavor in all the irradiated samples irrespective of the source used.

Numerous studies have reported the efficacy of irradiation treatments on microbial inactivation in low moisture foods in recent years. However, the advantages and the safety of food irradiation has been documented time and again; the labeling requirement is one of the major drawbacks limiting customer acceptance (Morehouse & Komolprasert, 2004). Lack of knowledge on the food irradiation process and fear of considering irradiated food to be radioactive are also regarded as the constraints affecting its wide acceptability. At recent times, the public attitude towards this technology has seen a positive change due to many awareness campaigns (Morehouse, & Komolprasert, 2004). Furthermore, public-industry partnerships can help in increasing customer acceptance of these treatments.

2.6 Combination treatments

Hydrogen peroxide (H_2O_2) and peracetic acid (PAA) are synergistic in nature for pure cultures of *P. aeruginosa, E. coli* and *S. aureus.* The best example for this synergism is the reaction of aqueous H_2O_2 with residual acetic acid to generate more PAA and is highly effective than individual treatments (Alasri, Roques, Michel, Cabassud, & Aptel, 1992). Since microorganisms are generally present on the surface of the spices, surface pasteurization needs

more attention (Cheon, Shin, Park, Chung, & Kang, 2015). Vacuum steam treatment is a mild thermal method for surface decontamination as a result of quick removal of condensate layer (containing microorganisms) formed on the surface of the product due to steam. Lilie, Hein, Wilhelm, & Mueller (2007) utilized the mechanical and thermal methods for microbial decontamination while producing very low thermal effects on whole black peppercorns. There was no significant change in the moisture content and the volatile profiles of the treated spice. Cheon et al. (2015) further explored the combined effect of UV-irradiation and mild heating (65°C) on the microbial inactivation in low moisture foods. They reported about 2.9 and 3.1 log reduction of *E. coli* O157:H7 and *S.* Typhimurium in red pepper powder in combination treatment, while for UV irradiation alone there was only 0.2 and 0.3 log reductions, respectively.

Bacillus species, common spore formers in low moisture foods was investigated by Zhang et al. (2006) for their susceptibility to combination treatments. It was shown that *B. atrophaeus* spores was more sensitive to CO_2 and H_2O_2 application, showing 6.3 log reduction, as compared to 4.7 log reduction recorded for *B. pumilus* spores over treatment to 200 ppm H_2O_2 in CO_2 at 27.5 MPa, 40°C for 4 h. The release of pyridine-2,6-dicarboxylic acid (DPA), a major chemical compound released from the spores is linked to the damage of spore coat. The treatment of *B. atrophaeus* spores with pure CO_2 for 4 h resulted in maximum release of DPA (1.40 nmol/mg spores). However, addition of 200 ppm of H_2O_2, increased the DPA release to 130 nmol/mg spores, demonstrating the degree of damage to the spore coat by combination treatment (Zhang et al., 2006). High pressure CO_2 treatment followed by high temperature (90°C) processing was found to be effective in completely eliminating *B. coagulans* and *B. lichenformis* spores. It was also indicated that high pressure CO_2 increased the sensitivity of bacterial spores to heat treatments.

In general, combination treatments are more advantageous compared to individual treatments, as many of the individual treatments do not comply with the food safety standard and are not stable (Raso & Barbosa-Canovas, 2003). The combination of heat treatments with the non-thermal processing might facilitate the eradication of microbial resistance to non-thermal processing (Patterson et al., 1995; Rademacher, Pfiffer, & Kessler, 1998). In addition, heat treatment at 90°C for 30 min, followed by cold plasma treatment with nitrogen at 900 W, 667 Pa for 20 min, exhibited synergistic inhibitory effects on *B. cereus* spores in red pepper powder (Kim, Kim, & Kim, 2013). However, the high cost of combination treatments is a barrier for industrial adoption.

2.7 Summary

Despite the common assumption that low-moisture foods are microbiologically safe, *Salmonella* contamination has occurred time and time again. This is particularly concerning because the long shelf life and enhanced survival of *Salmonella* in low-moisture foods allow the tainted food to sit on consumers' shelves for a long time and cause future infections that are difficult to trace back. Therefore, it is imperative to evaluate and validate the legacy and novel technologies as a kill step to improve the safety of low-moisture foods. Thermal processing methods such as radio frequency treatment, extrusion, and steam pasteurization have been extensively studied for microbial inactivation. Steam treatment or controlled condensation is very effective and commercial systems are available. However, the treated product may require drying operation to bring the moisture content to safe level. Moreover, it has a negative impact on the quality of low moisture foods such as spices and herbs, especially the loss of volatiles may occur due to high temperature treatment. Traditional Dry heat treatments have been found to be less effective due to larger come-up time because of lower thermal conductivity. Radio

frequency heating can create volumetric heating and reduce the come-up time considerably. RF has been shown to preheat egg white powder, wheat flour, milk powders rapidly, followed by holding in hot air oven at high temperatures to achieve desired kill. Shorter come-up allowed RF to heat the product to a higher temperature and lower holding time, resulting in minimal deterioration of food quality. In case of spices (black peppercorn, black pepper powder, cumin seeds, basil leaves), RF is very effective due to presence of antimicrobial properties. These products do not require a holding time and therefore the product can be pasteurized by RF heating alone in few minutes. However, lack of uniform heating is the main drawback of RF treatments; methods which can improve heating uniformity are intermittent mixing, using circular containers and/or packaging material/container with dielectric properties similar as food can be beneficial. Extrusion is another widely used technology which involves the production of food products using low-cost materials with quick processing time and also aid in microbial inactivation. Twin-screw extrusion is more effective than the single screw extrusion due to higher shear rate. Therefore, it is advantageous for the food industry to validate extrusion as a kill step, if extrusion is already used as a unit operation in the production of their specific low moisture food.

On the other hand, high pressure processing is not suitable for low moisture foods since it requires higher water activity for microbial inactivation. Plasma systems have been reported to possess strong potential to degrade mycotoxins. However, the research on mechanism of action exhibited by plasma ions on secondary metabolites such as aflatoxins and scale up process needs investigation. Also, it might be efficient only for single layer of granular food products. Since most pathogenic bacteria exhibit increased heat tolerance at low water activity conditions, gaseous technologies have gained interest recently due to their high penetration and diffusion

properties. Chlorine dioxide, hydrogen peroxide, and ozone are strong oxidizing agents and their use in aqueous form has been found effective in reducing microbial load in fresh produce. Ozone has a short active life and needs to be generated and supplied continuously during treatment. The main advantage of ozone treatment is that it does not leave any residues in treated food product. Chlorine-dioxide treatment, on the other hand, might produce chemical residues (ClO_2, chlorite, chlorate, and chloride) in low moisture foods and further research is required to quantitatively investigate the presence of residues. Moreover, studies on the efficacy of hydrogen peroxide and ozone gas to control pathogens in low moisture foods are limited and evaluation of quality attributes of gas treated food product is non-existent. Although, chlorine dioxide gas treatment of food produce might not gain consumer acceptance owing to the presence of chlorine which creates concerns in consumer's minds who associate it with a bleach. On the other hand, ozone and hydrogen peroxide decomposes rapidly in the environment and does not form any residues unlike chlorine dioxide. Hydrogen peroxide and ozone would be most probably seen as clean gases and thus, a synergistic approach to achieve higher microbial inactivation is required. In addition, studies pertaining to quality evaluation of foods treated with gaseous treatments is wanting.

Although propylene oxide is found to be effective on low moisture foods; its negative effects on environment and consumer health due to toxic residues/ biproducts are major concerns for its widespread usage. Efficacy of high-pressure CO_2 in low moisture foods is not as effective as in liquid foods. Though EtO is reported to be successful for microbial inactivation in low moisture foods, its use is variable to the industry. Therefore, lab studies are required to understand the interaction of various process parameters on microbial inactivation. Its use is strictly monitored considering the legal maximum residue levels in treated food produce.

Irradiation could be an alternative to EtO considering its residue free nature and high antimicrobial efficacy. However, concerns related to its impact on the nutritional and organoleptic quality and consumer unacceptability has led to its limited utilization in the food industries. Furthermore, there is also a need to evaluate newer intervention strategies and combination treatments to enhance the microbial inactivation in low-moisture foods without significantly altering their quality.

2.8 Conclusion

Assuring low-moisture food safety is challenging, due to its long shelf life and enhanced survival of *Salmonella*. It is imperative to evaluate and validate the legacy and novel technologies as a kill step to improve the safety of low-moisture foods. Extrusion is effective in pasteurizing products and therefore it is advantageous for the industry to validate this technology as a kill step if they are already using this unit operation. Steam treatment has been found to be effective; however, it may have detrimental effect on the quality of sensitive low moisture food products such as spices and herbs, mostly leading to the loss of volatiles when exposed to high temperature treatments. Radiofrequency processing has shown a niche in thermal processing of several low moisture food products due to shorter come-up time because of its volumetric heating. The chemical gaseous technologies such as propylene oxide and ethylene oxide has been shown to be effective in pasteurizing heat-sensitive low moisture products; however, there are consumer concerns. The milder chemical gases such as chlorine dioxide, ozone and hydrogen peroxide may be more acceptable for consumers; however, they have been least explored for their use as decontamination strategies against pathogenic organisms in low moisture foods. The application and maintenance of these interventions will minimize the occurrence of foodborne pathogens in low-moisture foods, which will in turn help to reduce the

foodborne illness and food losses due to recalls. Furthermore, adequate research is required to investigate the synergistic effect of combination treatments on microbial inactivation in low moisture foods and their probable mode of action on pathogens.

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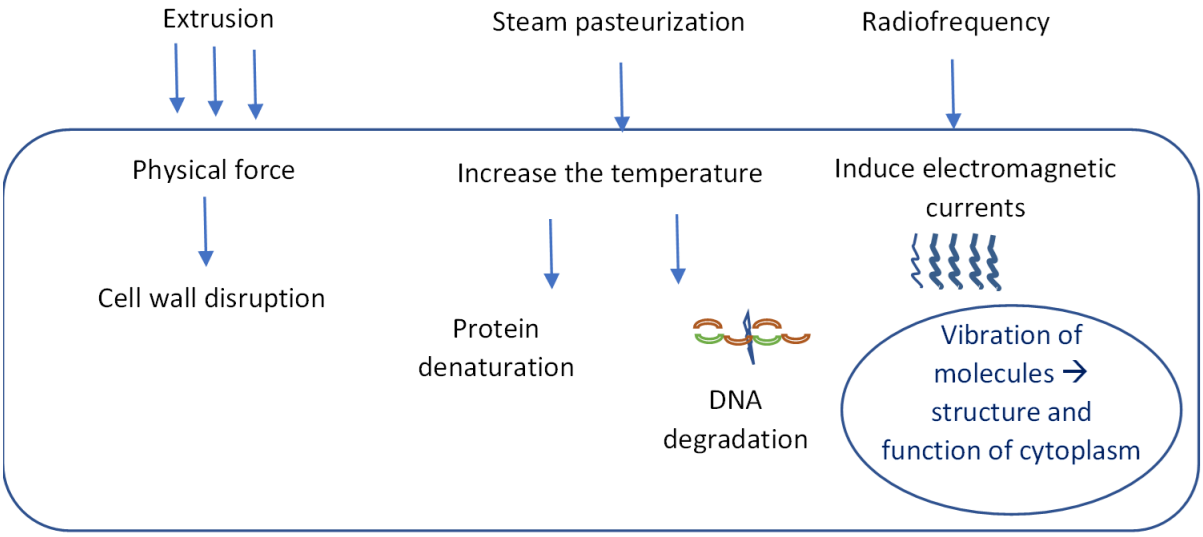

Fig 2.1. Mechanism of action of thermal technologies on microbial inactivation.

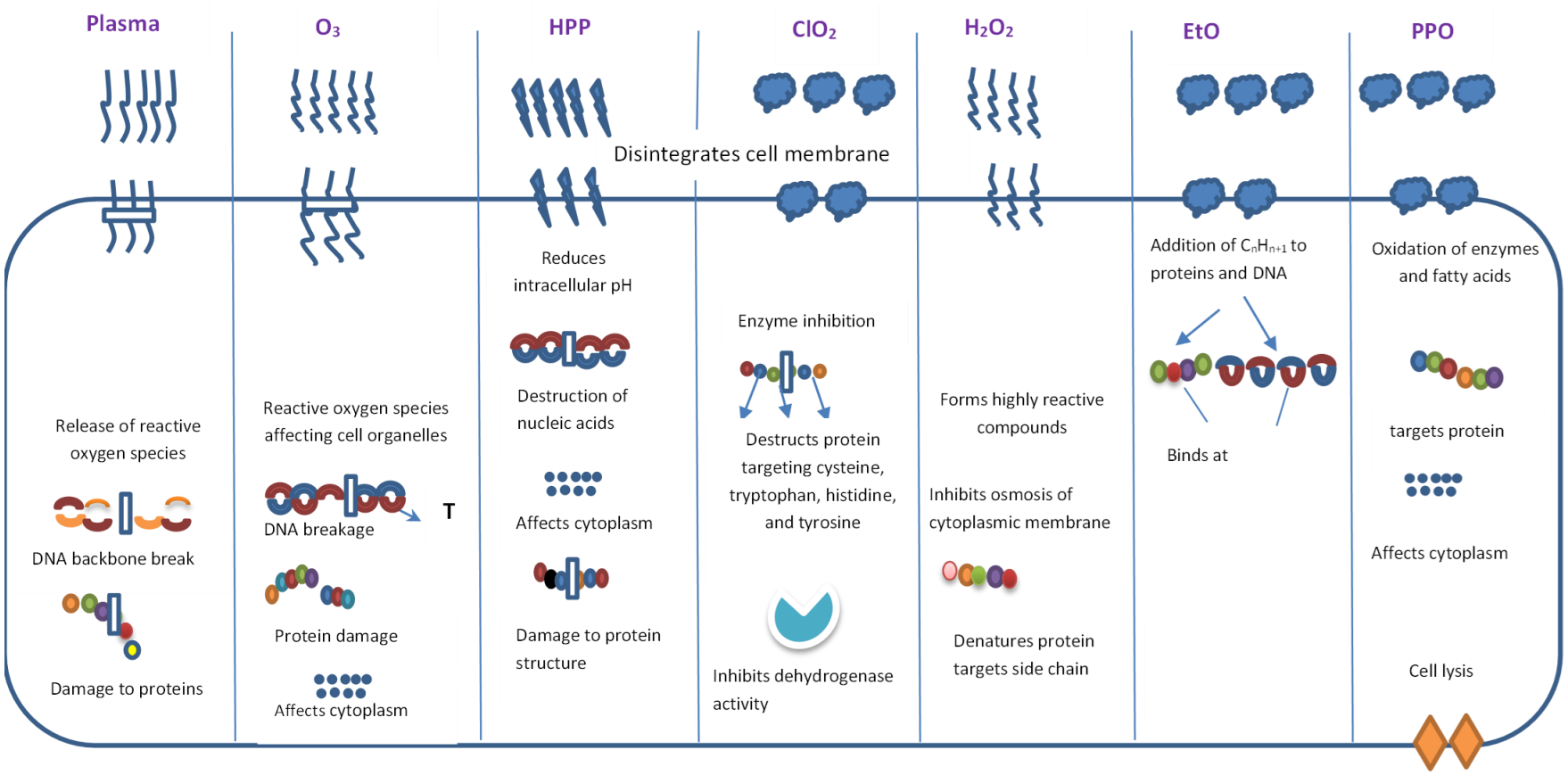

Fig 2.2. Mechanism of action of non-thermal technologies on microbial inactivation.

Table 2.1. Foodborne illness outbreaks reported in various low moisture foods.

Year	Product	Pathogen	Location	Comments	Reference
1973	Milk powder	*Salmonella* Derby	Trinidad	Approx. 3000 people were infected mostly infants and small children.	Weissman, Rmad, Miles, Swanston, & Ali (1977)
1993	Paprika	*S.* Saintpaul, *S.* Rubislaw, and *S.* Javiana	Germany	Children <14 years of age were mostly affected among 1000 total estimated cases. Main sources of illness were paprika powder, spice mixtures, and snacks.	Lehmacher et al. (1995)
1994	Infant formula powder	*S.* Virchow	Spain	48 cases were confirmed in 14 regions in Spain out of 17. Children under the age of 7 months were mostly affected.	Usera et al. (1996)
2001	Peanuts	*S.* Stanley	Australia	97 cases were estimated	Kirk et al. (2004)
2001	Peanuts	*S.* Newport	Australia	12 cases were estimated	Kirk et al. (2004)
2001	German chocolate	*S.* Oranienburg	Germany	439 notifications were registered within 6 months in Germany.	Werber et al. (2005)
2002-2003	Tahini	*S.* Montevideo	Australia and New Zealand	68 cases were reported, and sesame seed-based food was found to be the main source of illness.	Unicomb et al. (2005)
2005	Infant formula powder	*S.* Agona	France	Total of 141 confirmed cases were reported. Cross- contamination through the environment between two different production lines was reported to be the cause of the illness.	Brouard et al. (2007)

2005	Raw almonds	*S.* Enteritidis PT30	Canada	168 confirmed cases were identified, and raw whole almonds collected from home warehouses retail and distribution was detected to be the source.	Isaacs et al. (2005)
2007	Peanut butter	*S.* Tennessee	USA	715 cases from 48 states were reported.	Sheth et al. (2011)
2008	Infant formula powder	*S.* Kedougou	Spain	31 children under the age of one year were confirmed to be infected.	Rodríguez-Urrego et al. (2010)
2008-2009	Peanut butter	*S.* Typhimurium	USA	529 people from 43 states and 1 person from Canada were infected and 116 were hospitalized including 8 deaths.	Cavallaro et al. (2011)
2010	Black and red pepper	*S.* Montevideo	USA	272 cases from 44 states were confirmed and the strain was isolated from ready to eat salami and sealed containers of black and red pepper.	Gieraltowski et al. (2013)
2011	Rice cakes	*Shiga toxin Escherichia coli* (STEC) O157	Japan	142 confirmed cases were associated with the outbreak.	Nabae et al. (2013)
2011	In-shell hazelnuts	*Escherichia coli* O157:H7	USA	In-shell hazelnuts purchased from the retail food stores was found to be the main source of illness, infecting 8 people.	CDC (2011)
2012	Peanut butter	*S.* Bredeney	USA	442 people were reported to be infected in 20 states, majority of which were children under the age of 10 years.	CDC (2012)

2013	Tahini sesame paste	*S.* Montevideo and *S.* Mbandaka	USA	16 cases with 1 death were reported due to contamination in tahini sesame paste.	CDC (2013)
2014	Nut butter spread	*S.* Braenderup	USA	6 cases were reported and 1 hospitalization due to consumption of contaminated butter.	CDC (2014a)
2014	Chia powder	*S.* Newport, *S.* Hartford, and *S.* Oranienburg	USA	31 cases were confirmed in 16 states.	CDC (2014b)
2014	Cashew cheese	*S.* Stanley	USA	17 cases were reported in 3 states; a brand of cashew cheese was associated with the illness	CDC (2014c)
2015	Spice mix	*S.* Enteritidis PT13a	Sweden	174 cases were associated with the infection.	Jernberg et al. (2015)
2015	Nut butter spread	*S.* Paratyphi B variant L (+) tartrate (+)	USA	13 people were reported to be infected. People of all age group were found to be infected with age less than 1 year old.	CDC (2015a)
2015	Soft cheese	*Listeria monocytogenes*	USA	30 people got infected out of which 28 people got hospitalized and 3 died from the illness.	CDC (2015b)
2016	Flour	*E. coli* O121 or O26	USA	63 cases were reported in 24 states over exposure to raw flour.	CDC (2016a)

2016	Pistachios	*S.* Montevideo *and S.* Senftenberg	USA	11 cases were confirmed in several states.	CDC (2016b)
2017	Soynut butter	*E. coli* O157:H7	USA	32 people mainly younger ones lesser than 18 years of age were reported to be infected.	CDC (2017)
2018	Dried coconut	*S.* Typhimurium	USA	14 cases were reported from 8 different states.	CDC (2018)
2019	Tahini	*S.* Concord	USA	6 people got infected from consuming tahini.	CDC (2019a)
2019	Cake mix	*S.* Agbeni	USA	7 cases were reported, and the main source of illness was found to be cake mixes.	Ladd-Wilson et al. (2019)
2019	Flour	*E. coli* O26	USA	21 people were reported to be infected and source of infection is linked to flour mixes.	CDC (2019b)

Table 2.2. Summary of studies applying extrusion process for inactivation of pathogens in different foods.

Product	Pathogen	Moisture content (%)	Fat content (%)	Screw speed (rpm)	Temperature (˚C)	Reduction (log CFU/g)	Reference
Whey protein powder	*Streptococcus thermophilus*	4-5	NR[$]	50	143	4.2	Queguiner, Dumay, Cavalier, & Cheftel (1989)
Corn-soybean (70:30, w/w)	*Bacillus globigii*	18	NR	80-160	110-130	1.0-7.0	Likimani & Sofos (1990)
Mixture of deboned turkey and white corn flour	*Clostridium sporogenes*	NR	NR	NR	93-115	2.0-5.0	Li, Hsieh, Fields, Huff & Badding (1993)
Animal feed mash	*Salmonella*	28.5	NR	NR	83	< DL* (not indicated)	Okelo et al. (2006)
Carbohydrate-protein meal	*Enterococcus faecium* NRRL B-2354	25-31	NR	80-125	65-85	5.0	Bianchini et al. (2012)
Corn meal	*Escherichia coli*	35	NR	NR	>75	< DL* (<20 CFU/g)	Ukuku et al. (2012)
Whey protein	*E. coli*	36	NR	NR	95	< DL* (<20 CFU/g)	Ukuku et al. (2012)
Oat flour	*S.* Agona	14-28	8.5	500	70-100	5.0	Anderson et al. (2017)
Oat flour	*S.* cocktail	14-26	5-15	75-225	65-85	1.0-8.0	Verma et al. (2018a)
Oat flour	*E. faecium* NRRL B-2354	14-26	5-15	75-225	75-95	1.0-5.0	Verma et al. (2018b)

Oat flour (twin-screw extrusion)	*S.* cocktail	14-26	5-15	75-225	65	< DL* (<10 CFU/g)	Verma and Subbiah (2019)

[$] NR: Not reported; < DL*: Below detectable limit

Table 2.3. Summary of studies applying radiofrequency for inactivation of pathogens in various low-moisture foods.

Product	Pathogen	Treatment conditions (CUT[$])	Reduction (log CFU/g)	Reference
In-shell almonds	*S.* Enteritidis PT30	75°C (3.6 min)	5.0	Gao, Tang, Villa-Rojas, Wang and Wang (2011)
Broccoli powder	Total bacteria	80°C (5 min)	4.2	Zhao, Wei, Ruijin, Jaideep, and Fanbin(2017)
Whole black peppercorn	*S.* cocktail and *E. faecium*	96.5°C (2.5 min)	5.3 (Sal.), 5.3 (E.F.)	Wei et al. (2018)
Red pepper powder	*S. typhimurium*	70°C (1 min)	>5.0	Hu, Zhao, Hayouka, Wang and Jiao (2018)
Ground black pepper	*S.* cocktail and *E. faecium*	80°C (2 min)	6.0 (Sal.), 3.9 (E.F.)	Wei et al. (2019)
Wheat flour	*S.* Enteritidis PT30 and *E. faecium*	85°C (15 min), held for 18 min	5.0 (Sal.), 3.7 (E.F.)	Liu et al. (2018)
Cumin seeds	*S.* cocktail and *E. faecium*	100°C (1.3 min)	5.8 (Sal.), 3.3 (E.F.)	Chen et al. (2019)
Corn flour	*S.* Enteritidis PT30 and *E. faecium*	85°C, held for 10 min and stored at -20°C for 48 h	6.6 (Sal.), 4.8 (E.F.)	Ozturk et al. (2019)
Paprika	*Salmonella* cocktail and *Enterococcus faecium* NRRL B-2354	80°C (1.6 min)	4.2 (Sal.), 1.9 (E.F.)	Ozturk, Kong, and Singh (2020)
White pepper		80°C (4 min)	3.3 (Sal.), 1.4 (E.F.)	

Cumin powder		80°C (6 min)	2.8 (Sal.), 0.9 (E.F.)	
Dried basil leaves	*Salmonella* and *E. faecium*	100°C (1 min)	< DL* (<10 CFU/g)	Verma, Chaves, Howell & Subbiah (2021)

[$]CUT: Come-up time; Sal.: *Salmonella*; E.F.: *Enterococcus faecium;* < DL*: Below detectable limit

Table 2.4. Summary of studies applying steam treatment for inactivation of various pathogens in low-moisture foods.

Product	Pathogen	Temperature (°C)	Exposure time (s)	Reduction (log CFU/g)	Reference
Raw shelled almonds (Nonpareil and Mission)	*Salmonella* Enteritidis	93	65	>5.0 (Nonpareil), >4.0 (Mission)	Lee et al. (2006)
Almonds	*E. coli* O157:H7, *S.* Typhimurium, *S.* Enteritidis, *L. monocytogenes*	200	15	>5.8	Ban and Kang (2016)
Pistachio	*E. coli* O157:H7, *S.* Typhimurium, *S.* Enteritidis, *L. monocytogenes*	200	30	>5.5	
Whole flaxseed	*S.* PT 30, *E. coli* O157:H7	95	60	>6.0	Shah et al. (2017)
Quinoa	*S.* PT 30, *E. coli* O157:H7	95	60	< DL* (1.0 – 1.38 log CFU/g)	
Black peppercorn	*S.* PT 30, *E. coli* O157:H7	95	60	>6.4	
Milled flaxseed	*S.* PT 30, *E. coli* O157:H7	95	120	< DL* (1.0 – 1.38 log CFU/g)	
Sunflower kernels	*S.* PT 30, *E. coli* O157:H7			>6.2	
Black peppercorn	*S. enterica*	85	120	>5.0	Duncan et al. (2017)
Cumin seeds	*S. enterica*	85	60	>5.0	
Black peppercorn, pecans, and almonds	*S. enterica*	180	13	< DL* (1 log CFU/g)	Ban et al. (2018)

< DL*: Below detectable limit

Table 2.5. Summary of studies applying gaseous chlorine dioxide for inactivation of pathogenic bacteria in various foods.

Product	Pathogen	Gas conc. (mg/L)	Temp. (°C)	Relative humidity (%)	Exposure time (min)	Reduction (log CFU/g)	Reference
Almonds and black peppercorn	*Salmonella*	0.40 mg ClO_2/g	NR	80	360	2.6 (almonds), 3.7 (black peppercorn)	Rane, Bridges & Wu (2020)
Green pepper	*Escherichia coli* O157:H7	0.60	20	90-95	30	7.3	Han, Linton, Nielsen & Nelson (2000)
Green pepper	*E. coli* O157:H7	0.10-0.50	5-25	55-95	7-135	1.0-5.5	Han et al. (2001)
Apple	*L. monocytogenes*	4.00	21	90	30	>4.0	Du, Han, & Linton (2002)
Strawberries	*E. coli*, *L. monocytogenes*	4.00	22	90	30	>5.0	Han, Selby, Schultze, Nelson & Linton (2004)
Cabbage, Carrot, Lettuce	*S. enterica*	4.10	23	NR	30.8	4.4, 5.2, 1.6	Sy, Melinda, David & Larry (2005)
Cabbage, Carrot, Lettuce	*E. coli* O157:H7	4.10	23	NR	20.5	3.1, 5.6, 1.6	
Cabbage, Carrot, Lettuce	*L. monocytogenes*	4.10	23	NR	29.3	3.6, 5.9, 1.5	

Lettuce	*E. coli* O157:H7	5.00	NR	NR	14.5	5.0	Mahmoud and Linton (2008a)
	S. enterica	5.00	NR	NR	19.0	5.0	
Whole cantaloupe	*E. coli* O157:H7	5.00	22	90-95	10, 6	4.6	Mahmoud, Vaidya, Corvalan, & Linton (2008b)
	L. monocytogenes	5.00	22	90-95	10	4.3	
	S. Poona	5.00	22	90-95	6	>5.0	
Hydroponic tomatoes	*S. enterica, L monocytogenes*	0.50	22	90	12	>5.0	Bhagat, Mahmoud& Linton (2010)
Roma tomatoes	*S. enterica*	10.00	NR	NR	3	4.9	Trinetta, Morgan & Linton 2010)
Navel Oranges	*S. enterica*	0.50	NR	NR	14	>5.0	Bhagat, Mahmoud, & Linton (2011)
Spinach leaves	*E. coli* O157:H7, *S.* Typhimurium, *L. monocytogenes*	0.14	NR	90	15	<1.0	Park & Kang (2015)
Whole blueberries	*S. enterica*	5.50	NR	NR	60	5.6	Annous, Buckley & Burke (2020)

* NR: Not reported

Table 2. 6. Summary of studies applying gaseous ozone for inactivation of pathogenic bacteria in various foods.

Product	Pathogen	Gas conc. (mg/L)	Temp. (°C)	Relative humidity (%)	Exposure time (min)	Reduction (log CFU/g)	Reference
Black pepper	*E. coli* O157:H7, *Salmonella*	6.7	NR[$]	NR	60	>3.0	Zhao & Cranston (1995)
Pistachios	*E. coli* O157:H7	0.0021	20	70	360	3.5	Akbas and Ozdemir (2006)
Flaked red pepper	*E. coli* O157:H7	0.0021	20	70	360	2.0	Akbas and Ozdemir (2008)
Dried oregano	*Salmonella*	2. 8	NR	NR	120	2.8	Torlak et al. (2013)
	Salmonella	5.3	NR	NR	120	3.7	
Green pepper	*E. coli* O157:H7	7.0	22	85	40	>5.0	Han et al. (2002)
Cherry tomatoes	*S.* Enteritidis	30.0	NR	NR	15	< DL*(not indicated)	Daş et al. (2006)
Baby spinach	*E. coli* O157:H7	4.29	NR	95-100	15	1.8	Vurma, Ram, Sudhir & Ahmed (2009)
Tomato	*L. innocua*, *S.* Typhimurium, *E. coli*	2.11	22	NR	3	>3.0	Fan, Sokorai, Engemann, Gurtler & Liu (2012)

Shell egg	*S.* Enteritidis	160	55-58	NR	60	>6.0	Perry and Yousef (2013)
Parsley	*L. innocua*	0.95	21	85	20	1.3	Karaca and Velioglu (2014)
	E. coli O157:H7	0.95	21	85	20	1.1	

[$]NR: Not reported; < DL*: Below detectable limit

Table 2.7. Summary of studies applying aqueous hydrogen peroxide for inactivation of pathogenic bacteria in various foods.

Product	Pathogen	H_2O_2 conc. (%)	Temp. (°C)	Relative humidity (%)	Exposure time (min)	Reduction (log CFU/g)	Reference
Alfalfa seeds	*Salmonella* spp.	6	NR	NR	10	3.5	Beuchat (1997)
Lettuce	*E. coli* O157:H7, *S.* Enteritidis, *Listeria monocytogenes*	2	50	NR	1	>3.0	Lin et al. (2002)
Cantaloupe	*Salmonella* spp.	5	NR	NR	5	2.3	Ukuku (2004)
Honeydew	*Salmonella* spp.	5	NR	NR	5	3.0	
Baby spinach	*E. coli* O157:H7	2	50	NR	2	2.2	Huang and Chen (2011)
Romaine lettuce	*S.* Newport	3	NR	NR	2	0.4	Moore et al. (2011)
Iceberg lettuce	*S.* Newport	3	NR	NR	2	2.6	
Adult spinach							
Baby spinach	*S.* Newport	3	NR	NR	2	0.2	
	S. Newport	3	NR	NR	2	0.9	
Red bell peppers	*L. innocua* NCTC 10528	5	15	NR	2	2.3	Alexandre et al. (2012)

Button mushroom	*E. coli* O157:H7	3	NR	NR	0.5	0.8	Guan, Fan, & Yan (2013)

*NR: Not reported

Table 2.8. Summary of studies applying various non-thermal treatments for inactivation of pathogenic bacteria in various low moisture foods.

Product	Pathogen	Treatment conditions	Reduction (log CFU/g)	Reference
Pecans	*E. coli* k-12	800 ppm of PPO at 32-96% RH and 37°C for 4 hours	5.0 log MPN	Beuchat. (1973)
Red pepper powder	*B. cereus* spores	Heat treatment at 90°C for 30 min followed by cold plasma treatment using Nitrogen at 900 W, 667 Pa for 20 min	3.4	Mazzoni, Sharma, Demirci & Ziegler. R. (2001)
Whole almonds	*S.* Enteritidis PT 30	500 ppm of PPO for 4 hours	5.2-8.6	Danyluk et al. (2005)
Raw almonds	*S.* Enteritidis	HPP 413.68 MPa for 5 min at 25°C	0.1	Goodridge et al. (2006)
	S. Enteritidis	HPP 413.68 MPa for 5 min at 50°C	0.8	
	S. Enteritidis	HPP 482.63 MPa for 5 min at 50°C	0.5	
Infant milk powder	*Cronobacter sakazakii*	Cold plasma treatment at 900 W for 20 min	0.9	Calvo et al. (2007)
Onion powder	*Bacillus cereus* spores	Cold plasma treatment at 900 W for 20 min	0.4	
Ginseng	Total aerobic	CO_2 at 10 MPa for 15 h at 60°C	2.7	Dehghani, Annabi, Titus, Valtchev, & Tumilar (2009)
	Total aerobic	CO_2 at 15 MPa for 15 h at 30°C	4.3	
	Total aerobic	CO_2 along with 0.02 mL of water/ethanol/H_2O_2 at 15 MPa for 6 h at 60°C	< DL* (not indicated)	
	Total aerobic		< DL* (not indicated)	

		CO_2 along with 0.1 mL of water/ethanol/H_2O_2 at 17 MPa for 2 h at 30°C		
Paprika	Mesophilic aerobic	CO_2 at 30 MPa for 45 min at 90°C	5	Calvo and Torres (2010)
Garlic powder	Total aerobic	HPP 600 MPa for 5 min	2	Park et al. (2019)

< DL*: Below detectable limit

Table 2.9. Summary of studies applying various combination treatments for the inactivation of pathogenic microorganisms in various foods.

Product	Pathogen	Treatment conditions	Reduction (log CFU/g)	Reference
Alfalfa, mung bean, and radish seeds	*E. coli* O157:H7	Dry heat treatment at 50°C for 1 h followed by irradiation treatment at 2-2.5 KGy	4.0-5.0	Bari, Sabina, Isobe, Uemura, & Isshiki (2003)
Alfalfa seeds	*Salmonella*	300-600 Pa HPP for 2 or 5 min with heat treatment from 40-50°C	<1.0	Neetoo and Chen (2010)
Radish seeds	Total aerobic	ClO_2 gas at 200 mg/mL for 5 min followed by drying at 25°C for 24 h	3.1	Kim et al. (2010)
	E. coli O157:H7	ClO_2 gas at 200 mg/mL for 5 min followed by drying at 25°C for 24 h	3.8	
Radish seeds	Total aerobic bacteria	200 or 500 µg/mL ClO_2 followed by air treatment for 2h and 48 to 24 h of dry heat treatment	5.1	Bang, Kim, Kim, Beuchat, Kim & Ryu. (2011)
	E. coli O157:H7	200 and 500 µg/mL ClO_2, air dried followed by heat treatment for 12 and 6 h	5.7	
Alfalfa seeds	*Salmonella* and *E. coli* O157:H7	600 Pa HPP for 2 min with dry heating at 55, 60, 65 and 70°C for 6, 12, 24, 96 h	5.0	Neetoo and Chen (2011)
Red pepper powder	*E. coli* O157:H7	Ultraviolet irradiation with mild heating at 65°C	2.9	Cheon et al. (2015)
	Salmonella Typhimurium	Ultraviolet irradiation with mild heating at 65°C	3.1	
Raw pistachios kernel	*Enterococcus faecium*	Infrared heating for 2 h followed by tempering at 70°C for 2 h	6.1	Venkitasamy et al. (2017)

Chapter 3. Thermal inactivation kinetics of *Salmonella enterica* and *Enterococcus faecium* NRRL B- 2354 as a function of temperature and water activity in fine ground black pepper

3.1 Abstract

Fine ground black pepper generally consumed as a seasoning without any further processing has been associated with *Salmonella enterica* outbreaks. Thermal inactivation kinetics data is necessary to develop a pasteurization process for fine ground black pepper. This study investigates the influence of temperature and water activity on thermal inactivation kinetics of *Salmonella* in fine ground black pepper. It also assesses the suitability of *Enterococcus faecium* as a surrogate for *Salmonella.* Fine ground black pepper of varying water activities, a_w (0.40, 0.55, 0.70) was subjected to isothermal treatments at different temperatures (65-80°C) for five equidistant time points with intervals ranging from 18 s – 250 min. The survival data were used to fit two primary models (log-linear and Weibull) and two secondary models (response surface and Modified Bigelow). Results indicated that among the two primary models, the Weibull model explained the thermal inactivation kinetics better with lower RMSE (0.24 – 0.56 log CFU/g) and AIC_c values at all a_w and temperatures. Water activity and treatment temperature significantly enhanced the thermal inactivation of *Salmonella*. *E. faecium* NRRL B- 2354 was found to be a suitable surrogate for *Salmonella* in fine ground black pepper at all tested treatment conditions. The developed modified Bigelow model based on the Weibull model could be applied to predict the inactivation kinetics of *Salmonella* in black pepper and would benefit the spice industry in identifying process parameters for thermal pasteurization of fine ground black pepper.

Keywords: Process validation, Thermal lethality, water activity, temperature, *E. faecium*

Wason, S., Verma, T., Wei, X., Mauromoustakos, A., & Subbiah, J. (2022). Thermal inactivation kinetics of *Salmonella enterica* and *Enterococcus faecium* NRRL B-2354 as a function of temperature and water activity in fine ground black pepper. *Food Research International*, *157*, 111393.

3.2 Introduction

Low moisture foods (LMFs) account for about 30 % of total foodborne illness outbreaks linked to *Salmonella* contamination between 2012-2016 (CDC, 2016). Black pepper, a common low moisture food ingredient or seasoning, has been often associated with various foodborne illness outbreaks (CDC, 1982; CDC, 2010) and recalls (Wason et., 2021; Food Safety News, 2016; USFDA, 2020). Around 14 foodborne illness outbreaks were reported between 1973-2010 in Canada, New Zealand, Germany, Norway, Denmark, France, Serbia, UK, and the USA linked to *Salmonella* contamination in black pepper (FDA, 2013). Because black peppercorns are dried outdoors, it is often exposed to environmental contaminants such as dust, insects, contaminated water, and animal waste(ASTA, 2017; Farakos et al., 2014). Hara-Kudo et al. (2006) analyzed 40 spice types for *Salmonella* contamination and the total microbial population in the spice samples. They found that *Salmonella enterica* contamination was only detected in black and red pepper among the 40 different spices tested. In addition, aerobic and spore-forming bacterial populations were higher in black and red pepper samples when compared to other spice varieties. In black pepper, *Salmonella* was observed to grow rapidly at a permissive water activity (0.9888) at ambient temperature conditions without the requirement of any additional nutrient (Keller et al., 2013). Because *Salmonella* is resistant to desiccation, it can survive longer, even up to a year in black pepper with a very low decay rate of 0.002 log CFU/g/day at 25 °C (Beuchat et al., 2011; Keller et al., 2013). In addition, the infectious dose of *Salmonella* is estimated to be very low (13.0 CFU/g) in most LMFs, and therefore any potential contamination in LMFs can be concerning (Finn et al., 2013).

Due to increasing food-borne illnesses and outbreaks in LMFs, the Food Safety and Modernization Act (FSMA) requires the food processing plants to identify the hazards and

deploy adequate process control protocols to ensure microbial food safety. Thermal processing is the most common method used to inactivate microorganisms in foods, including LMFs (Anderson, 2019; Wei et al., 2021; Zhang et al., 2020). However, the mitigation of pathogens from foods with lower water activities is challenging due to the enhanced heat resistance prevalent among bacterial pathogens such as *Salmonella* (He et al., 2013; Mattick et al., 2000; Podolak et al., 2010). Thermal resistance of *Salmonella* dramatically changes with water activity (Verma et al., 2021; Zhang et al., 2020). Therefore, modeling the influence of water activity and temperature on the thermal inactivation kinetics of *Salmonella* will be helpful to design an effective thermal process control. In addition, the food matrix and its composition affect the change in water activity during thermal treatment and thus can also influence the thermal inactivation kinetics of *Salmonella* in a specific food product (Bell & Kyriakides, 2002; Shachar & Yaron, 2006). Therefore, comprehensive inactivation kinetics of *Salmonella* in a particular food product at various water activities is required to evaluate process control efficiency.

Mathematical models can reduce the financial and time constraints involved in validation studies and have better scope in industrial applications (Marks, 2008). Log-linear and Weibull models are the most common primary models applied to explain the inactivation kinetics of *Salmonella* in LMFs (Santillana Farakos et al., 2013). Weibull model can be used to describe the linear as well as non-linear inactivation data due to the inclusion of shape parameter (p), which indicates the linear ($p = 1$) or concavity/convexity ($p>1/p<1$) of the inactivation curve (Marks, 2008). At the same time, secondary models such as the modified Bigelow model and response surface model are helpful to assess the effect of a_w and temperature on the thermal inactivation in LMFs. Hence, comparing various primary and secondary models can be crucial to identify the robust model to predict thermal inactivation (Gautam et al., 2020; Rachon et al., 2016).

However, comparing different published microbial models can be often difficult due to variation in the thermal inactivation protocols, serovars of *Salmonella* used in the study, and the modeling approach followed. For instance, Gautam et al., (2020) developed two secondary models to predict the effect of either temperature or water activity (but not both simultaneously) on inactivation of *Salmonella* in black pepper powder. For easier application of the developed models to determine the process parameters for thermal pasteurization, our study was aimed to develop a combined model for predicting the effect of both the temperature and water activity on thermal inactivation simultaneously. Earlier studies with black pepper (Gautam et al., 2020; Wei et al., 2021), did not report the effect of particle size on thermal inactivation of *Salmonella*, even though the particle size was reported to impact the heating rate and inactivation kinetics of *Escherichia coli* ATCC 25922 in red pepper powder (Zhang et al., 2020). Hence, we included the particle size characterization of the black pepper samples in this study. Furthermore, previous inactivation study with ground black pepper carried out by Wei et al. (2021) stated that black peppercorns were inoculated first and then ground into black pepper powder rather than inoculating ground black pepper. However, this procedure might affect the heat sensitivity of *Salmonella* as reported by Limcharoenchat et al. (2018), who found that inoculation of wheat grains prior to grinding of the samples increased the heat sensitivity of *Salmonella* as compared to inoculation of wheat flour.

Validation studies in the food industry would require a suitable surrogate to prevent the potential contamination from *Salmonella* through the food supply chain. *Enterococcus faecium* NRRL B-2354 is reported to be an appropriate surrogate for *Salmonella* for the thermal processing of many low moisture foods such as chicken meat powder, savory seasoning (Rachon et al., 2016); wheat flour (Smith et al., 2016); Oat flour (Verma et al., 2018); cocoa powder (Tsai

et al., 2019); milk powder (Wei et al., 2021). However, *E. faecium* NRRL B- 2354 was found to be less heat resistant than *Salmonella* in confectionary (Rachon et al., 2016) and paprika powder (a_w = 0.55) at temperatures higher than 70 °C and rice flour at a high water activity (a_w = 0.55) irrespective of temperature conditions (Rachon & Gibbs, 2015). Thus, it is crucial to evaluate the surrogate for different product matrices. Therefore, the present study was aimed to i) establish thermal inactivation kinetic models for *Salmonella* in fine ground black pepper as a function of a_w and temperature and ii) assess whether *E. faecium* NRRL B-2354 can be used as a potential surrogate for *Salmonella* in the validation of thermal processing of fine ground black pepper.

3.3 Materials and methods

3.3.1 Black pepper samples

Pre-sterilized fine ground black pepper samples from three variable production lots were obtained from McCormick & Company, Inc (Sparks, MD, USA). All the samples were held inside a walk-in deep freezer maintained at -12°C until further use. Before inoculation, the background microorganisms were enumerated by the aerobic plate count method as described in Verma et al. (2021). Black pepper's particle size analysis was performed using RO-TAP sieve shaker (Rx-29, W.S. Tyler, OH, US) based on the ASABE standard (ASABE, 2017). A series of 8 sieves were arranged according to sieve size with the maximum size of 0.425 mm and the minimum size of 0.075 mm. The sample (100 g) was placed on the top sieve and shaken for 1 min, followed by a measurement of the weight of the sample retained on each sieve. The analysis was performed thrice for each lot to calculate average geometric particle size.

3.3.2 Bacterial strains

Salmonella cocktail was prepared from five serotypes of *Salmonella enterica*, namely, *Salmonella enterica* serovars Montevideo 488275, *S.* Mbandaka 698538, *S.* Agona 447967, *S.* Tennessee K4643, and *S.* Reading Moff 180418. The choice of the serotypes was linked to their involvement in food-borne illness outbreaks and recalls specific to LMFs in the past. *Salmonella* Agona (447967), *Salmonella* Mbandaka (698538), and *Salmonella* Montevideo (488275) were obtained from the Food and Drug Administration, Office of Regulatory Affairs, Regional Laboratory in Jefferson, AR. *Salmonella* Reading (Moff 180418) and *Salmonella* Tennessee (K4643) was obtained from the FDA culture collection in Bedford Park, IL. and University of Georgia in Griffin, GA, respectively. *Enterococcus faecium* NRRL B-2354 was chosen as a potential surrogate for comparing microbial inactivation in *Salmonella* and was obtained from the United States Department of Agriculture, Agriculture Research Services (USDA, ARS) in Peoria, IL. The acquired cultures were supplemented with 40% (v/v) glycerol and stored in an ultra-freezer at -80°C until further use.

3.3.3 Inoculum preparation

The inoculum was prepared according to the method described by Verma et al. (2021). In brief, the frozen stock (1 mL) was thawed at ambient temperature for 5 min and then added to tryptic soy broth with 0.6% (w/w) yeast extract (10 mL) (TSBYE; Becton, Dickinson and Company, Franklin Lakes, NJ) followed by incubation at 37°C for 24 h. Further, working stock plates were prepared by streaking a loopful (10 µL) of TSBYE onto tryptic soy agar supplemented with 0.6 % (w/w) yeast extract (TSAYE; Becton, Dickinson and Company, Franklin Lakes, NJ) followed by incubation at 37°C for 24 h. Upon incubation, one isolated bacterial colony was propagated in 10 mL of TSBYE. Further, 100 µL was plated on TSAYE and

incubated at 37°C for 24 h. Using a sterile L-shaped spreader, the bacterial lawns were then extracted in 3 mL of 0.1 % (w/w) buffered peptone water (BPW; Becton, Dickinson and Company, Sparks, MD). An equal volume of inoculum from each *Salmonella* serotype was combined to prepare a *Salmonella* cocktail. A similar procedure was followed to prepare the *E. faecium* NRRL B- 2354 inoculum. The *Salmonella* and *E. faecium* populations in the inoculum were about ca. 10.4 and 10.7 log CFU/mL, respectively. The procedure was repeated with new frozen cultures to inoculate samples from different lots to provide biological replication.

3.3.4 Inoculation of Sample

Fine ground black pepper samples drawn from the walk-in freezer were held at room temperature for 24 h before inoculation. Briefly, 300 g of black pepper sample was taken in a polypropylene bag and spray inoculated with 6 mL of bacterial inoculum separately for *Salmonella* and *E. faecium* NRRL B- 2354 inside a biosafety cabinet. The bacterial inoculum was sprayed 10 cm away from the samples to avoid the formation of clumps. Post inoculum spray, the sample was shaken manually for 5 min to ensure uniform distribution of inoculum. The sample was then placed on a sanitized aluminum tray and held inside a relative humidity (RH) equilibration chamber (Lau & Subbiah, 2020). The moisture content and a_w of fine ground black pepper samples were recorded using halogen moisture analyzer (Model: HR73, Mettler Toledo, Greifensee, Switzerland) and dew point water activity meter (Model: 4 TE, Meter Group, Pullman, WA) for 14 days after inoculation. For the aw measurement, the black pepper powder (~0.5 g) was drawn from the RH chamber in a sealed plastic cup. The lid of the sample cup was removed, and the sample was placed in the water activity meter for the a_w measurement at 25 °C. Stability and homogeneity tests were performed every day until one week and on the 12^{th} and

14^{th} days to assess the effect of the inoculation method on the distribution of bacteria in the sample.

After inoculation, homogeneity and stability tests were performed by drawing 2 g of samples at five different tray positions and aseptically transferred to sterile Whirl-Pak style bags for bacterial enumeration. Buffered peptone water was used for the serial dilution of the sample in 1:10 ratio and spread plated on m-TSAYE (TSAYE supplemented with 0.05 % (w/w) ammonium iron (III) citrate (Sigma Aldrich, St. Louis, MO), and 0.03 % (w/w) sodium thiosulfate pentahydrate (Fisher Scientific, Fair Lawn, NJ) for *Salmonella* and e-TSAYE (TSAYE supplemented with 0.05% (w/v) ammonium iron citrate, and 0.025% (w/v) esculin hydrate (Acros Organics, NJ, USA) for *E. faecium* followed by incubation at 37°C for 24 h. *Salmonella* has the ability to reduce sodium thiosulphate pentahydrate to form hydrogen sulphide which then reacts with ammonium iron citrate to create a complex producing black or black centred colonies which helps in *Salmonella* enumeration (Soria & Bueno, 2016). To consider the black pepper samples to be homogenous, the standard deviation of the bacterial population from five samples chosen at random must be < 0.3 log CFU/g. These samples were further used for the isothermal treatments. Similar enumeration procedure was followed for uninoculated black pepper samples to check for the presence of *Salmonella* and *E. faecium* in control sample.

3.3.5 Isothermal treatment

The experimental design used was a 3x3 factorial design, with water activity (0.40, 0.55, and 0.70) as the hard-to-change factor and the temperature regimes (65, 70, 75, and 80°C) as the easy- to-change factor for each of the two bacteria. Each experiment was replicated thrice with three different lots of samples. Heat-sealable aluminum pouches (7.62 x 7.62 cm; IMPAK

Corporation, Los Angeles, CA) were used to pack and treat the sample (2 g) in a custom-designed thermal death time sandwich system.

The come-up time was calculated based on the average time required to achieve within 0.5 °C of the target treatment temperature plus twice the standard deviation of the sample replicates. For this purpose, a T-type thermocouple (Omega Engineering Inc., Norwalk, CT) was inserted to the geometric center of the pouches containing the fine ground pepper samples treated at different a_w and temperature conditions. The microbial population at the come-up time was counted as time 0 population. Isothermal treatments were conducted for at least five-time points equidistantly spaced for each temperature and water activity combination. Post treatment, sample pouches were placed in ice-water bath for at least 1 minute to terminate the thermal inactivation. It was followed by diluting the sample and homogenizing it using a stomacher for 1 minute. The diluent was then serially diluted, spread plated on mTSAYE for *Salmonella* and eTSAYE for *E. faecium* NRRL B- 2354 and incubated at 37°C for 24 h. The bacterial counts were expressed as log CFU/g. The treatments (3 a_w X 3 temp X 2 bacteria) were replicated for three batches of sample which was inoculated with new frozen cultures.

3.3.6 Model analysis

a) Primary Model fitting

Two primary models, Log-linear (Eqn. 1) and Weibull (Eqn. 2) were used to fit the survival data of *Salmonella* or *E. faecium* NRRL B- 2354 in fine ground black pepper as shown below:

Log-linear model: $$\log_{10}\left(\frac{N}{N_0}\right) = -\frac{t}{D} \quad (1)$$

Weibull model: $$\log_{10}\left(\frac{N}{N_0}\right) = -\left(\frac{t}{\delta}\right)^{p} \quad (2)$$

Where N and N_0 (CFU/g) is the microbial population at time t and after the come-up time, respectively, t (min) is the isothermal treatment time, *D* (min) is the time required to reduce the microbial population by 90 % at a specific temperature (°C), δ is the scale parameter representing the overall steepness of the survival curve and p is the shape parameter, which describes whether the inactivation rate is constant, (n = 1), decreasing (n < 1) or increasing (n > 1) with time. Parameters were estimated for all the treatment conditions sets separately using the GInafit – Version 1.6 freeware add-in for Microsoft Excel (Geeraerd et al., 2005).

The goodness-of-fit of the models were quantified by the root mean square error (RMSE) (log CFU/g) and corrected Akaike information criterion (AIC_c):

$$\text{RMSE} = \sqrt{\frac{\sum_{i=1}^{n}[log_{10}(N)_{observed,i} - log_{10}(N)_{predicted,i}]^2}{n}} \quad (3)$$

where $log_{10}(N)_{observed,i}$ is the experimental log population, $log_{10}(N)_{predicted,i}$ is the estimated log population from the model parameters, and *n* is the total number of observations. RMSE is a measure of deviation of the experimental values from predicted values obtained from the model. In other words, RMSE tells us how well a regression model can predict the value of the response variable (Motulsky & Cristopoulos, 2004).

$$\text{AICc} = \text{nln}\left(\frac{SS}{n}\right) + 2\text{K} + \frac{2K(K+1)}{n-K-1} \quad (4)$$

where n is the total number of observations, SS is the sum of squares of the residuals, and K is the number of parameters being estimated plus 1. AIC_c provides information on whether a drop in the residual sum of squares is explained by adding parameters in the model. Lower the value

for AIC_c and RMSE, more likely that the determined model is correct for the data (Dolan et al., 2013; Smith et al., 2016).

The time (t_S) required for 1, 3, and 5-log reduction were predicted by the following equation

$$t_S = \delta * S^{1/p} \quad (5)$$

where S = $\log_{10}\left(\frac{N}{N_0}\right)$, is the log reduction required and p is the fixed shape parameter of 0.67 (*Salmonella*) and 0.73 (*E. faecium*).

A fixed value approach was used to re-estimate the δ-value (delta*) by fixing the p-value as the average value from all the survival curve to remove any structural variations (Gautam et al., 2020; Zhang et al., 2020). The re-estimated δ*-values were further used for model analysis.

b) Secondary Model fitting

Secondary models were fitted to assess the influence of initial water activity and temperature on D- and δ-value. First secondary model evaluated in this study was a modified version of a Bigelow-type relationship (Gaillard et al., 1998):

Modified Bigelow model

$$D(T, a_w) = D_{ref} \cdot 10^{\frac{a_{wref} - a_w}{z_{a_w}}} \cdot 10^{\frac{T_{ref} - T}{z_T}} \quad (6)$$

where D_{ref} is the decimal reduction time (min) at T_{ref} and a_{wref}, T is the temperature (°C), T_{ref} and a_{wref} are the optimized reference temperature and water activity, z_{aw} or $z_{T\,(°C)}$ is the a_w or temperature increment needed to reduce the D and δ -values by 90%. For the secondary model based on Weibull distribution, δ is used in place of D as the time required for 1st log reduction

All parameters for the Bigelow-type model were estimated using OLS minimization with nlinfit in MATLAB 2019 (The Mathworks, Inc., Natick, MA).

The z_T was also calculated for different water activities using equation 4.

$$z_T = \frac{T_2 - T_1}{\log\left(\frac{D_1}{D_2}\right)} \quad (7)$$

Response surface model (Eqn. 8) was developed for the primary models and only those parameters were included, which had a significant effect at $p < 0.05$. Response surface model was fitted in an open-source statistical software R (https://www.R-project.org/; Lenth, 2009).

$$D_{(T,a_w)} = \beta_o + \beta_1 * T + \beta_2 * a_w + \beta_3 * T^2 + \beta_4 * a_w^2 + \beta_5 * T * a_w \quad (9)$$

The goodness of fit of the secondary models for *Salmonella* and *E. faecium* were evaluated by AIC_c and RMSE values.

3.3.7 Statistical analysis

Inactivation data was analyzed by performing ANOVA for the response variable based on 2x3x3 factorial design. There were 18 treatment combinations considering two bacteria (*Salmonella* and *E. faecium* NRRL B- 2354) at three different a_w and three temperatures. Each treatment condition was repeated with three different lots of samples (three replications). Means of each treatment group were compared by Tukey-Kramer adjustment (Tukey's Honestly Significant Difference test) at 5% significance level.

3.4 Results & Discussion

The mean ± standard deviation of initial a_w and the geometric particle diameter of fine ground black pepper samples were 0.55 ± 0.002 and 0.24 ± 0.05 mm respectively. Considering

the initial water activity of the samples, a_w =0.55 was selected to be the inherent water activity of black pepper samples throughout the study. The background microbiota of the black pepper samples estimated by aerobic bacterial counts was below the detection limit (1 log CFU/g). Bacterial enumeration of uninoculated sample on m-TSAYE and e-TSAYE did not show the presence of *Salmonella* and *E. faecium,* respectively. Since the background aerobic microbiota count for the black pepper was low enough compared to the inoculated *Salmonella* levels, it was not expected to interfere with the microbial inactivation.

3.4.1 Stability and homogeneity

The mean ± standard deviation of the initial population of *Salmonella* and *E. faecium* NRRL B- 2354 were 8.13 ± 0.02 and 8.06 ± 0.03 log CFU/g respectively. The inoculated samples were adjusted to three different water activities (0.40, 0.55, and 0.70) to determine the effect of water activity on the thermal inactivation kinetics of *Salmonella* and *E. faecium*. Fig. 1. shows the water activity and moisture content values recorded in black pepper for 14 days. The sample held in the RH chamber achieved target a_w and moisture content within five days of equilibration. To achieve a conservative estimation of the thermal inactivation kinetics of *Salmonella*, it is crucial to provide sufficient time for the bacteria to completely adapt to a desiccated environment and reach a stable population (Wei et al., 2018). Also, it is vital to consider the influence of the inoculation method on bacterial stability and thermal resistance in LMFs. For instance, Hildebrandt et al. (2016) reported the lawn-based method (wet inoculation) to be more suitable for providing a stable bacterial population with repeatable D-values amongst the various inoculation methods tested. This study also found that the lawn-based (wet) inoculation method is suitable for providing a high and stable bacterial population (Fig. 1). This

finding agrees with reported results in the literature for various LMFs including, black pepper (Wei et al., 2021), and oat flour (Anderson et al., 2017; Verma et al., 2018).

It is evident from Fig. 1. that there was 0.70 and 0.05 log CFU/g reduction in *Salmonella and E. faecium* populations, respectively, after three days of equilibration. After a slight initial reduction in population, *Salmonella* (7.6 ± 0.06 log CFU/g) and *E. faecium* (8.0 ± 0.05 log CFU/g) populations remained stable over an extended period from 5 to 14 days. Therefore, isothermal treatments were performed only after five days of equilibration. Smaller standard deviations between sub-samples of the inoculated samples (< 0.3 log CFU/g, as shown by error bars in Fig. 1) indicate that the inoculation procedure resulted in a homogenous distribution of the inoculum.

3.4.2 Thermal inactivation of Salmonella in fine ground black pepper

The come-up times (CUTs) to reach target temperatures of 65, 70, 75, and 80°C were 68, 76, 72, and 83 s respectively. Based on the preliminary trials, at least five equidistant time points were selected such that the maximum time achieved ca. 5 log reduction. Surviving bacterial population after CUTs at each water activity and temperature was considered as the time 0 population and used for further analysis of survival curves. There was a considerable decrease (3.05 and 3.96 log) in the population of *Salmonella* and *E. faecium* NRRL B- 2354 in black pepper after CUTs, for treatment at 70 and 75°C in samples with a high initial water activity of 0.70 (Table 1). However, Gautam et al. (2020) observed comparatively less reduction (0.56 and 1.35 log) in *Salmonella* population in black pepper powder over a longer (3 min CUT) at same treatment temperatures of 70 and 75°C even at a higher a_w of 0.75. The differences in reduction of the bacterial populations may be explained by the high heat resistance of *S.* Typhimurium and

S. Senftenberg (Mercer et al., 2017) used in the five strain *Salmonella* cocktail to inoculate black pepper in their study.

The two primary models, the log-linear (Eqn. 1) and Weibull (Eqn. 2), were fit to the survival data at various initial water activities and treatment temperatures (Fig. 2). The parameter estimates of the log-linear (D-value) and Weibull (δ and p-value) model are provided in Table 2. $D_{70°C}$, $D_{75°C}$, $D_{80°C}$ of *Salmonella* and *E. faecium* at 0.55 a_w were 7.57, 3.86, and 1.76 min and 36.94, 9.49, and 2.78 min, respectively. The survival curve was steeper at higher treatment temperature, indicating a higher inactivation rate. For instance, at 0.40 a_w, the obtained D-value of 20.36 min significantly decreased to 3.86 min when the treatment temperature was increased from 70 to 80°C. This result is in agreement with (Wei et al. 2021)) who observed a reduction of D-value for *Salmonella* (46.0 to 7.8 min) and *E. faecium* (34.1 to 5.2 min) in black pepper powder at 0.45 water activity as temperature increased from 65 to 75°C. As expected, the D and δ-values of the *Salmonella* in black pepper decreased, in general, as initial water activity was increased from 0.40 to 0.70 at a particular temperature. Similar observations were reported in other LMFs, such as dried basil leaves (Verma et al., 2021), chia seeds (Lau et al. 2021), milk powder (Wei et al., 2020), red pepper powder (Zhang et al., 2020), and wheat flour (Smith et al., 2016). Smith et al. (2016) observed a higher $D_{75°C}$ value for *Salmonella* in wheat flour (3.25 min) as compared to black pepper (1.34 min) at 0.70 a_w recorded in the present study. This variation could be attributed to antimicrobial compounds in black pepper rendering *Salmonella* to lose its cell wall integrity, making them more sensitive to heat inactivation (Tang et al., 2017). The z_T-value of *Salmonella* and *E. faecium* in black pepper at 0.40, 0.55, and 0.70 a_w were determined from the log-linear model and are presented in Table 2. The lower z_T values obtained at higher

water activity show that *Salmonella* and *E. faecium* tend to become more sensitive to heat requiring relatively lower treatment temperature for inactivation at higher water activities.

In the present study, δ-value for *Salmonella* reduced from 2.0 to 0.65 min as treatment temperature increased from 70 to 80°C at initial water activity of 0.55 a_w. The corresponding values reported by (Gautam et al. 2020) were 1.34 to 0.19 min for the same conditions for different high heat resistant strains *S.* Typimurium and *S.* Senftenberg in black pepper. In a study involving red pepper powder, Zhang et al. (2020) reported δ-values of 12.42, 3.59, 1.68 and 2.23 min for thermal inactivation of *E.coli* ATCC 25922 with an a_w of 0.55 at their respective treatment temperatures of 62, 65, 68, and 71°C. The minor differences in δ-values for similar products could be attributed to the variation in a food matrix, bacterial strains (Ma et al., 2009; Quintavalla et al., 2001) or inoculation methods (Bowman et al., 2015; Liu et al., 2019).

3.4.3 Primary model comparison

The log-linear model and Weibull model fitted the data well, while the goodness of fit for Weibull model was relatively better for thermal inactivation of both the *Salmonella* and *E. faecium* NRRL B- 2354 in black pepper considering lower RMSE (0.24-0.55 log CFU/g) and AIC_c values (-15.1 to -42.81) as compared to RMSE (0.30-0.63 log CFU/g) and AIC_c values (-18.59 to -49.13) obtained for log-linear model (Table 2.). The inactivation curves show a non-linear trend which can be better explained by the Weibull model. The Weibull model can describe linear as well as non-linear inactivation curves with shoulder or tailing. The Weibull model can take biological variation among the bacterial cells within the population by using an additional parameter, shape, p-value (Peleg, 2006). For the Weibull model, the convexity was observed in all the survival curves with p values <1 except for black pepper with 0.70 a_w treated at 75°C. This suggested that the heat-sensitive bacterial cells reduced at a faster rate initially, while more

resistant cells survived during the heating (Peleg, 2006). Weibull model has been preferred over log-linear model to explain the thermal inactivation kinetics in several other LMFs such as almonds (Villa-Rojas et al., 2013), chia seeds (Lau et al. 2021), cumin seed powder (Ozturk et al., 2020), red pepper powder (Zhang et al., 2020) and wheat flour (Smith et al., 2016).

As depicted in Fig. 3, there appears to be no relationship between shape parameter, p-value and temperature at various water activities. These results are in accordance with Garcia et al. (2019)) and Zhang et al. (2020) who also indicated no significant influence of a_w and temperature on the estimated p-value (shape parameter). Thus, the δ-values were re-estimated by fixing the p-value as the average value (shape parameter (p) = 0.67 for *Salmonella* and 0.73 for *E. faecium*) from all the survival curves to remove any structural variations (Gautam et al., 2020; Zhang et al., 2020). The re-estimated δ-values (δ*) (not shown) were further used for model analysis. The data obtained from the Weibull model was utilized to develop secondary models such as modified Bigelow and response surface models to assess the impact of a_w and temperature on the thermal inactivation kinetics of *Salmonella.*

3.4.4 Comparison of secondary models

Thermal processing of food involves evaporation of water from the food product leading to loss of moisture. This results in the reduction of water activity leading to the enhanced thermal resistance of *Salmonella* in the sample as has been reported in some studies (Verma et al., 2021; Zhang et al., 2020). Therefore, understanding the effect of water activity on thermal inactivation kinetics is crucial in designing a thermal pasteurization process. Recent literature review showed that no secondary model is available to explain the relationship of the Weibull model with water activity and temperature. In this study, we attempted to use response surface model and the modified Bigelow model as secondary models based on the Weibull model parameters.

3.4.4.1 Salmonella Modeling

a) Modified Bigelow model

Modified Bigelow model was used to fit D and δ* values as a function of temperature and water activity as shown below.

$$D\,(T, a_w) = 3.58 \cdot 10^{\frac{0.55-a_w}{0.36}} \cdot 10^{\frac{75-T}{14.78}} \quad (10)$$

RMSE = 0.86 min; adjusted R^2 = 0.98; AIC_c = -1.61

$$\delta^{*}(T, a_w) = 1.91 \cdot 10^{\frac{0.55-a_w}{0.43}} \cdot 10^{\frac{75-T}{16.43}} \quad (11)$$

RMSE = 0.68 min ; adjusted R^2 = 0.91; AIC_c = -14.46

Eqn. 11 can be incorporated into Eqn. 5 to predict the time required for a desired log reduction (sterility value) at any temperature and water activity.

b) Response surface model

$$\delta^* = 267.89 - 4.7646^*T - 267.82^*a_w + 0.020667^*T^2 + 66.685^*{a_w}^2 + 2.4744^*{a_w}^*T \quad (12)$$

RMSE = 0.59 min; adjusted R^2 = 0.92; AIC_c = 62.26

3.4.2 E. faecium Modeling

a) Modified Bigelow model

$$D(T, a_w) = 12.37.10^{\frac{0.55-a_w}{0.70}} \cdot 10^{\frac{75-T}{11.45}} \quad (13)$$

RMSE = 3.95 min; adjusted R^2 = 0.96; AIC_c = 80.76

$$\delta^{*}(T, a_w) = 6.28.\,10^{\frac{0.55-a_w}{0.67}} \cdot 10^{\frac{75-T}{10.84}} \quad (14)$$

RMSE = 2.22 min; adjusted R^2 = 0.96; AIC_c = 49.82

b) Response surface model

$$\delta^* = 1595.745278 - 36.575744^*T - 532.374630a_w + 0.212587^*T^2 + 5.465111^*a_w{}^*T \quad (15)$$

RMSE = 1.84 min; adjusted R^2 = 0.96; AIC_c = 123.63

The response surface model developed for δ* values obtained for *Salmonella* and *E. faecium* had significant ($p<0.05$) linear and quadratic water activity and temperature effects. Water activity had a negative linear effect on the δ* values obtained for *Salmonella* and *E. faecium*. Based on lower AIC_c values, modified Bigelow model provided a better fit than the response surface model for this data. Smith et al. (2016) and Verma et al. (2021) also recommended a modified Bigelow model over response surface model to predict thermal inactivation in dried basil leaves and wheat flour, respectively. Among the two models, the modified Bigelow model based on Weibull distribution fit the data better as compared to log linear distribution as indicated by lower AIC_c values. Villa-Rojas et al. (2013) reported similar observations in the case of almond kernels. Values of z_T or thermosensitivity and z_{aw} or water activity sensitivity of *Salmonella* in almond kernels were reported to be 8.28°C and 0.187 which were smaller than the same values observed for *Salmonella* in this study. The contour plots were developed based on the modified Bigelow model to predict δ* as shown in Fig. 4. It represents that both temperature and water activity had a positive effect on bacterial inactivation. The gaps between contour lines tend to widen as water activity and temperature increase. At lower water activity, a minimal decrease in temperature substantially increased the δ* compared to high water activity. At 65°C,

~ 0.05 decrease in a_w increased the δ* by 4 mins (*i.e.* from 12 to 16 min). On the other hand, a 0.05 decrease in a_w (*i.e.* from 0.65 to 0.60) increased the required time by less than 2 min. Similarly, at 0.55 a_w, a 5°C decrease in treatment temperature (70 to 65°C) increased the time required for a 5-log reduction by around 4 min; while a 5°C decrease in temperature from 75°C to 70°C increased the time by only around 2 min. The same trend was observed for *E. faecium* as well. Thus, high water activity and the temperature had a considerable impact on the inactivation rate of *Salmonella* and *E. faecium* in black pepper. This contour plot can be beneficial for the spice industry to identify suitable processing conditions for the pasteurization of black pepper powder.

3.4.5 Prediction Analysis

The modified Bigelow model can predict δ* values at any given water activity and temperature within the tested range. Predicted δ* values were compared with observed/experimental δ* values and the plot between them showed good correlation (Fig. 5(a)). Gautam et al. (2020) also observed a strong correlation between experimental and predicted δ* values based on high R^2 values (0.86-0.99) in black pepper powder. The goodness of fit parameters based on squared differences may be deceptive sometimes if the model is constantly predicting higher or lower values than experimental values. Therefore, experimental values vs residuals were plotted as shown in Fig. 5(b). The random distribution of residuals around zero suggests that the model is appropriate to predict δ*. Points above the diagonal in Fig. 5(a) and the negative points in the residual plot (Fig. 5(b)) are fail-safe. The predicted values of δ* from the modified Bigelow model (Eqn. 11 for *Salmonella*) were substituted into the Weibull model (Eqn. 2) to predict log survival of *Salmonella* at all treatment conditions using a fixed p-value of 0.67 for *Salmonella*. These predictions were denoted as the Weibull-modified Bigelow model.

As seen in Fig. 6, the comparison of mean experimental values and predicted survival curves from Weibull-modified Bigelow model showed a good fit at lower water activities. Overall, the RMSE ranged from 0.31 to 0.70 log CFU/g and were within acceptable range. The experimental errors were calculated as RMSE of 3 reps from its mean value and ranged from 0.25 to 0.47 log CFU/g. The comparison of experimental errors, RMSE of the Weibull model (primary model) and the Weibull-modified Bigelow model is shown in Fig. 7. The predicted values from Weibull-modified Bigelow had higher RMSE values at higher water activity and lower temperature values. A similar observation was reported by Wei et al. (2020) in milk powder with a higher percentage error of residuals in the prediction of D-values at higher water activity and temperature using the modified Bigelow model. When the product is in a stressful condition of low water activity, it induces various metabolic processes in *Salmonella,* such as accumulating compatible solutes and osmoprotectants, modifying the cell membrane, etc which makes it more heat resistant (Finn et al., 2013). Thus, it is more challenging to kill *Salmonella* at lower water activities and our model predicts well for these worst-case conditions. Compared to the experimental errors, these errors in model predictions can be considered acceptable for the industry.

There are a variety of parameters that determine the target log reduction, such as persistence and initial level of contamination. However, these parameters tend to vary for spice type and have not been reported yet. FDA considers a 5-log reduction of *Salmonella* appropriate in some low moisture foods such as almonds (ASTA, 2017). Table 3. presents the 1, 3 and 5 log reduction times for *Salmonella* in black pepper extrapolated from Eqn. 5. at different a_w and temperature conditions. *Salmonella*'s 3-log reduction times increased from 6.53 to 9.26 and 20.75 min when a_w reduced 0.70 to 0.55 and 0.40, respectively. A similar trend was observed for

5-log reduction times. Predicted 3 and 5 log reduction times were comparable to the observed treatment times for *Salmonella* inactivation at different temperature-a_w combinations. Although various time-temperature and a_w combinations did not achieve more than 3 log reduction of *Salmonella* in black pepper reported by Gautam et al. (2020), the trend of predicted 3 and 5 log reduction times to time was similar as observed in this study. Higher temperatures were required at lower water activities to achieve 5-log reduction when compared to higher water activity where lower temperatures are sufficient at similar treatment times.

3.4.5 *E. faecium* NRRL B- 2354 as a surrogate for *Salmonella*

FSMA passed in 2016 has become a mandate for all food manufacturers to formulate a food safety plan and validate their lethality step. However, it is impractical to use *Salmonella* for process validation due to the potential risk of contamination in the food facility. Therefore, a suitable non-pathogenic surrogate with similar heat and desiccation resistance characteristics as *Salmonella* must be evaluated. Because *E. faecium* NRRL B- 2354 has comparable genomic and functional characteristics to *Salmonella* (Kopit et al., 2014), we investigated its suitability as a potential surrogate to *Salmonella*. Several studies have confirmed *E. faecium* as a suitable surrogate for *Salmonella* in many LMFs such as black pepper powder (Wei et al., 2021), chia seeds (Lau et al. 2021), dried basil leaves (Verma et al., 2021) and white pepper (Ozturk et al., 2020). Because the surrogates are product and process specific, it is imperative to evaluate their suitability in validating the thermal pasteurization in black pepper. It was found that *E. faecium* was stable and achieved a consistent population in black pepper with a high initial population. D and δ*-values of *E. faecium* was either significantly higher or on par with *Salmonella* at all experimental processing conditions indicating its suitability as a surrogate of *Salmonella* for black pepper. Furthermore, *E. faecium* displayed a similar inverse relationship between thermal

inactivation and water activity/temperature as *Salmonella* in black pepper. The developed modified Bigelow model showed that δ* value for *E. faecium* (6.28 min) is higher than that of *Salmonella* (1.91 min) at 75 °C and 0.55 a_w. Z_{aw} for *E. faecium* (0.67) is higher than that for *Salmonella* (0.43). Interestingly, the estimated z_T value for *E. faecium* (10.84 °C) was lower than *Salmonella* (16.43 °C). This resulted in a steeper contour line for *E. faecium* than *Salmonella*, when T is plotted on x axis with a_w as y-axis (Fig. 4). This shows that *E. faecium* may be less resistant than *Salmonella* under different processing conditions, most probably at very higher water activity and temperature conditions than experimental range. Similarly, Rachon & Gibbs. (2015) observed that *E. faecium* was less resistant than *Salmonella* at treatment temperatures higher than 75°C in paprika powder. *E. faecium* had lower z_T value of 11.90 and 12.72 than *Salmonella,* with z_T values of 15.43 and 16.22 at 0.45 and 0.55 a_w respectively. The reference D value at 75 °C and 0.55 a_w is much higher for *E. faecium* (12.37 min) than *Salmonella* (3.58 min). Fig. 8. shows the cross-over temperatures after which *E. faecium* will be less resistant than *Salmonella*; these cross-over temperatures varied with a_w (92, 102, 112°C for 0.40, 0.55, and 0.70 a_w, respectively). As the quality of black pepper will be considerably affected beyond 90°C, the industry is expected to use temperatures less than or equal to 80°C. Therefore, *E. faecium* is still a suitable surrogate for *Salmonella* in black pepper in the studied temperature and aw range.

3.5 Conclusion

Thermal inactivation of *Salmonella* and *E. faecium* NRRL B- 2354 at the tested a_w of 0.40, 0.55, and 0.70 revealed a non-linear trend and could be explained better by the Weibull model. *Salmonella* acquired higher thermal resistance at lower water activities and temperatures. Modified Bigelow model performed better than response surface model. The modified Bigelow model combined with the Weibull model could be applied effectively to predict the thermal

inactivation kinetics at a given treatment temperature and initial water activity. The outcome of the present study will be beneficial for the spice industry to identify treatment conditions and develop a pasteurization process for black pepper. Additionally, *E. faecium* is a suitable surrogate for *Salmonella* for performing validation studies on the thermal pasteurization process due to its higher thermal resistance at the tested treatment conditions and non-pathogenic nature.

3.5 References

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Table 3.1 Log reduction of *Salmonella* and *E. faecium* NRRL B- 2354 during come-up time.

Water activity	**Temperature (°C)**	**Come-up time (s)**	**Log reduction (log CFU/g) ± SD**	
			Salmonella	***E. faecium***
0.40	70	76	0.45±0.44	0.12±0.06
	75	72	0.71±0.26	0.22 ±0.15
	80	83	1.30±0.36	0.23 ±0.20
0.55	70	76	0.89±0.08	0.41±0.35
	75	72	1.49±0.18	0.40±0.19
	80	83	2.83±0.30	0.17±0.42
0.70	65	68	1.7±0.36	0.26±0.09
	70	76	3.05±0.34	0.29±0.11
	75	72	3.96±0.36	0.90 ±0.13

Table 3.2 Parameter estimates for log-linear and Weibull model for inactivation of *Salmonella* and *E. faecium* NRRL B-2354 in fine ground black pepper.

Pathogen	Sample a_w	Temperature (°C)	Log-linear Model				Weibull Model			
			D (min) ± SD	RMSE (log CFU/g)	AIC_C	z_T	δ (min) ± SD	P ± SD	RMSE (log CFU/g)	AIC_C
Salmonella	0.40	70	20.36± 0.83^{cd}	0.52	-22.22	13.85	10.77±1.97^{de}	0.73±0.06^{bcd}	0.47	-25.35
		75	8.89±0.80^{e}	0.57	-18.44		4.74±1.22^{de}	0.71±0.05^{cd}	0.53	-21.05
		80	3.86±0.18^{efg}	0.40	-31.89		1.73±0.41^{e}	0.64±0.07^{cd}	0.28	-43.75
	0.55	70	7.57±0.30^{ef}	0.58	-18.16	15.78	2.0± 0.36^{e}	0.53±0.05^{cd}	0.32	-38.87
		75	3.86±0.20^{efg}	0.57	-18.99		1.20±0.68^{e}	0.56±0.13^{cd}	0.37	-34.22
		80	1.76±0.03^{fg}	0.41	-30.62		0.65±0.14^{e}	0.54±0.06^{cd}	0.31	-40.64
	0.70	65	5.82±0.60^{efg}	0.63	-15.1	15.65	2.13±0.35^{e}	0.58±0.04^{cd}	0.39	-32.14
		70	4.81±0.79^{efg}	0.33	-38.27		2.29±0.09^{e}	0.54±0.08^{cd}	0.28	-44.44
		75	1.34±0.15^{g}	0.3	-42.81		1.36±0.14^{e}	1.19±0.21^{a}	0.30	-41.26
E. faecium	0.40	70	52.46±4.40^{a}	0.39	-32.05	13.07	52.61±13.32^{a}	1.00±0.12^{ab}	0.41	-30.39
		75	25.73±1.53^{c}	0.49	-24.27		15.49±5.29 ^{cd}	0.78±0.14^{bcd}	0.41	-30.27
		80	9.02±0.96^{e}	0.61	-16.47		5.30±2.01^{de}	0.76±0.08^{bcd}	0.55	-19.16
	0.55	70	36.94±1.92^{b}	0.44	-28.28	8.89	22.00±1.48^{c}	0.78±0.04^{bcd}	0.38	-33.14
		75	9.49±0.44^{e}	0.58	-18		6.21±2.06^{de}	0.80±0.11^{bc}	0.56	-18.59
		80	2.78±0.27^{fg}	0.44	-27.86		1.14±0.51^{e}	0.67±0.08^{cd}	0.25	-48.22
	0.70	65	57.37±6.03^{a}	0.44	-28.04	9.22	33.88±3.09^{b}	0.75±0.03^{bcd}	0.37	-33.8
		70	17.11±0.86^{d}	0.53	-21.59		3.75±1.39^{e}	0.51±0.06^{b}	0.24	-49.13
		75	4.73±0.37^{efg}	0.55	-20.32		1.21±0.43^{e}	0.53±0.05^{cd}	0.27	-44.54

Table 3.3 Calculated minimum times for thermal inactivation of Salmonella in fine ground black pepper based on Weibull model (Eqn. 5).

Water activity	Temperature (°C)	Predicted time for 1-log reduction (min) ± SD	Predicted time for 3-log reduction (min) ± SD	Predicted time for 5-log reduction (min) ± SD
0.40	70	8.87±0.51^{a}	45.71±2.62^{a}	97.99±5.63^{a}
	75	4.03±0.56^{b}	20.75±2.89^{b}	44.48±6.19^{b}
	80	1.85±0.13^{cd}	9.57±0.64^{cd}	20.51±1.38^{cd}
0.55	70	3.48±0.19^{b}	17.95±0.97^{b}	38.48±2.07^{b}
	75	1.80±0.07^{d}	9.26±0.34^{d}	19.85±0.72^{d}
	80	0.98±0.02^{d}	5.03±0.12^{d}	10.79±0.26^{d}
0.70	65	2.92±0.42^{bc}	15.07±2.18^{bc}	32.29±4.68^{bc}
	70	3.11±0.73^{b}	16.03±3.74^{b}	34.36±8.02^{b}
	75	1.27±0.21^{d}	6.53±1.09^{d}	13.99±2.33^{d}

*Values presented in the table mean ± standard deviation (n = 3). a-d within a column, values with different letters are significant different ($P < 0.05$).

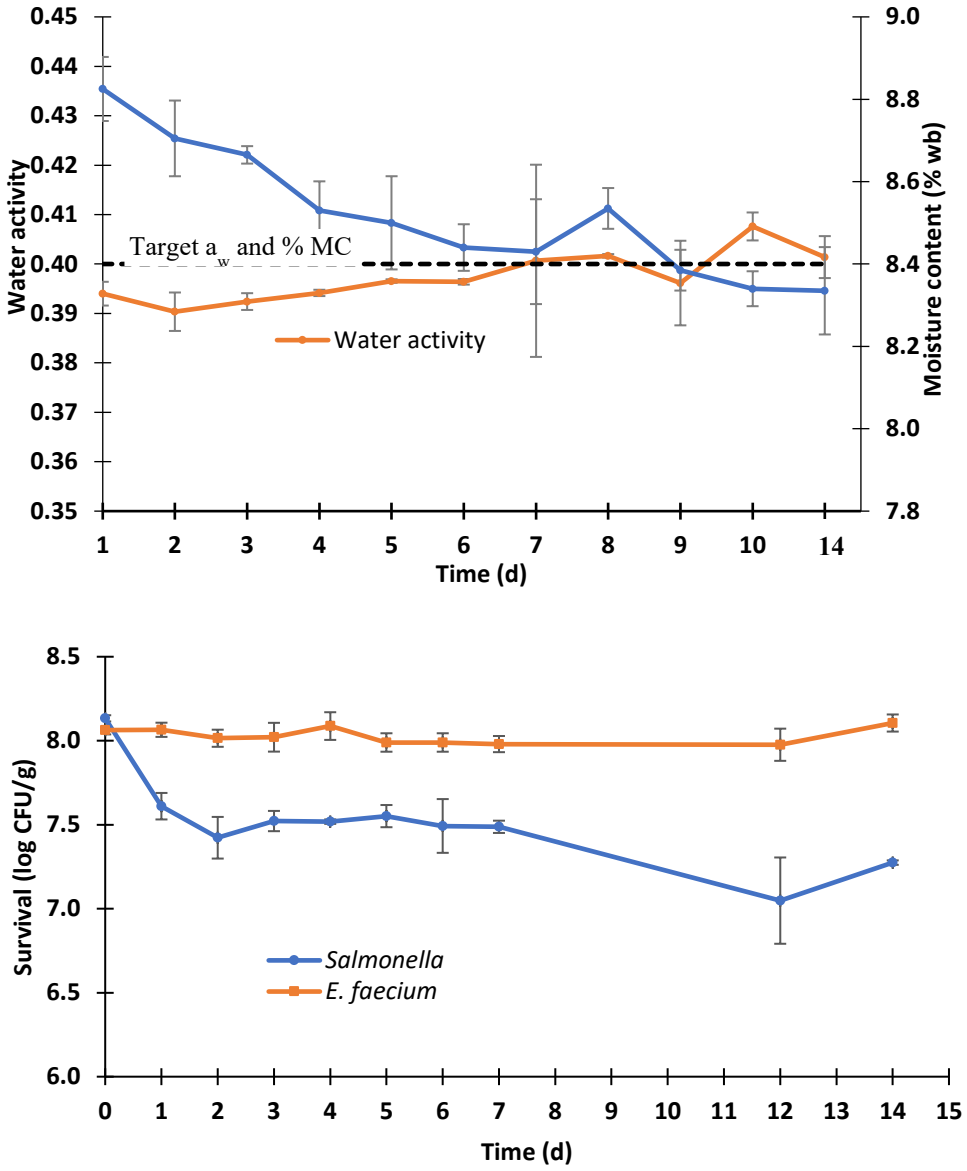

Fig 3.1. (A) Monitoring water activity and moisture content of fine ground black pepper in RH chamber equilibrated to 0.40 a_w. (B) Viability and homogeneity (±one standard deviation as error bars) test of *Salmonella* and *E. faecium* NRRL B-2354 in fine ground black pepper for 14 days at a_w = 0.40.

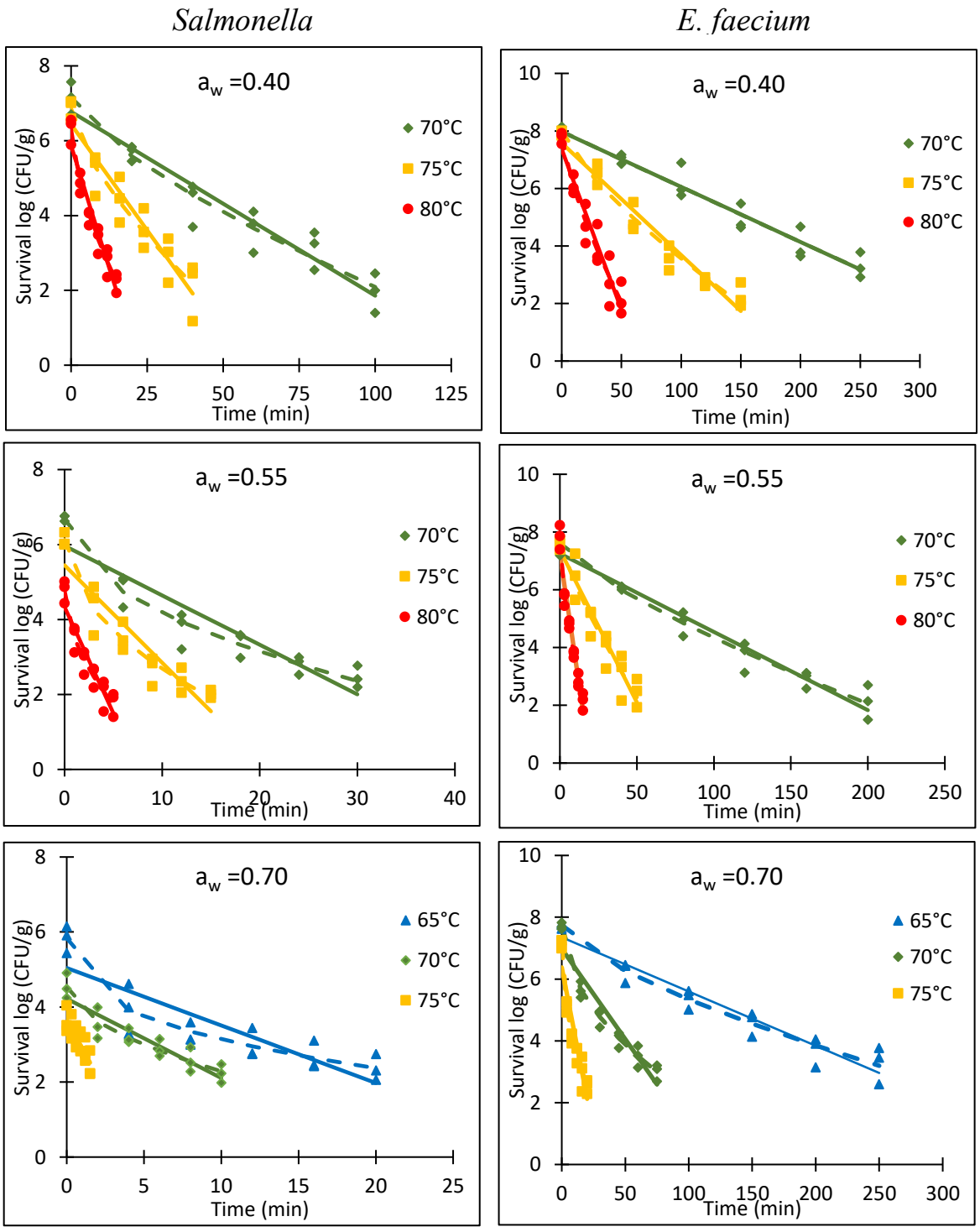

Fig 3.2. Survival curves for *Salmonella* and *E. faecium* NRRL B- 2354 using log-linear (solid line) and Weibull model (broken line) at different initial water activities and treatment temperatures. Each isothermal treatment was conducted in three replications.

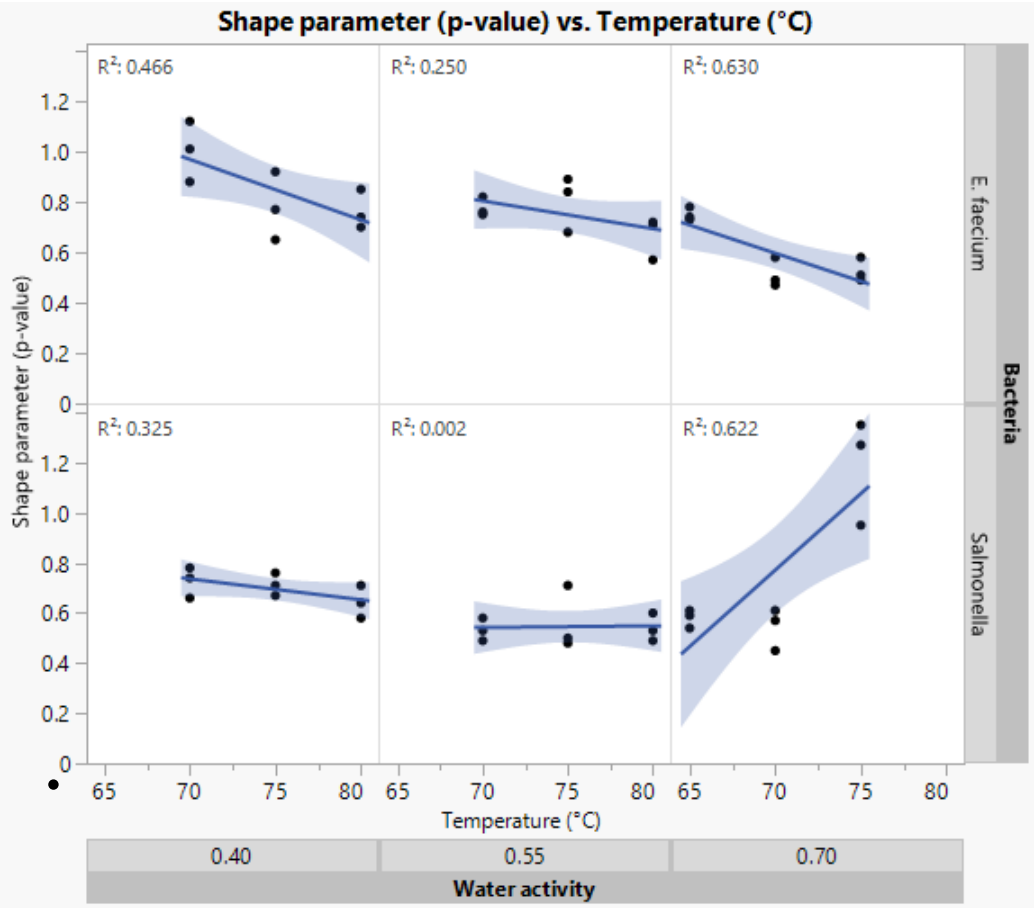

Fig 3.3. The p parameters and their 95% confidence interval obtained for the various combinations of temperature-a_w tested by fitting the Weibull model to experimental data. The shaded area represents the 95% confidence interval.

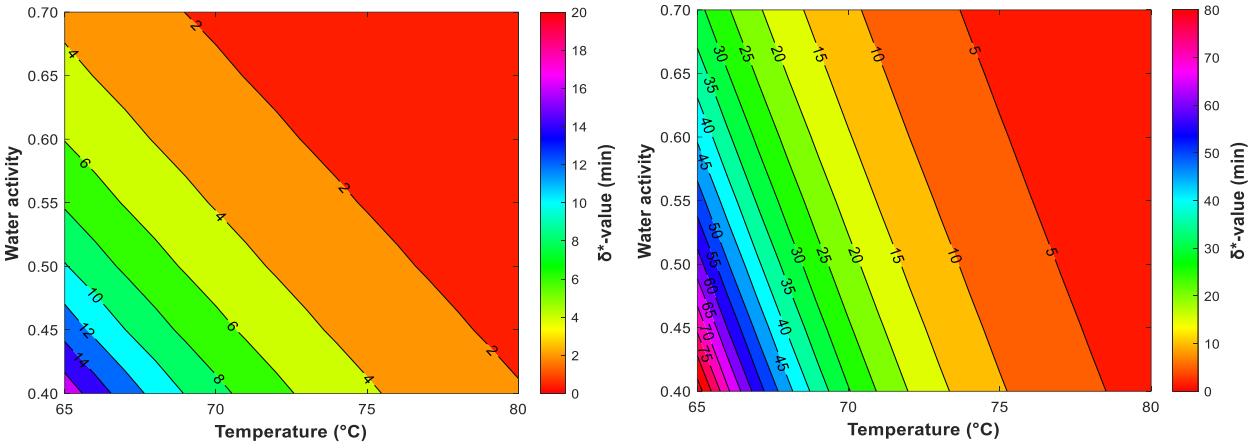

Fig 3.4. Contour plots showing the δ* for A. Salmonella (fixed shape parameter= 0.67) and B. E. faecium NRRL B- 2354 (fixed shape parameter = 0.73) using the modified Bigelow model.

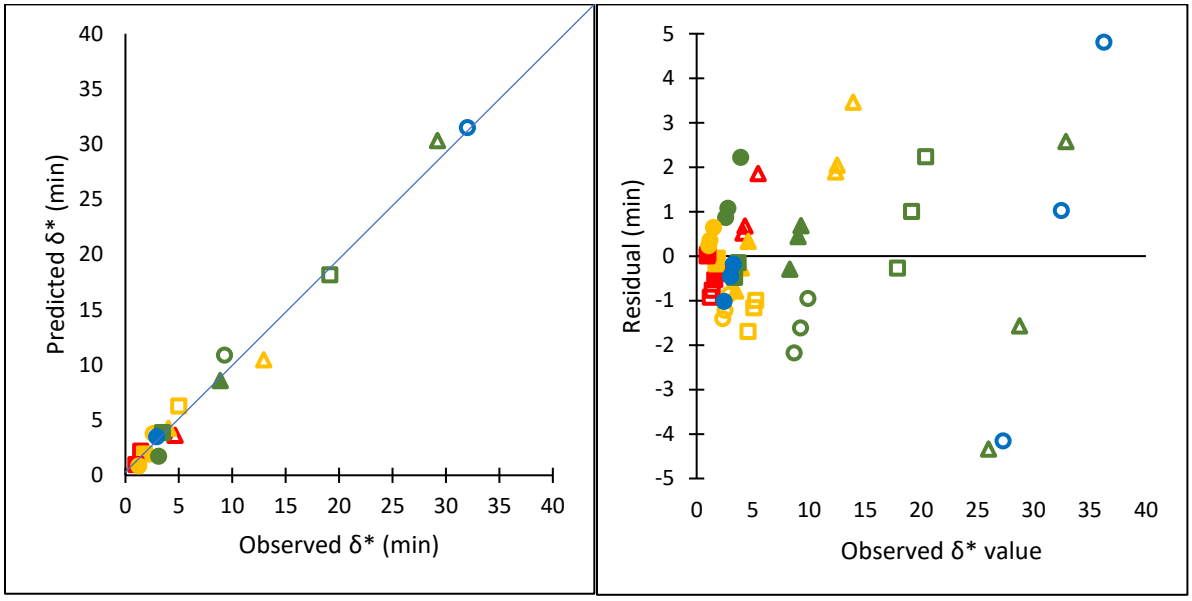

Fig 3.5. Comparison of predicted and experimental delta values of *Salmonella* (fill; at fixed shape parameter of 0.67) and *E. faecium* NRRL B- 2354 (open; at fixed shape parameter of 0.73) in fine ground black pepper with 0.40 a_w (triangle), 0.55 a_w (square) and 0.70 a_w (circle) at 65 °C (Blue), 70 °C (Green), 75 °C (Orange) and 80 °C (Red) using modified Bigelow model.

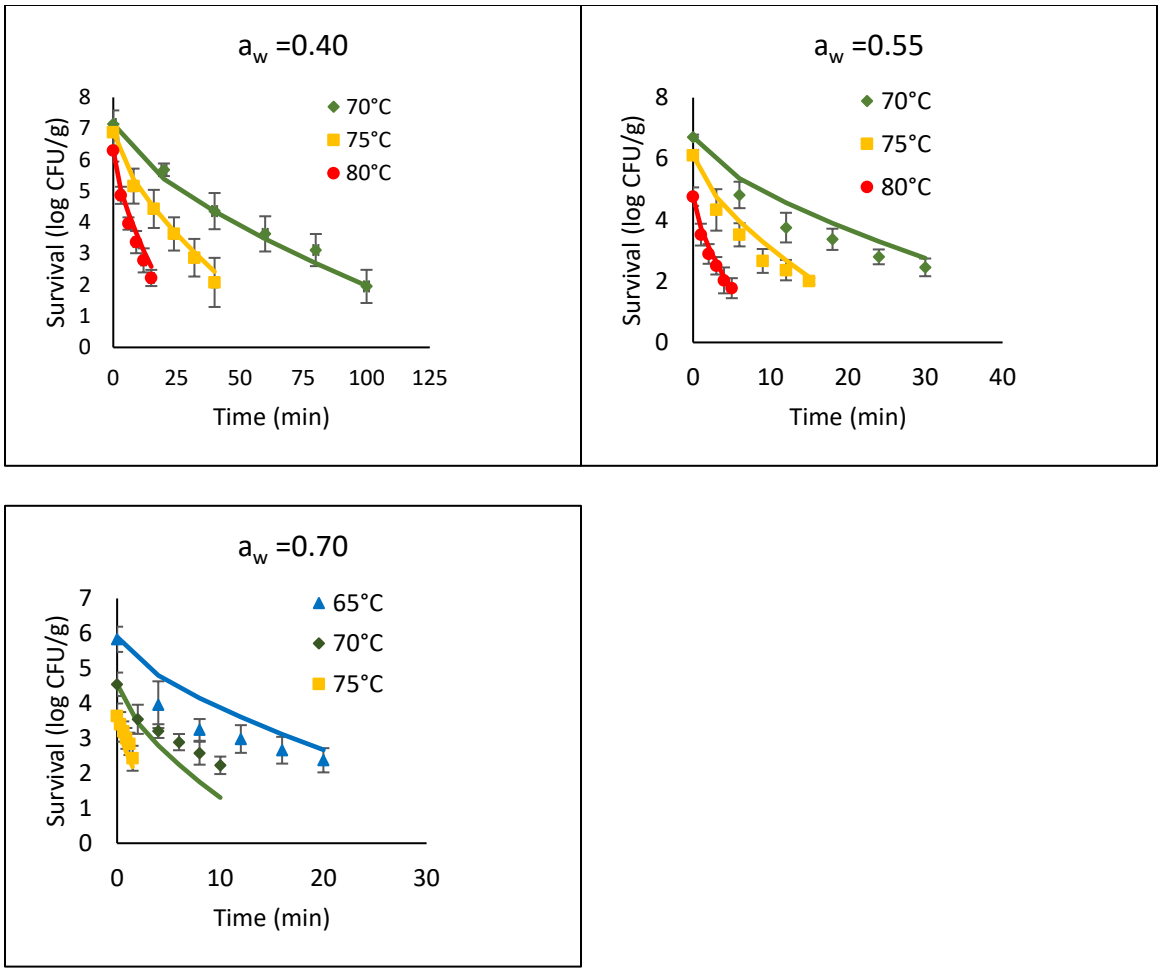

Fig 3.6. Predictions of the thermal death of *Salmonella enterica* inoculated in black pepper powder at different initial water activities and treatment temperatures using parameters from the Weibull-modified Bigelow model.

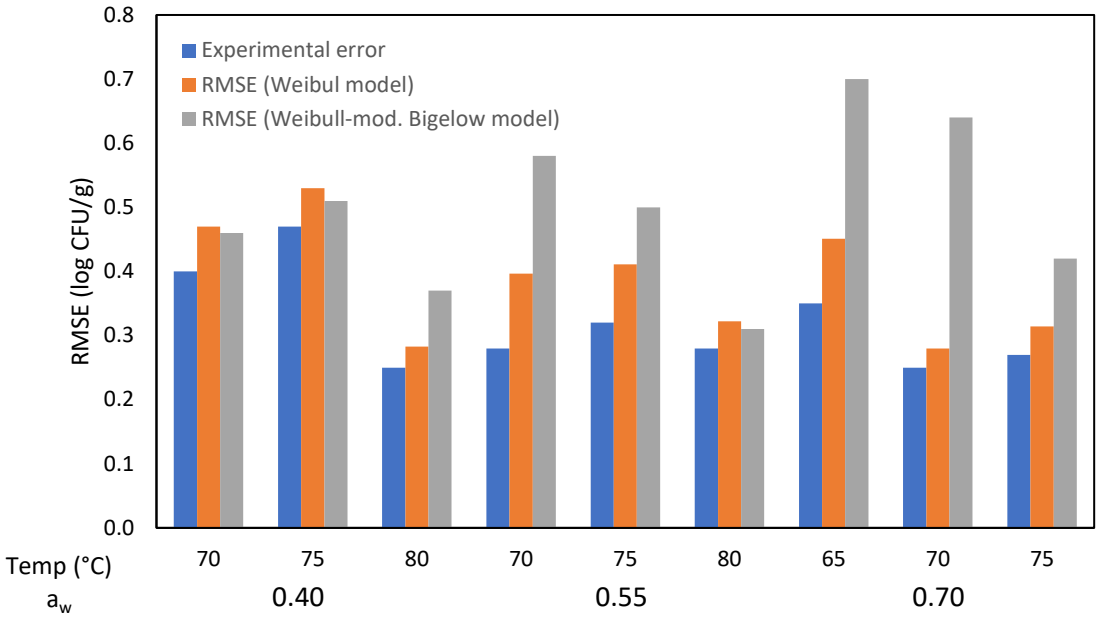

Fig 3.7. Comparison of RMSE of log survival calculated from Weibull model, experimental errors and the Weibull-modified Bigelow model arising from lot variation and enumeration process of *Salmonella* in fine ground black pepper.

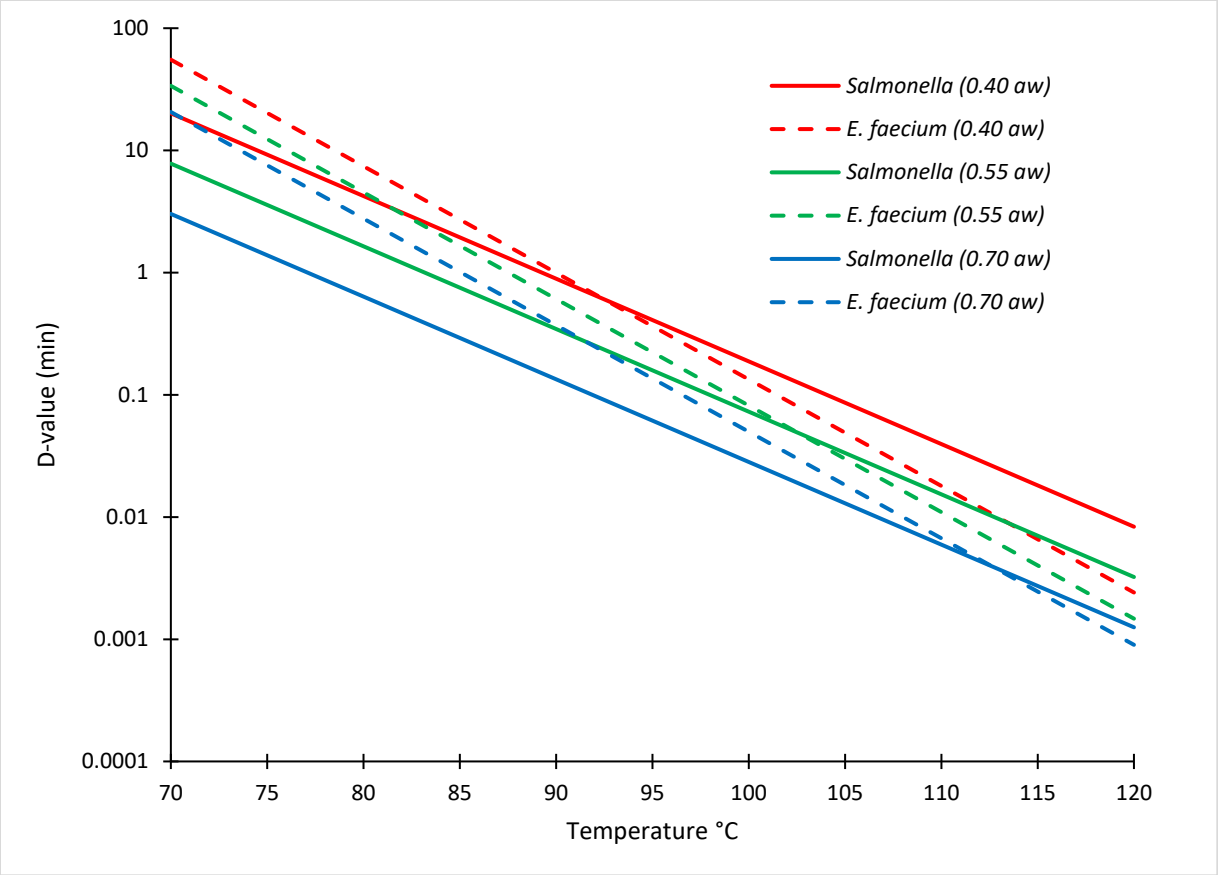

Fig 3.8. Comparison of predicted D-values for *Salmonella* (solid line) and *E. faecium* NRRL B-2354 (dash line) at different initial water activities and treatment temperatures using modified Bigelow model.

Chapter 4. Effect of Inoculation (pre- versus post-grinding) of black pepper on decimal reduction time of *Salmonella* spp. and *Enterococcus faecium* NRRL B-2354

4.1 Abstract

Black peppercorn is the most extensively used spice across the globe and is generally consumed as a seasoning without prior processing. Contamination of the spice likely happens during harvest and post-harvest processing would elevate food safety risks. The laboratory inoculation techniques should ideally mimic the real-life environment to reliably estimate the decimal reduction time (D-value) of bacteria for process validation. This study aims at investigating the influence of the inoculation method on the D-value of *Salmonella* in black pepper powder. In addition, *Enterococcus faecium* NRRL B-2354 was tested for its efficiency as a surrogate for *Salmonella*. *Salmonella* cocktail and *E. faecium* were inoculated separately on ground black pepper by either pre-grinding or post-grinding inoculation procedure. In the pre-grinding procedure, whole black peppercorns were inoculated, equilibrated to 0.40 a_w, ground, and re-equilibrated. In the post-grinding procedure, ground black pepper was inoculated and stabilized at a water activity of 0.40 before thermal treatment. The thermal treatment was conducted at 80°C for a time ranging from 0-30 min to determine the D-value of *Salmonella* and *E. faecium*. In general, the *Salmonella* and *E. faecium* populations were high and stable in ground black pepper. Based on lower Akaike Information Criteria (AICc) values, log-linear model was selected over the Weibull model to determine the inactivation kinetics. The $D_{80°C}$ values of *Salmonella* inoculated by pre-grinding and post-grinding procedures were 5.5 ± 0.8 and 3.9± 0.3, respectively. *Salmonella* and *E. faecium* were significantly ($p<0.05$) more thermally resistant in ground black pepper when inoculated pre- rather than post-grinding. Therefore, inoculation protocol must be considered by spice industries while validating the pasteurization

process. *E. faecium* is a suitable surrogate for *Salmonella* because of its higher decimal reduction time for both inoculation methods.

Keywords: Inoculation procedure, *Salmonella*, Black pepper, Thermal inactivation

4.2 Introduction

Low-moisture foods (LMFs) are those that either naturally contain water activity (a_w) below 0.85 or high-moisture foods that have been dehydrated (Batz et al., 2014). LMFs pose food safety risks as evidenced by recent foodborne outbreaks and recalls in the US (Wason et al., 2023, 2021; Xie et al., 2021; Bowman et al., 2015; Bowman et al., 2015; Bianchini et al., 2014). According to a consumer survey report collected from 1973 to 2010, 86% of American households used herbs and spices and 12% of the imported spices were adulterated with filth. Fourteen outbreaks were reported during this period in several countries including Canada, Denmark, Germany, France, Norway, New Zealand, Serbia, and the USA because of *Salmonella* contamination in spices leading to 1946 infections, 128 hospitalizations, and two deaths (FDA, 2017). Moreover, *Salmonella* contaminating spice was identified as the cause of 95% of food recalls linked with spices in the U.S.A. between 1980-2000 (Vij et al., 2006). Acuff et al. (2023) reported that contaminated spices were associated with one outbreak but led to a much higher number (82) of recalls between 2012-2020 in the USA, Canada, Europe, New Zealand, and Australia. Therefore, spice decontamination is critical for public health as well as food industries. Black peppercorn (*Piper nigrum* L.) is the most commonly used spice worldwide as a food and pharmaceutical ingredient (Abdulazeez, 2016). It is used as a food taste enhancer or a seasoning (Singletary, 2010) and is generally not cooked before consumption (Santillana Farakos & Frank, 2014; Waje et al., 2008). The persistence of *Salmonella* on black peppercorns could be over a year under unfavorable conditions such as low ambient storage RH (<40%) at 25 °C even

without any supplementary nutrients (Keller et al., 2013). Therefore, contaminated black peppercorns pose a huge public health concern.

Black pepper may get contaminated in the farms during production. When black peppercorns are ground to powder, any *Salmonella* contamination could transfer to the ground black pepper. Ground black pepper can also get cross-contaminated post processing if proper sanitary conditions are not maintained in the manufacturing plant. In the first scenario, the grinding process may affect the thermal sensitivity of *Salmonella* necessitating to identify appropriate thermal treatment to ensure the safety of spices. Spice industries must consider the impact of the inoculation method (pre-grinding and post grinding) on the D-value of *Salmonella* for the thermal processing of black pepper since it is commonly used as a seasoning without cooking.

Thermal pasteurization is commonly used to decontaminate LMFs (Wason et al., 2022a). However, there is limited information on the impact of inoculum preparation(Hildebrandt et al., 2016; Keller et al., 2013; Komitopoulou & Peñaloza, 2009; Uesugi et al., 2006), inoculation protocols (Limcharoenchat et al., 2018) and particle size (Zhang et al., 2020) on the thermal resistance of *Salmonella* in LMFs at a laboratory scale. Zhang et al. (2020) noted an increase in particle size of red pepper enhanced the D-value of *Escherichia coli* ATCC 25922. Hildebrandt et al. (2016) reported inoculation with bacterial lawns of the *Salmonella* population in wheat flour sample to be stable with consistent D-values. On the other hand, broth-based inoculum achieved a 4.4 log reduction of *Salmonella* population during equilibration and gave higher D-values. Similarly, Uesugi et al. (2006) and Komitopoulou & Peñaloza (2009) reported that broth-based inoculum in dry confectionary material was more resistant to drying as compared to lawn-based inoculum. The inoculation techniques should be selected to achieve bacterial responses

that correspond to real contamination and processing circumstances. Previous studies have determined the thermal inactivation kinetics of *Salmonella enterica* in ground black powder and obtained different D-values. Different inoculation procedures were used; Wason et al. (2022a) inoculated ground black pepper directly while Wei et al. (2018, 2021a) and Gautam et al. (2020) inoculated black peppercorns. Further, Wason et al. (2022a) studied fine ground black pepper and reported its particle size as 0.24 ±0.05 mm while Wei et al. (2021a) used ground black peppercorn that passed through a US 20 Sieve (0.85 mm) The difference in particle size could impact the D-value and therefore, the effect of inoculation procedure of ground black pepper cannot be clearly determined. Moreover, Limcharoenchat et al. (2018) investigated the influence of inoculation procedures on the thermal inactivation of *Salmonella* in three different LMFs and found varying results. The inoculation of almonds and dates before fabrication into almond meal and date paste increased the D-value of *Salmonella* as compared to inoculation after fabrication. In contrast, increased heat susceptibility of *Salmonella* was observed when wheat kernels were inoculated and ground than inoculating wheat flour directly (Limcharoenchat et al., 2018). This suggests that the impact of inoculation protocol on the D-value of *Salmonella* is product specific and therefore must be determined for a particular food product to get reliable data beneficial for the industry. Ground black pepper lacks a conservative estimation of the D-value of *Salmonella*.

According to the Food Safety Modernization Act (FSMA), food manufacturers must verify the inactivation procedure to make sure the products are safe for consumption (CDC, 2015; Hu & Gurtler, 2017; FDA, 2001). *Enterococcus faecium* NRRL B-2354 has been demonstrated as a promising surrogate for *Salmonella* in several different food matrices including wheat flour in the heat process (Liu et al., 2018), peanuts in dry air heating (Brar & Danyluk, 2019), black peppercorns in vacuum steam pasteurization (Shah et al., 2017),

radiofrequency heating (Wei et al., 2018), and steam treatment (Zhou et al., 2019). It is imperative to assess the impact of the inoculation procedure on the surrogate of *Salmonella* i.e. *E. faecium*. Therefore, the objectives of the present study were to i) investigate the influence of pre and post-grinding inoculation methods on the of *Salmonella*'s D-value in black pepper and ii) assess the surrogate efficacy of *E. faecium Salmonella* in thermal processing.

4.3 Materials and methods

4.3.1 Black pepper samples and Bacterial Strains

Three different batches of black peppercorn samples were procured from McCormick & Company, Inc (Sparks, MD, USA) and kept in a freezer at -12 °C until use.

4.3.2 Bacterial strains:

Five serotypes of *Salmonella enterica* were used to prepare a *Salmonella* cocktail. For this study, serovars, *S.* Montevideo 488275, *S.* Agona 447967, and *S.* Mbandaka 698538 and were acquired from the Food and Drug Administration, Office of Regulatory Affairs, Regional Laboratory in Jefferson, AR; *Salmonella* Reading (Moff 180418) from FDA culture collection in Bedford Park, IL. The University of Georgia in Griffin, GA, provided *Salmonella* Tennessee (K4643) while the United States Department of Agriculture, Agriculture Research Services (USDA, ARS) in Peoria, IL provided *Enterococcus faecium* NRRL B-2354 strain Glycerol (40% (v/v)) was used to supplement the obtained cultures and were stored at -80°C in an ultra-freezer.

4.3.3 Inoculum preparation

The inoculum was prepared by the lawn method as per the method described in Wason et al. (2022a).

4.3.4 Sample inoculation

Fig 1. provides a schematic representation of the two different types of inoculation procedures followed for the inoculation of black pepper. Pre-grinding procedure denotes inoculation of whole black peppercorns which are then ground to powder form, while post-grinding is described as the inoculation of ground black pepper powder. The whole black peppercorns and ground black pepper powder were inoculated by following the same procedure as described below. Prior to inoculation, black peppercorns were taken out of the refrigerator and kept at ambient temperature for at least 24 h. Ground black pepper powder was produced as described in section 2.5.

About 300 g of black peppercorns was weighed in a Ziplock bag and inoculated with either 6 mL of bacterial culture (*E. faecium* NRRL B- 2354 or *Salmonella* cocktail). The inoculum was sprayed on a layer of sample using a centrifuge tube (339650, Thermo Fisher Scientific, Waltham, MA) with a manual spray head connection (ps20-410-natural, Midwest Bottle, Garrison, KY). To ensure an equal dispersion of inoculum throughout the sample, the sample bag was manually shaken for an additional ten minutes. The same procedure was followed to inoculate ground black pepper. The inoculated black peppercorns and ground black pepper were put on sanitized stainless-steel trays and adjusted to a water activity of 0.40 inside a relative humidity (RH) chamber (Lau & Subbiah, 2020).

After equilibration and providing enough time for the bacterial population to stabilize, black peppercorns were ground in a biosafety cabinet as described in section 2.5 below. The ground samples were placed inside the RH chamber to re-equilibrate to target a_w.

4.3.5 Grinding of black peppercorns.

Grinding of black peppercorns was done using a food grinder (3-cup Power Grinder, 120 V, 750 W, Waring Commercial, Torrington, CT, U.S.A) by running the samples twice for 30 s with 10-seconds break. The sample was sieved through a US No. 20 mesh sieve manually by shaking it for at least 3 minutes. A U.S. 20-mesh sieve was used to filter out fine particles (P1, in grams) from the grinding process, while coarse particles (R1, in grams) were held on the sieve. The coarse particles (R1) were crushed and sieved using a 20-mesh sieve to create a second pass-through fine ground black pepper sample (P2) in order to make sure that crucial components were not lost in the final sample (G2). The P2 was then mixed with P1 in a specific proportion, using the eqn 1 (Vasquez, 2018) to ensure the preservation of the inoculum and antimicrobial components in the black pepper powder. The finely ground black pepper (G2) was placed on the sanitized stainless-steel tray inside the RH chamber.

$$G2 = P2 * (1 + \frac{P1}{R1}) \quad (1)$$

ASABE standard (ASABE, 2017) was followed to determine the particle size of ground black pepper (G2) using RO-TAP sieve shaker (Rx-29, W.S. Tyler, OH, US) as explained in Wason et al (2022a). The stack of sieves ranged from 0.075 mm to 0.6 mm.

4.3.6 Stability and Homogeneity test

The homogeneity and stability of whole black peppercorns (and later ground) and ground black pepper powder were evaluated for two weeks post-inoculation to evaluate the uniformity in the distribution of inoculum and stability of the bacteria. Two grams of sample from three different locations on the sample tray was serially diluted in 1:10 ratio using 0.1% buffered peptone water. The dilution was spread plated on m-TSAYE for *Salmonella* and e-TSAYE for *E.*

faecium enumeration and incubated at 37°C for 24 h (Wason et al., 2022a; Garcia et al., 2022). Water activity and moisture content were monitored throughout the study using a dew point water activity meter (Model: 4 TE, Meter Group, Pullman, WA), and a halogen moisture analyzer (Model: HR73, Mettler Toledo, Greifensee, Switzerland) respectively.

4.3.7 Isothermal treatment:

The material was subjected to isothermal treatments in a thermal death time sandwich (Lau and Subbiah, 2020) after the bacterial population reached stability. The sample's come-up time required to achieve the desired temperature (80°C) was determined by the following procedures described by Wason et al. (2022a). The pre- and post-ground samples (2 g) were weighed in heat-sealable pouches of dimensions 7.62 x 7.62 cm (IMPAK Company, Los Angeles, CA) for thermal treatment. Six different exposure times of 80°C were used to treat the samples. Black pepper powder inoculated with *Salmonella* were heat treated for 0 to 20 minutes with a four-minute interval, whereas *E. faecium* inoculated samples were heat treated for 0 to 35 minutes with seven-minute time intervals. The sample was immediately put into an ice-cold water bath following heat treatment for at least a minute to stop the thermal treatment. The treated samples were diluted in 0.1% BPW for bacterial enumeration as explained in section 2.6.

4.3.8 Microbial modeling

Log-linear (Eqn. 2) and Weibull model (Eqn. 3) were used to fit the survival data of *Salmonell*a or *E. faecium* NRRL B-2354 in fine ground black pepper.

Log-linear model: $$\log_{10}\left(\frac{N}{N_0}\right) = -\frac{t}{D} \quad (2)$$

Weibull model: $$\log_{10}\left(\frac{N}{N_0}\right) = -\left(\frac{t}{\delta}\right)^{p} \quad (3)$$

Where N and N_0 (CFU/g) are the microbial populations at time t (isothermal treatment time, min) and after the come-up time, respectively; *D* (min) is the time needed for 90% inactivation of the microbial population at a certain temperature (°C); δ is the scale parameter representing the time required for first log reduction and p is the shape parameter which represents the concavity (n < 1) or convexity (n > 1) of the inactivation curve with respect to time. Estimation of parameters was performed using the GInafit - Version 1.6 add-in freeware (Geeraerd et al., 2005).

The corrected Akaike information criterion (AIC_c), adjusted R^2, and RMSE (root mean square error, log CFU/g) were used to measure the models' goodness-of-fit (Wei et al., 2021b). Lower AIC_c and RMSE value indicates that the model prediction is better (Dolan et al., 2013; Smith et al., 2016). The following ratio was used to calculate each model's likelihood of being the right model (Motulsky and Christopoulos, 2004).

$$\text{Relative likelihood of log-linear over Weibull model} = \frac{e^{\frac{AIC_{c,log-linear\ model} - AIC_{c,Weibull\ model}}{2}}}{1 + e^{\frac{AIC_{c,log-linear\ model} - AIC_{c,Weibull\ model}}{2}}} \quad (4)$$

4.4 Results & Discussion

4.4.1 Stability and homogeneity of *Salmonella* and *E. faecium* in black pepper

The mean population of *Salmonella* and *E. faecium* in black peppercorns immediately after inoculation were 7.8 ± 0.1 and 8.1 ± 0.2 log CFU/g respectively (Fig 2a). Throughout the storage period, the population of both bacteria on the three samples drawn from different locations in the RH chamber was below 0.3 log CFU/g standard deviations. This suggests a homogenous distribution of bacterial population in the sample. As shown in Fig 3., the samples in the humidity chamber were equilibrated to the target water activity within four days after inoculation. Thus, the inoculated black peppercorns were ground on the fourth day. The *E.*

faecium population reduced slightly from 8.1 to 7.8 log CFU/g while *Salmonella* population reduced from 7.8 to 7.0 log CFU/g in four days. A small decrease in *Salmonella* (0.3 log CFU/g) and *E. faecium* (0.6 log CFU/g) populations was observed after grinding. After five days, both *Salmonella* and *E. faecium* showed a drop in population (log CFU/g) of less than 0.2 indicating that the bacterial population was well stabilized. Similarly for the post-grinding procedure (Fig 2b.), *E. faecium* population remained stable at 7.9 log CFU/g and did not significantly change after day five. In the case of *Salmonella*, the population remained stable after day six. Studies by Wei et al. (2021a), Verma et al. (2021), and Chen et al. (2019) observed a similar finding with respect to the stability of *Salmonella* in black pepper, dried basil leaves, and cumin seeds, respectively. In these studies, samples were stored after inoculation for at least five days before thermal treatment.

Previous researchers have shown that inoculation methods significantly affect the stability and D-value of bacteria (Hildebrandt et al., 2016; Keller et al. (2013); Uesugi et al., 2006; Komitopoulou & Peñaloza, 2009). Hildebrandt et al. (2016) evaluated five different *Salmonella* inoculation procedures on wheat and observed D-values of *Salmonella* to be significantly different at similar a_w and treatment temperatures. The authors further reported that the population of lawn-based inoculum in wheat flour remained stable and provided repeatable D-values while broth-based inoculum resulted in a 4.4 log reduction of *Salmonella* during equilibration and significantly high D-values. A similar lawn-based method was used in our study for inoculum preparation to ensure the stability of the bacterial population. Moreover, comparing the heat inactivation of *Salmonella* between the two separate studies (Jeong and Kang, 2014; Wei et al., 2019), it can be concluded that *Salmonella* was less resistant to thermal treatment immediately after inoculation than after sufficient days of stabilization. Therefore,

thermal treatment was carried out only after the bacteria has stabilized following inoculation to attain a conservative estimation of the D-value.

The population of *Salmonella* and *E. faecium* in ground samples inoculated pre-grinding and post-grinding were similar. When comparing the *Salmonella* population in ground black pepper produced from grinding inoculated black peppercorns, the population did not show a significant difference from ground black pepper powder which was directly inoculated. On the other hand, *the E. faecium* population was consistently higher in post-ground black pepper powder. The difference in *Salmonella* and *E. faecium* population was more prominent on day four of storage. Grinding could disrupt the bacterial cells leading to a slight reduction in *Salmonella* and *E. faecium* population as previously reported by Wei et al. (2021a). However, previous research has shown that initial inoculation level does not affect the *Salmonella* Enteritidis PT 30's D-value in low-moisture products; thus, these variations in the original population should not influence comparisons between pre- and post-ground samples. (Hildebrandt et al., 2016). The moisture content and water activity of pre-ground and post-ground black pepper powder were similar throughout 14 days of storage.

4.4.2 Model Comparison

Table 1. lists the model parameters for the two main models that were used to fit the survival data: log-linear and Weibull model. With adjusted R^2 ranging between 0.89 and 0.95 for the log-linear model and 0.92-0.96 for the Weibull model, the data was well fit by both models (Vashishth et al., 2021). Villa-Rojas et al. (2013) found the Weibull model had higher R^2 than log-linear model for predicting *Salmonella* inactivation in almond meal. Other studies have used RMSE values to explain the efficacy of the prediction model in LMFs (Farakos et al., 2013; Liu et al., 2018; Ozturk et al., 2020). Dolan et al. (2013) and Smith et al. (2016) recommended the

use of AICc values to determine model fit. We used the AICc values to determine if a decline in the residual sum of squares may more effectively explain the inclusion of parameters to the model. Lower AICc values suggest a better model fit for the kinetics of heat inactivation. Based on the AICc value, log-linear model has a 100% chance of being reliable for data on *Salmonella* before grinding, 99% for *Salmonella* after grinding, 100% for *E. faecium* before grinding, and 79% for *E. faecium* after grinding (Table 1.). The inactivation curves (Fig 4.) depicted a linear trend and the log linear model most likely performed more accurate data prediction than Weibull model. Previous researchers have also found log-linear model accurately predicts the microbial inactivation in LMFs (Verma et al., 2020; Smith et al., 2016). Therefore, D-values were utilized to compare the influence of pre-grinding and post-grinding processes on the heat inactivation of both bacteria in black pepper powder.

4.4.3 Effect of inoculation protocol

The D-values for both *Salmonella* and *E. faecium* inoculated in black pepper powder pre-grinding were significantly ($p<0.05$) higher than the post-grinding procedure (Table 1). The D-value of *Salmonella* in ground black pepper when inoculated pre-grinding was 5.5 ± 0.8 min and it significantly ($p<0.05$) reduced to 3.9 ± 0.3 min for post-grinding procedure. The mean ± standard deviation of particle size of ground black pepper was calculated as 0.32 ± 0.004 mm. The D-value (3.9 ± 0.3 min) of *Salmonella* in ground black pepper post grinding was similar to the D-value (3.9 ± 0.2 min) reported by Wason et al. (2022a) in ground black pepper with a particle size of 0.24 ± 0.005 mm for the same treatment conditions. The inoculation procedure followed in both studies was similar and eventually provided the same results. It was expected that the particle size of black pepper powder would influence the inactivation rate (Zhang et al., 2020), however, the small difference in particle size did not affect the D-value. Zhang et al.,

(2020) investigated the impact of three particle size (0.45–1.00 mm, 0.20–0.45 mm, and < 0.20 mm) on the thermal inactivation of *E. coli* in red pepper powder. The authors reported an increase in heat resistance of bacteria with an increase in the particle size of red pepper. In Wason et al. (2022a) study with ground black pepper inoculated post-grinding, a $D_{65°C}$-value of 5.8 min was reported for *Salmonella* at 0.70 a_w as compared to 13.3 min reported by Wei et al. (2021a) at 0.65 a_w who used pre-grinding procedure of inoculation. Even though lower water activity could enhance the heat resistance of bacteria, the increase in D-value by more than two times provides some evidence that inoculating black peppercorns first, followed by grinding increases the heat resistance of *Salmonella* in black pepper powder.

The trends in these studies show that *Salmonella* was more resistant to heat treatment when inoculated on black peppercorns before grinding as compared to direct inoculation in black pepper powder. In the pre-grinding procedure, after inoculation of black peppercorns, the coarse particles (outer layer) and seed (inner layer) portions were ground and mixed. The coarse particles contain most of the bacterial inoculum while the seed constitutes a majority of antimicrobial components. During grinding, the seed portion may get cross-contaminated with the bacteria. However, in post-grinding procedure, all black pepper particles would have an equal chance of contamination and bacterial attachment. The introduction of live cells into a low moisture environment with antimicrobial components readily available in ground black pepper could contribute to the sensitivity of *Salmonella* to heat (Tang et al., 2017). During the grinding process, the physiological state of *Salmonella* can be affected and therefore a change in the physicochemical characteristics is expected at the interface between bacteria and the surrounding environment (Young et al., 1991) leading to extra stress. This stress along with the desiccation stress provides *Salmonella* with enhanced heat tolerance. Moreover, *Salmonella* cells in

inoculated black peppercorns may undergo change during the grinding process thereby affecting the microstructure and physical characteristics of black pepper. About 1 log reduction of both bacteria was observed collectively during grinding and stabilizing in ground black pepper inoculated pre-grinding while the bacterial population did not show significant changes in samples inoculated post-grinding. The initial reduction of sensitive bacterial cells could have led to an increase in D-value due to relatively more heat resistant cells remaining. Hildebrandt et al. (2016) reported similar findings where broth-based inoculation resulted in about a 4 log reduction of *Salmonella* in wheat flour during equilibration but yielded high D-values.

The effect of the inoculation procedure on *Salmonella*'s D-value is found to depend on the low moisture food product by Limcharoenchat et al. (2018). They observed that grinding inoculated dates into paste and inoculated almonds into almond meal and almond butter increased the D-value of *Salmonella* compared to post-ground inoculated products. However, the same trend was not observed for wheat grains. It was found that *Salmonella* was less resistant to heat in wheat flour when it was inoculated pre-grinding, than post-grinding. A difference in inactivation resistance dependent on the inoculation procedure has been reported for other technologies. Steinbrunner et al. (2019) assessed the impact of various physical forms of low moisture foods on *Salmonella* resistance to x-ray irradiation. The study showed a high D-value of *Salmonella* in whole dates and wheat kernels compared to date paste and wheat flour respectively. Surprisingly, the D-value of *Salmonella* increased after grinding almond kernels into a meal but decreased when it was further ground to almond butter. However, this study compared the *Salmonella* inactivation in whole vs ground low moisture foods, while the present inactivation study was performed only on ground black pepper. These differences suggest the fact that the inoculation protocol can influence the heat resistance of *Salmonella.*

The inoculation protocol used in this study represents various routes of *Salmonella* contamination. Wei et al. (2021a) reported a higher D-value (7.8 ± 0.8 min) for *Salmonella* in black pepper powder at 75°C at an a_w of 0.45 when inoculation was performed according to the pre-grinding procedure. The difference in D-values (5.5 ± 0.8 min) in our study and Wei et al. (2021a) was due to differences in the a_w of black pepper powder. Newkirk et al. (2018) studied the influence of inoculum preparation on *Salmonella* inactivation. They reported that *Salmonella* grown into biofilm on black peppercorn required a longer treatment time for a 5-log reduction of *Salmonella* compared to inoculum grown in tryptic soy agar and inoculated to the product. Moreover, Wei et al. (2019 & 2020) found that when radiofrequency heating was applied to crushed black pepper, *Salmonella* was inactivated more quickly (130 s, 5.98 log CFU/g reduction) than in whole black peppercorns (150 s, 5.31 log CFU/g reduction). These findings imply that the D-value of *Salmonella* in LMFs can be significantly affected by the inoculation method. These findings will be beneficial to spice processors irrespective of the mechanism responsible for variation in thermal resistance. Designing a suitable thermal inactivation procedure requires a systematic understanding of variations in the D-value of *Salmonella* when inoculated on black pepper in two different physical forms (whole and ground). Because inoculation protocol significantly impacted the D-value of both bacteria, spice industries must consider the impact of inoculation procedures while designing process validation studies.

4.4.4 *E. faecium* as a suitable surrogate

Fig 2. shows that the *E. faecium* population was high and stable in pre-ground and post-ground black pepper powder throughout 14 days of storage. Further, the standard deviation of *E. faecium* population in samples from three different locations in the chamber was < 0.3 log CFU/g indicating that the population was homogenously spread in the sample. The D-values of *E.*

faecium were significantly greater than *Salmonella* for thermal treatment. For the pre-grinding and post-grinding procedures, the D-values of *E. faecium* were 10.3 ± 0.4 min and 7.5 ± 0.2 min while corresponding D-values for *Salmonella* were 5.5 ± 0.8 min and 3.9 ± 0.3 min. Wei et al. (2021) reported a $D_{80\,^{\circ}C}$-value of *E. faecium* inoculated on ground black pepper post-grinding as 5.2 ± 0.3 min at 0.45 a_w which is less than the 7.5 ± 0.2 min which could be due to a higher water activity of sample used by Wei et al. (2021) *E. faecium* NRRL B2354 has been widely studied as a suitable surrogate for *Salmonella* in thermal treatments (Wason et al., 2022b; Verma et al., 2021, 2018; Ozturk et al., 2020). *E. faecium*'s higher D-values compared to *Salmonella* and its stability during storage suggest that it can be used as a conservative surrogate to assess the effect of inoculation protocol on *Salmonella* inactivation by the spice industry.

4.5 Conclusions

Thermal inactivation of ground black pepper when inoculated pre-grinding provides a conservative estimation of D-value. In other words, the ground black pepper obtained from inoculated black peppercorn enhanced the D-value of *Salmonella* when compared to direct inoculation on ground black pepper. In real-life scenarios, contamination occurs most frequently during harvest and drying of the whole black peppercorns. The D-values acquired by post-grinding procedures may be suitable if ground black pepper is cross-contaminated in the industry during grinding or storage and is pasteurized. The conservative approach (pre-grinding) is the safest one because no one could predict when the contamination happened, and it is more probable that the spices get contaminated in the field than in the industry. As most of the heat inactivation studies by researchers used post-grinding procedures, those findings do not adequately reflect the actual circumstances in which a food product gets contaminated on the farm and subsequently manufactured into a final product. As black pepper is frequently taken as

a condiment without being cooked, it can contaminate the product when used as an ingredient in other processed foods. To ensure the safety of black pepper, the spice industry must consider the effect of inoculation protocol/source of contamination while identifying the processing parameters for effective decontamination of ground black pepper. The mechanism by which *Salmonella* exhibits high or low thermal resistance needs further exploration.

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Table 4.1. Comparison of log-linear and Weibull model for determining the inactivation kinetics of *Salmonella* spp. and *E. faecium* in black pepper.

	Salmonella Pre-grinding	*Salmonella* Post-grinding	*E. faecium* Pre-grinding	*E. faecium* Post-grinding
D-value (min)	5.5 ± 0.8	3.9 ± 0.3	10.3 ± 0.4	7.5 ± 0.2
Adj R^2	0.90	0.94	0.89	0.95
RMSE (log CFU/g)	0.43	0.35	0.47	0.41
AICc	-27.79	-34.9	-24.28	-29.19
δ (min)	3.09	2.12	3.47	4.69
p	0.72	0.71	0.58	0.84
Adj R^2	0.92	0.96	0.94	0.96
RMSE (log CFU/g)	0.39	0.28	0.34	0.4
AICc	-32.28	-43.75	-36.92	-31.8
Relative likelihood of log-linear over Weibull model	1.0000	0.9882	0.9982	0.7867

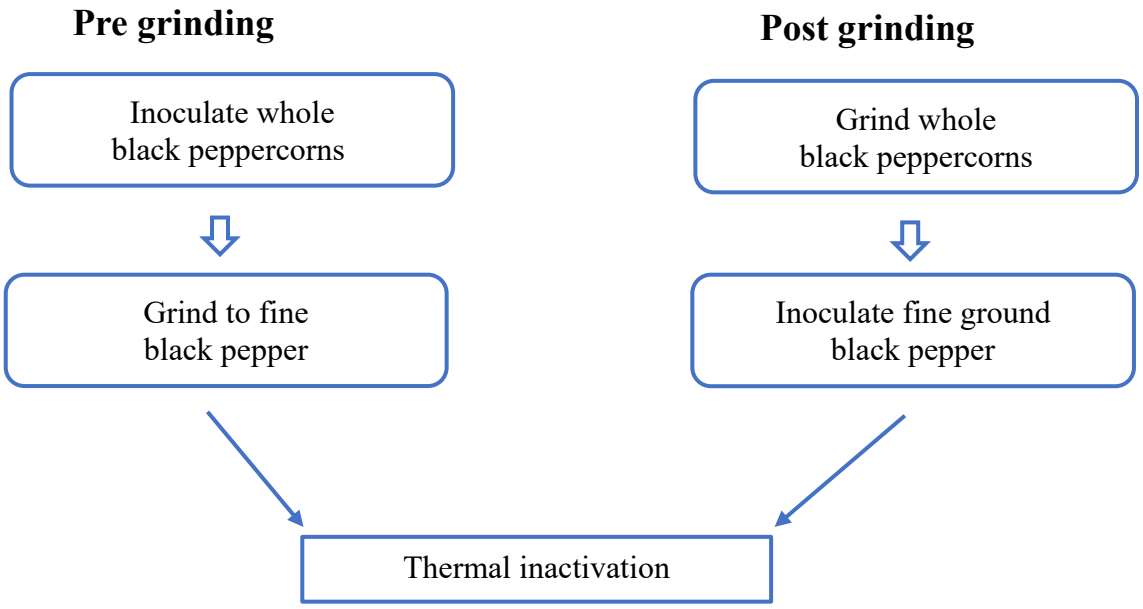

Fig 4.1. Pre-grinding and post-grinding inoculation procedure followed for inoculation of black pepper with *Salmonella* and *E. faecium.*

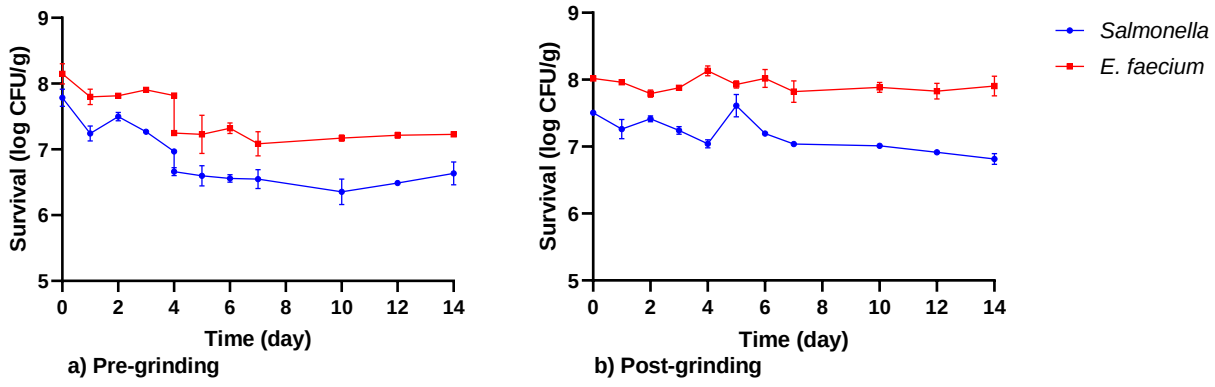

Fig 4.2. Stability & homogeneity (± 1 standard deviation as error bars) of *Salmonella* and *E. faecium* in black pepper during equilibration for 14 days for two methods of inoculation. a) Pre-grinding b) Post-grinding.

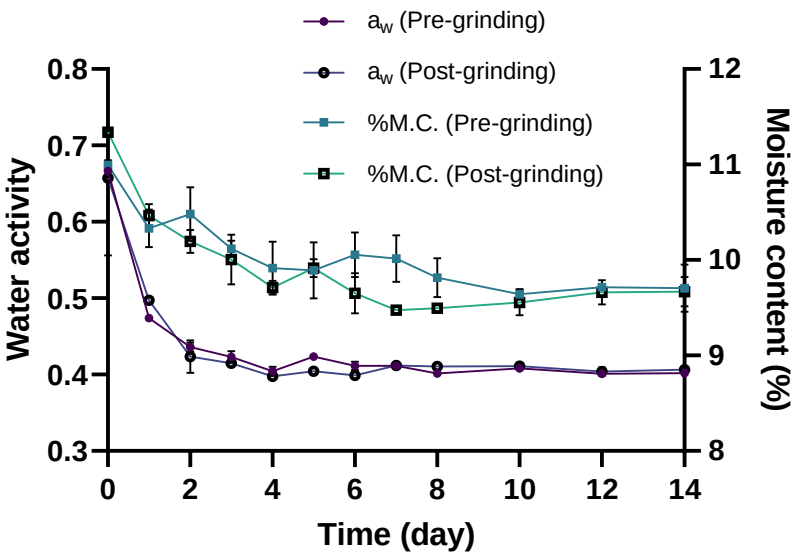

Fig 4.3. Monitoring moisture content (M.C. wb %) and water activity (a_w) of black pepper in RH chamber during equilibration at a_w=0.40.

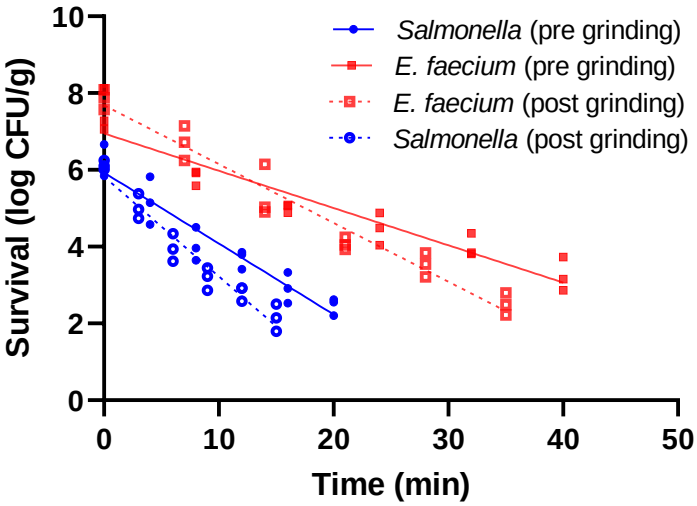

Fig 4.4. Inactivation of *Salmonella* spp. and *E. faecium* during isothermal heat treatment at 80 °C.

Chapter 5. In-package pasteurization of dried basil leaves using radiofrequency heating

5.1 Abstract

Recent foodborne illness outbreaks associated with the consumption of low moisture foods such as spices have emphasized concerns over microbial food safety. In-package RF pasteurization method was evaluated for inactivation of *Salmonella enterica* in dried basil leaves. Samples were packed in polystyrene bottle provided with a steam venting film and was either positioned vertically (electrode gap = 16 cm) or horizontally (electrode gap = 10.5 cm) for RF treatment. The horizontally placed container achieved rapid heating of dried basil leaves as compared to the vertical placement and achieved more than six log reduction of both microorganisms within 35 s of RF treatment. *E. faecium* was more heat resistant than *Salmonella,* indicating its suitability as a surrogate for RF pasteurization of dried basil leaves. Quality analysis revealed no significant ($P>0.05$) change in color, total phenolics, antioxidants, and volatile composition of dried basil leaves after RF treatment. Pasteurization of final packaged food product with steam vent minimizes cross-contamination and improves food safety.

Keywords: Process lethality, *Salmonella*, Radiofrequency processing, Steam-vent nut, in-package steaming

Wason, S., Verma, T., Irmak, S., & Subbiah, J. (2022). In-package pasteurization of dried basil leaves using radiofrequency heating. *Food Control*, *141*, 109179.

5.2 Introduction

Low moisture foods (LMFs) typically have water activity (a_w) below 0.85 and do not promote the growth of many bacterial pathogens (FAO/WHO, 2014). However, bacteria can remain viable for longer periods posing a risk of product contamination (Mermelstein, 2018). This is well evidenced by multi-state outbreaks of salmonellosis reported in LMFs such as spices, nuts, and dried fruits in the past decades (Wason et al., 2021). Spices and herbs are used as seasonings or ingredients in cooked or ready-to-eat foods. If pathogens are present in spices and herbs, they may potentially grow after being added to high-moisture cooked product where the competition from other bacteria is low. Sweet basil is an aromatic herb most popularly used as a food seasoning, in teas, and as a supplement to promote human health. Though they are reported to possess antimicrobial and antifungal activities (Meyers, 2003); two outbreaks reported in 2006 and 2007 were linked to *Salmonella* contamination in fresh basil leaves. In 2007, *Salmonella* Senftenberg outbreak was related to the consumption of contaminated fresh basil leaves. Initially, 51 cases were reported in England, Wales and Scotland, which later spread through Denmark, Netherlands, and the United States (Pezzoli, 2008). Any associated foodborne outbreak would require the recall of all products since the last cleaning of the processing facility leading to a significant economic impact for the food industries. Fresh basil leaves have a shorter shelf life and are gradually dried using a forced air drier at ≤ 50°C to minimize quality deterioration. The slow drying process can assist in maintaining the color, aroma, and oil content of basil leaves (Baczek et al., 2019); however, the lower temperature does not offer complete pasteurization.

Thermal pasteurization has been extensively used for the inactivation of the bacterial population in a variety of LMFs (Anderson, 2019; Verma et al., 2018; Wei et al., 2018). However, the development of enhanced heat resistance among the *Salmonella* strains in desiccated conditions limits the efficacy of the thermal inactivation process (Podolak et al., 2010). Furthermore, high-temperature treatment of spices and herbs could negatively impact the product quality leading to a loss in volatiles. The use of ethylene oxide (EtO), steam, and irradiation have been recognized by the American Spice Trade Organization to enhance the microbial safety of spices and herbs (ASTA, 2017). However, EtO treatment of dried basil leaves is not recommended due to the possible formation of ethylene chlorohydrin residues during the treatment (U.S. Environmental Protection Agency, 2009). On the other hand, steam pasteurization can potentially increase the moisture of low moisture foods such as dried basil leaves and would require an additional drying step to bring down the moisture. Radiofrequency heating is a novel thermal processing method used extensively for the pasteurization of many food products including spices and herbs (Chen et al., 2019; Jeong & Kang, 2014; Kim et al., 2012; Verma et al., 2021a; Wei et al., 2018). It offers rapid heating and deeper penetration unlike the conventional heating methods (Jiao et al., 2014). However, most of the RF treatment studies report pasteurization of bulk products prior to packaging, which does not eliminate the potential cross-contamination issue. The cross-contamination between the product lots, tainted equipment, packing operations, spillage, and the packaging materials have been identified as the sources for contamination of LMFs (Podolak et al., 2010). Hence, treatment of the packaged products can further improve microbial food safety.

Non-uniform heating is one of the limitations concerning the use of RF heating, which can challenge the microbial safety of LMFs due to under heating or over heating at specific

regions of the food product (Jiao et al., 2014; Piyasena et al., 2003; Tiwari et al., 2011). Hot air assisting, intermittent mixing, surrounding with PEI blocks and immersion in soybean oil have been proposed to increase the heating uniformity of RF treatment (Dag et al., 2021; Jiao et al., 2014; Wang et al., 2005; Wang et al., 2007). However, these approaches can complicate the RF heating process and limit its commercial viability. Treatment of dried basil leaves in the packaging container provided with a steam vent is a potential alternative. The movement of steam inside the package would enhance the heat distribution throughout the container leading to a better heating uniformity as compared to an open container. The excess steam will be released when the vent opens at a particular threshold steam pressure. Because RF heating is a complex physical process, the heating rate is affected by the food composition, the size of the material (Orsat & Raghavan, 2014), and the orientation of the sample container inside the RF cavity (Bedane et al., 2021; Romano & Marra, 2008). Thus, due consideration must be given to evaluate the effect of different orientations of the sample container on the RF pasteurization efficacy.

The U.S. Food and Drug Administration (FDA) Food Safety Modernization Act passed in 2011 requires the food industry to validate their process control and document all the data in their validation report. For the industrial validation of the RF heating of dried basil leaves with steam vent packaging, a surrogate i.e., a non-pathogenic bacteria with either similar or higher heat resistance is essential. Various reports have suggested the use of *Enterococcus faecium* NRRL R-2354 as a suitable surrogate for *Salmonella* during validation of thermal pasteurization technique on spices and herbs such as dried basil (Verma et al., 2021b), black peppercorns (Wei et al., 2018), white pepper (Ozturk et al., 2020) and cumin seeds (Chen, et al., 2019). Because the surrogates are specific to the products and processes, the suitability of *E. faecium* as a potential

surrogate to *Salmonella* in dried basil leaves needs to be evaluated for RF treatment. Therefore, the present study was aimed to i) optimize the RF heating method for efficient microbial inactivation in steam vent packaged dried basil leaves, ii) evaluate the suitability of *E. faecium* as a surrogate to *Salmonella* in dried basil leaves specific to RF pasteurization with steam vent package, and iii) evaluate the impact of RF heating on the quality of dried basil leaves.

5.3 Materials & Methods

5.3.1 Bacterial strains and inoculum preparation

Salmonella enterica strains such as Agona 447967, Mbandaka 698538, Montevideo 488275, Reading Moff 180418, and Tennessee K4643 were used to prepare the cocktail. All the supplies required for media preparation were purchased from Becton, Dickinson, and Company (Sparks, MD). The strains were individually grown overnight in trypticase soy broth supplemented with 0.6% (w/w) yeast extract (TSBYE,) and spread plated on tryptic soy agar supplemented with 0.6 % (w/w) yeast extract (TSAYE) followed by incubation at 37°C for 24 h. The bacterial lawns were then harvested with 3 mL of 0.1 % (w/w) buffered peptone water (BPW) and mixed in equal proportions to form a *Salmonella* cocktail. The *E. faecium* inoculum was also prepared in the same way.

5.3.2 Sample inoculation and moisture equilibration

Three batches of dried basil leaves from different production lots were procured from McCormick & Co., Inc. (Hunt Valley, MD) and stored at room temperature. Dried basil leaves (100 g) were aseptically measured in a Ziploc bag to which either 2 mL of *Salmonella* cocktail or *E. faecium* inoculum was sprayed using a hand operated spray head (ps20-410-natural, Midwest Bottle, Garrison, KY) fixed on a sterile 15-mL centrifuge tube (339650, Thermo Fisher

Scientific, Waltham, MA). The sample bag was closed and hand massaged for 10 min to make sure the bacterial population is uniformly distributed in the sample. The inoculation steps were repeated with new frozen stock to inoculate a different batch of the sample. The inoculated sample was placed in a sanitized aluminum tray (23 × 30 × 1.5 cm) and kept in a relative humidity (RH) chamber. Based on the stability and homogeneity tests on *Salmonella* and *E. faecium* inoculated dried basil leaves reported by Verma et al. (2021b), bacterial population dropped on the first day after inoculation and then gradually stabilized within 5 days after which no significant reduction was observed until 14 days. Therefore, the inoculated samples were moisture equilibrated for a minimum of 5 days, and these samples were used in subsequent experiments within 2 weeks. Further, radiofrequency (RF) heating causes evaporation of water from the sample leading to a decrease in water activity. To maintain the natural water activity of the sample after RF processing, the initial water activity was adjusted to a higher level in the RH chamber.

5.3.3 Radiofrequency treatment

The material of the package was chosen based on its dielectric properties and industrial applicability. Polypropylene has a dielectric constant of 2.10 at 1 MHz and a low dielectric loss factor (Mark, 1999). A polypropylene bottle of dimensions diameters - 6 cm Height - 14.2 cm was used for processing dried basil leaves (McMaster-Carr Catalog no. 6702T74. The effect of the two bottle orientations were evaluated for heating efficiency during RF processing. The bottle was either placed in horizontal orientation, when the bottle is laid down parallel to the bottom electrode, or in vertical orientation, when the bottle stands perpendicular to the bottom electrode. The bottle was placed at the center of the bottom electrode between the two electrodes in both cases. The electrode gap is an important factor affecting the heating rate and uniformity

(Orsat & Raghavan, 2014). The gap between the top and bottom electrode which gave the best combination of high heating rate and good heating uniformity was selected based on preliminary trials. The electrode gap was optimized independently for each sample orientation. This procedure would be followed in industrial setting to get high coupling to achieve the highest energy efficiency for each sample orientation. However, it is important to note that the discussion of the effect of sample orientation factor is confounded by the varying electrode gap factor, which is known to impact heating rate and heating uniformity. The electrode gap was set at 10.5 cm for horizontal orientation while the electrode gap was increased to 16 cm for vertical orientation of the bottle. The difference in temperature profiles of wheat kernels was negligible within the round polypropylene container of 6.8 cm diameter during RF heating (Jiao et al., 2015a). Because the diameter of the bottle is very small, the temperature was only measured at the center of the top, middle, and bottom layer as depicted in Fig. 1. The sample was heated until all the points reached the target temperature of 100°C. Three fiber optic temperature probes (Neoptix, Inc., Quebec City, Quebec, Canada) with an accuracy of ±0.6 °C were inserted into the bottle through equidistant pre-drilled holes on one of the bottle sides as shown in Fig. 1. Each fiber optic probe was sealed using duct tape outside on the bottle to avoid heat escape during treatment. A pre-weighed homogenous uninoculated sample of dried basil leaves (60 g) was then placed in the bottle and sealed with the plastic wrap (Press'n Seal, The Glad Products Co.). A steam venting nut was provided in the middle of the film which enabled the escape of excess steam produced during in-package RF pasteurization. The time-temperature profile during RF heating was recorded every second using the probe. The hot and cold spots were estimated as the points reaching the target temperature of 100°C at the fastest and slowest rate respectively, based on the time-temperature profile of dried basil leaves.

The heating uniformity of the sample placed in two orientations during RF processing was determined as non-uniformity index (λ) by the following equation (Wang et al., 2005)

$$\lambda = \frac{\Delta\sigma}{\Delta\mu}$$

where $\Delta\sigma = \sqrt{\sigma^2 - \sigma_0^2}$ and $\Delta\mu = \mu - \mu_0$ are the rise in standard deviation and average temperature, respectively of different locations in the sample during RF treatment. The smaller λ values indicate better heating uniformity in the sample.

The coefficient of variation (CV) was calculated to evaluate the variation in temperature distribution within the bottle in relation to the mean temperature according to the following formula.

$$CV = \frac{\sigma}{\mu}$$

The inoculated pack method used by Liu et al. (2018) and Wei et al. (2018) was also followed in this study. For the microbial challenge study, the samples inoculated with *Salmonella* were packed in paper bags and placed at the identified cold spot as shown in Fig 2. Further*, the E. faecium* inoculated sample was placed adjacent to the *Salmonella* inoculated sample to compare its inactivation behavior. Post RF treatment, the paper bags were immediately transferred to a sterile whirl bag and immersed in an ice-water bath to stop the thermal inactivation. The samples were enumerated by serially diluting in 0.1% BPW and plating on m-TSAYE for *Salmonella* and e-TSAYE for *E. faecium* (Wei et al., 2021a). The experiments were conducted in triplicates with samples from different production lots which were inoculated using a new frozen bacterial stock.

5.3.4 Quality analysis

Quality analysis was performed to evaluate effectiveness of RF treatment and alterations in the quality of the basil samples in *conjunction.* Quality analysis was conducted on uninoculated samples treated with RF conditions that achieved more than a 5-log reduction of *Salmonella* in the microbial challenge study. The RF treated samples were transferred to Ziploc bags, cooled at room temperature slowly, and analyzed for their quality to represent the worst-case scenario for quality loss. The dried basil leaves were ground to a uniform particle size of ≤ 850 μm and were used for the quality analysis. About 1.0 ± 0.1 g of ground sample was diluted with 100 mL of 200 proof ethanol (Decon Labs Inc., PA) to prepare an extract. The mixture was then stirred using a magnetic stirrer at 300 rpm for 18 ± 2 h and was filtered using filter paper (20-25 μm; Fisherbrand 09795G). The filtered extract was later transferred to 50 mL conical tubes. The test tubes were wrapped using an aluminum foil to avoid photosensitivity of the samples and stored at 4°C until the quality analysis was performed. The total phenolics and antioxidant activity of the dried basil leaves were determined using these prepared extracts.

a) Moisture content and water activity

The moisture content and the water activity of the dried basil leaves pre- and post RF treatment were measured using a dew point water activity meter (Model: 4TE, Meter Group, Pullman, WA) and halogen moisture analyzer (Model: HR73, Mettler Toledo, Greifensee, Switzerland), respectively.

b) Color

The color of the RF treated ground dried basil leaves was measured using a colorimeter (Konica Minolta, model: BC-10, Osaka, Japan). The samples were placed on a petri dish in a flattened manner and the color components L*, a*, and b* (L* represented the lightness index,

"a*" represented red-green, while "b*" represented yellow-blue) were measured at five random locations. Prior to the color analysis, the colorimeter was calibrated using a white tile. The total color difference (ΔE) of the pre- and post-RF treated dried basil leaves was calculated using the equation given below (Robertson, 1977).

$$\Delta E = \sqrt{\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2}}$$

c) Total volatile analysis

The volatile composition analysis of the dried basil leaves following RF treatment was performed by using a Thermo Scientific headspace gas chromatography (Trace 1300) equipped with ISQ Mass Selective Detector (MS). Briefly, 1 g of dried basil leaves was taken in a 20 mL headspace vial and placed in the autosampler. The conditions were pre-set to incubate the samples at 72°C agitator temperature for 15 min. The gas (0.7 mL) from the headspace of the vial was injected into GC-MS with a 1:5 split ratio. A capillary column with dimensions of 30 m x 0.25 mm ID x 0.25 dF was used for the analysis. The GC conditions used for the analysis were: oven temperature from 40°C to 290°C with a 30°C/min heating rate and maintained for 2 min, mass transfer line and ion source temperatures for the detectors were 250°C and 200°C, respectively. The ionization energy of 70 eV, and mass range of 10-650 amu were used for the analysis. All the components of the samples were identified by comparing their mass using a NIST 11 mass spectral library.

d) Total phenolic content

The total phenolics in the dried basil leaves were determined by following the Folin-Ciocalteu method (Singleton & Rossi, 1965) with slight modifications. Gallic acid (Acros Organics) standard solutions prepared at 1, 2, 3, 4 and 5 µg/mL were used to establish calibration

curves. For estimation of total phenolics in dried basil leaves, 0.2 mL extract was taken. Both for the gallic acid standards and for dried basil leaf extracts, 2 mL of the diluted Folin-Ciocalteu reagent (20% v/v, Sigma-Alrdich) was added and the solutions were vortexed for 5 s and later stored in dark. After 10 min, 2 mL of 7.5% (w/v) sodium carbonate was added to each solution and vortexed for another 5 s. The total volume of the solutions was made to 5 mL using distilled water and was stored under dark for 2 h at room temperature. The absorbance of the standards, samples were measured at 765 nm using a UV-1800 spectrophotometer (Shimadzu Corp., Kyoto, Japan) using ethanol (0.2 mL) as the blank. All the analyses were conducted in triplicates and the total phenolic content was expressed as gallic acid equivalent (GAE).

e) Antioxidant activity

The antioxidant activity of the basil extract was estimated by recording the scavenging activity of the 1,1 Diphenyl-2-picrylhydrazyl (DPPH). A stock solution of 30 µg/mL of DPPH (Alfa Aesar, 98% purity) was prepared by diluting 3 mg of DPPH in 100 mL of ethanol. The prepared DPPH solution was then covered with an aluminum foil and stored at 4°C until further use. From this solution, 1 mL was added to each of the tubes containing basil extract concentrations, *viz.,* 0.0, 0.2, 0.4, 0.6, 0.8 and 1.0 mL. The total volume of each of the solutions was brought to 5 mL by using ethanol. All the tubes were vortexed for 5 s and then later stored at room temperature under dark conditions. Ethanol was used as blank. After 30 min of incubation, the absorbances were measured at 517 nm using a UV-1800 spectrophotometer (Shimadzu Corp., Kyoto, Japan) using ethanol as the blank. The scavenging activity of the DPPH radical in each sample (Bersuder et al., 1998) was estimated using the equation given below.

$$DPPH\ Radical - Scavenging\ Activity\ (\%) = \left[\frac{A_{control} - A_{sample}}{A_{control}}\right] x100$$

where $A_{control}$ is the absorbance of control and A_{sample} is the absorbance of solutions containing 0.2-1.0 mL of dried basil leaf extracts. All the analyses were performed in triplicated, and the values were expressed as mean ± SD.

5.3.5 Statistical analysis

Inactivation data and quality parameters were analyzed by performing one-way ANOVA for the microbial log reduction, color, total phenolics, DPPH radical scavenging activity and volatile composition as a function of orientation. Each treatment condition (horizontal orientation with a treatment time of 35 s and vertical orientation with a treatment time of 115 s) was repeated with three different lots of samples (three replications). Means of each treatment group were compared by Tukey-Kramer adjustment (Tukey's Honestly Significant Difference test) at 5% significance level. The data analysis was done using JMP Pro 16.0.0.

5.4 Results and Discussion

5.4.1 Heating profile of dried basil leaves at two different package orientations during RF treatment

The average temperature of dried basil leaves during RF heating at points, P1, P2, and P3 in the polypropylene bottle is shown in Fig 2. The temperature difference between the three points at the end of the heating was minimal (1°C) in both orientations. As shown in Fig. 2, the coefficient of variation (CV) of temperature of dried basil leaves in the bottle ranged from 0.01-0.10 for both horizontal as well as vertical orientations. During the heating process, the evaporation of moisture present in dried basil leaves resulted in the generation of steam. The steam vent film helped in retaining and accumulating the steam inside the bottle. During heating at vertical orientation, the steam was generated within the package after 90 s and the package

started bulging at around 100 s and the vent started releasing the steam at around 105 s. Because the rate of steam release was higher than the steam generation, the package started returning to the normal condition at around 110 s. RF energy was applied for additional 5 s for the cold spot temperature to reach the target temperature of 100 °C and then the treatment was stopped at 115 s. Similarly, for horizontal orientations, the stream generation started at around 22 s and the packaged bulged at around 25 s, steam released at 26 s, packaged returned to normal at 30 s. We continued to apply RF energy for another 5 seconds, which improved the heating uniformity and stopped the RF treatment. The total treatment time was 35 s.

The CV values peaked when the temperature was between 45- 90 °C after which they dropped significantly (Fig. 2). It indicates that the movement of steam within the steam vent package helped in heat distribution within the sample to achieve uniform heating. Wei et al. (2019) reported a non-uniformity index of 0.076 ± 0.004 for ground black pepper during RF treatment for 130 s. In the present study, a comparatively lower heating non-uniformity index of 0.054 ±0.010 and 0.013 ±0.005 for dried basil leaves was achieved for vertical and horizontal orientations, respectively. In addition, the temperature at the three layers (P1, P2 and P3) had a lower standard deviation of 0.78°C, while Verma, et al. (2021a) reported a much higher standard deviation of 2.80°C among the three layers of dried basil leaves packed in the laminated paper tray and heated under RF with the similar electrode gap of 10.5 cm. These results indicated that dried basil leaves packed in polypropylene bottles and placed horizontally under RF heats more uniformly. However, the amount of dried basil leaves used by Verma et al., 2021a) for RF heating in laminated paper trays was higher (125 g) than the present study (60 g), which could also be the probable reason for differences in heating uniformity. The higher uniform heating of the sample achieved in a polypropylene bottle with a steam vent will help deliver better

pasteurization results and preserve the quality of the RF pasteurized samples. Because a better heating uniformity was achieved inside the package during radiofrequency processing, the use of hot air assistance, intermittent mixing, surrounding with PEI blocks (Jiao et al., 2015b; Wang et al., 2005, Wang et al., 2007) and immersion of soybean oil (Dag et al., 2021) was not necessary. These approaches make the RF process more complicated and may impede its industrial adoption. Hence, RF pasteurization of dried basil leaves using steam vent packaging can aid in overcoming these limitations in industrial applications.

It can be observed from Fig. 3 that dried basil leaves placed horizontally with a smaller electrode gap heated up much faster than the vertically placed bottles which required a larger electrode gap. Further, it took only 35 s to reach 100°C at the coldest spot for the samples placed horizontally while 115 s of treatment time was required for the samples placed vertically. When the height and diameter of luncheon roll meat emulsion (in cylindrical shape) was maintained equal in size, Romano & Marra (2008) reported that RF heating in vertical orientation achieved a faster heating rate and heating uniformity compared to the horizontal orientation of the cylinder. A similar observation was also observed in tylose-based food stimulants (in cylindrical shape) used to imitate lean beef meat with a high moisture content of 73% as compared to dried basil leaves (Bedane et al., 2021). When the diameter and height of the cylinder are same, the surface area exposed to electrodes are same in both cases. However, the vertical orientation had a flat top surface area closer to electrode. In case of horizontal orientation, only a line on the curved cylinder was closer to the electrode, while most of the curved surfaces are farther away from the electrode surface. This may explain why vertical orientation had a faster heating rate than horizontal orientation, when the diameter and height are equal with constant electrode gap between two orientations. In the present study, the diameter to height ratio is considerably lower

than those studies, which may be the reason for differences in results. In this study, the vertical orientation had a larger electrode gap (16 cm) than the horizontal orientation (electrode gap of 10.5 cm). A smaller electrode gap with larger surface area resulted in faster heating for horizontal orientation in this study.

The probable reason for the variation in heating rate between the vertical and horizontal orientation of the samples could be due to the difference in surface area, electrode gap, air gap between sample and top electrode and electric field deflection. Faster heating rate in horizontal could be attributed to smaller electrode gap which is inversely related to heating rate (Orsat & Raghavan, 2014). Further, a larger surface area exposed at the top when the bottle was placed horizontally as compared to vertical orientation led to higher energy absorption resulting in a faster heating rate. The fringe effect of the electric field causes more heating at the edges of the food container (Chen et al., 2017). The higher electric field magnitude at the bottom corners resulted in higher temperatures at point 3 as compared to other measured points in the bottle. The sample at the top edge in this study lost heat to the surrounding air in the form of steam which lowered the heating rate at P1. Further, in horizontal orientation, electric fields deflected from the points on the curved surface led to higher electric field strength (Tiwari et al., 2011) all through the length of the bottle thereby providing rapid heating. For vertical orientation, the heat was well conserved in the middle layer, which contributed to the higher heating rate at P2 whereas the heat loss was high in the top and bottom layers to the air and ground electrode, respectively. In general, faster heating rates have lower heating uniformity; however, we found horizontal orientation with a higher heating rate to provide slightly better heating uniformity as compared to vertical orientation. In case of horizontal orientation, the temperature at all the points increased exponentially and then asymptotically converged at around 100 °C at the end of the heating. For

vertical orientation, when the cold spot (P1) temperature reached 100 °C (115 s), the hot spot temperature at the middle location (P2) increased to 109 °C. Therefore, the heating uniformity was better for horizontal orientation, even though the heating rate was higher. Hence, it is beneficial to use horizontal orientation not only for rapid heating but also for uniform heating.

The location closer to steam-vent (P1) took the longest time to reach 100°C and therefore, it was regarded as the cold spot in both cases. The lowest heating rate of the sample at the top center (P1) could be due to its proximity to the air leading to the loss of heat from the sample to the surrounding air. Similarly, the top center has been reported to be the cold spot in other studies with ground black pepper (Wei et al., 2019), dried basil leaves (Verma, et al., 2021a), cumin seeds (Chen et al., 2019) wheat flour (Villa-Rojas et al., 2017). No significant difference ($P< 0.05$) between the mean temperature at points 1 and 3 was observed for both orientations. Therefore, inactivation study was carried out at two locations, P1 and P3 which experienced a lower heating rate to ensure a 5-log reduction of *Salmonella* in dried basil leaves by RF processing. The inoculated pack studies carried out earlier showed no impact on the heating behavior of the low moisture food under RF (Ozturk et al., 2019; Verma, et al., 2021a), therefore, the same method was followed in this study. Sample inoculated with either *Salmonella* or *E. faecium* packed in a paper bag was placed at the selected locations inside the bottle as shown in Fig. 2 for microbial inactivation study.

5.4.2 Microbial inactivation

The initial *Salmonella* and *E. faecium* population in dried basil leaves after five days of equilibration at a_w of 0.621 ± 0.002 were 7.5 ± 0.1 and 7.9 ± 0.1 log CFU/g, respectively. The bacterial population was allowed to stabilize during the equilibration time, because the addition of inoculum to low moisture dried basil leaves could induce a shock to microorganisms and

allowing adaptation time would provide a more conservative estimate of treatment efficacy. In accordance, Jeong & Kang (2014) observed a higher bacterial inactivation in ground black pepper following thermal treatment when carried out immediately after inoculation as compared to the results observed by Wei et al. (2019) who provided equilibration time. Therefore, it is imperative to give sufficient time for the bacteria to acclimatize to the outside environment, so the experimental results are not influenced by the microbial shock.

The log reductions of *Salmonella* and *E. faecium* population in dried basil leaves packed inside the polypropylene bottles placed at vertical or horizontal positions during RF heating are represented in Fig. 4 and 5, respectively. After 25 s of heating, the *Salmonella* was reduced by 2.60 and 4.30 log CFU/g at P1 and P3 of horizontal orientation, respectively (Fig. 5). As RF heating time was increased to 35 s, both *Salmonella* and *E. faecium* were reduced to below the limit of detection (<10 log CFU/g). However, in case of vertical orientation, 95 s of RF heating reduced *Salmonella* by 3.00 and 4.10 log CFU/g at their respective locations, P1 and P3. The target 5-log reduction was not achieved after increasing the RF heating time to 105 s; therefore, the treatment time was extended to 115 s to achieve more than 5 log reductions of both microorganisms (Fig. 4). The results of the heating profile and microbial inactivation data indicate that the horizontal orientation of the bottle under RF provides a better heating rate, more uniform heating, and lower time required for 5 log reduction than the vertical orientation of the bottle. The efficient microbial inactivation observed in horizontally oriented bottles in the RF cavity can also be beneficial to scale up the process. The bottles can be easily rolled on a conveyor belt and pasteurized continuously using radiofrequency processing. Rotation of bottles might improve product mixing and could result in more uniform heating. Further studies are

recommended to evaluate the improvement in heating uniformity and treatment efficacy of horizontally oriented bottles rotating under the RF cavity.

Several studies have recommended RF heating as a pasteurization technology for corn flour (Ozturk et al., 2019); red pepper powder (Hu et al., 2018); ed and black pepper (Jeong & Kang, 2014); shelled almonds (Jeong et al., 2017); cumin seeds (Chen et al., 2020). Most of those studies are conducted in open containers to allow for escaping of steam generated during heating. This study used a steam-vent that allows for venting steam, while preventing cross-contamination during treatment. The film with venting nut used in this study can be easily replaced with a one-way steam valve to prevent potential cross-contamination issues post pasteurization. Thus, pasteurization of the final packaged food product will eliminate the cross-contamination problem thereby improving food safety considerably. Because the RF pasteurization using in-package steaming technology will serve as a kill step for the packaged products, it can be easily incorporated at the end of current manufacturing lines without affecting other food manufacturing processes.

Studies on non-thermal methods of decontamination for spices and herbs have been reported to be effective but required longer treatment times to achieve the desired log reduction. For instance, (Verma et al., 2022) found ClO_2 treatment to be effective in inactivating *Salmonella* in dried basil leaves at a maximum tested concentration of 15 mg/L exposed for 2 hours at 80 % RH. In addition, studies on black peppercorns and cumin seeds at the same gas concentration and RH required an even longer treatment time of 5 hours to achieve 5 log reduction (Wei et al., 2021b). While our study with RF required a considerably shorter time (35 s) to inactivate *Salmonella* in dried basil leaves.

As per FSMA, food industries must conduct process validation steps for the lethality treatment. This requires a non-pathogenic surrogate to be deployed due to the potential food safety risk in using *Salmonella* within the facility. The suitability of *E. faecium* as a surrogate was evaluated for process validation in the food industry. At highest treatment times of 115 s and 35 s for vertical and horizontal orientation, both *Salmonella* and *E. faecium* populations were reduced below the detection level. Figures 4 and 5 show that the reduction of *E. faecium* population was significantly lower than S*almonella* at all other treatment times indicating its suitability as a good surrogate for *Salmonella.* Similar observations were reported for paprika, white pepper and cumin powder (Ozturk et al., 2020), cumin seeds (Chen et al., 2019) and dried basil leaves (Verma, et al., 2021a).

5.4.3 Quality analysis

Radiofrequency processing of dried basil leaves packed in polypropylene bottle and placed horizontally (115 s) and vertically (35 s) achieved more than 5 log reduction and therefore these treatment conditions were analyzed for their effect on the quality of dried basil leaves. The quality parameters such as the water activity, moisture content (%, wb), color values, total phenolics, antioxidant activity, and volatile composition were determined for RF treated dried basil leaves and compared with untreated control samples (Table 1).

RF heating process results in loss of moisture due to the steam generation during heating, which not only influences the quality of the final product but also causes economic loss due to the loss of mass. Therefore, in this study, the moisture content of the sample was increased before the treatment to achieve close to native moisture content after RF heating and avoid the reduction in mass due to moisture loss. The same technique has been followed in several other studies in the past with various low moisture foods such as cumin seeds (Chen et al., 2019),

black peppercorns (Wei et al., 2018), dried basil leaves (Verma et al., 2021a). It was observed that the a_w of the untreated control sample significantly decreased ($P<0.05$) upon treatment of dried basil leaves to RF treatment for 35 and 115 s. In addition, the water activity was found to be specific to the duration of RF treatment i.e., longer treatments leading to a significant decrease in water activity. The water activity of dried basil leaves reduced from 0.620 ± 0.003 to 0.540 ± 0.009 and 0.500 ± 0.005 upon exposure to RF treatment for 35 s and 115 s, respectively. Similarly, the moisture content (wb, %) of the dried basil leaves dropped by more than 1% when treated by RF for 35 and 115 s. The final water activity and moisture content of the sample after treatment were 0.540 ±0.009 and 9.56 ±0.16 % which were very close to its native values and are acceptable. The increase in moisture content before the treatment, in turn improved the microbial inactivation by increasing the heating rate as well as heat sensitivity of *Salmonella* and *E. faecium* due to the moist environment in the package.

It is difficult to procure raw untreated dried basil leaves for this study. Because the samples used in our study had been previously steam-sterilized, the comparison of other quality parameters such as color, total phenolics, antioxidant activity, and volatile composition before and after treatment may not indicate the actual variation due to RF treatment. The steam treatment could have caused quality deterioration in the sample due to long exposure to heat. For instance, (Javanmardi et al., 2003) reported total phenolic content and antioxidant activities of 23 accessions of dried basil leaves from Iran which ranged from 23.0 to 65.5 mg GAE/g and 13.8 to 35.7 □mol Trolox equivalent/g. These reported values are much higher than those observed for the control samples in this study (5.46 ±0.85 mg GAE/g). However, the total phenolic content of the dried basil leaves did not significantly vary between the untreated and RF-treated samples. The total phenolics was slightly lower for dried basil leaves treated to RF in horizontal

orientation (4.50 ± 0.94 mg GAE/g) than that in the vertical orientation (5.16 ± 0.79 mg GAE/g) despite a shorter treatment time even though the values are not significantly different. Similarly, studies by Chen et al. (2019) and Wei et al. (2018) on black pepper and cumin seeds did not notice any significant change in the total phenolics between the untreated and treated samples.

Phenolic compounds are well known to have antioxidant properties such as reactive oxygen species scavenging and inhibition (Kähkönen et al., 1999). DPPH (2,2-diphenyl-1-picrylhydrazyl) assay is an acceptable and most widely used technique to evaluate the antioxidant activity of the extracts (Dudonné et al., 2009). The reported studies showed that there was a strong correlation between DPPH antioxidant activity with total phenolics (Aryal et al., 2019). It is possible to suggest that phenolics are highly responsible for the antioxidant activity of the extracts.

Therefore, antioxidant activity of the basil extracts was determined using DPPH free radical scavenging assay and correlated with total phenolics. The DPPH antioxidant activity of the untreated and RF-treated dried basil leaves for 35 s (horizontal) and 115 s (vertical) are given in Fig. 6. It was observed from the DPPH radical scavenging activity assay that the antioxidant activity was concentration-dependent. Furthermore, it was noticed that the antioxidant activity in both untreated and RF treated dried basil leaves reached the optimum activity when extract concentration of 1.2-2.0 mg/mL was used for the assay. In general, the antioxidant activity between the untreated and the RF treated dried basil leaves did not significantly differ irrespective of the RF processing time that is in accordance with total phenolics results.

The color of dried basil leaves was measured by a colorimeter in terms of L*, a*, and b* values, where L* represented the lightness index, “a*” represented red-green, while “b*” represented yellow blue. The color values (L*, a*, and b*) of dried basil leaves were not

significantly (P>0.05) affected by the RF treatment for 35 and 115 s. Telfser & Gómez Galindo. (2019) reported that the greenness of fresh basil leaves denoted with 'a*' value of −13.8 ± 1.4 reduced to −4.3 ± 0.2 post air drying. Dried basil leaves used in our study had a much higher 'a*' value (less green) (2.47 ± 0.23) which did not change post RF treatment. The L* and b* values for the untreated and RF treated dried basil leaves were on par with each other. Though the b* values for the RF-treated dried basil leaves increased from 13.97 ± 0.31 to 14.57 ± 0.57 upon RF treatment for 115 s, no significant difference was observed between the untreated control and treated samples. Similarly, studies by (Verma et al., 2021a) did not observe any significant effect of RF treatment (65 s) on the color of the dried basil leaves. Interestingly, it was observed that though the dried basil leaves were treated to high-temperature RH treatment, no significant change in the color values was obtained due to a shorter treatment time. The color difference (ΔE) values for the dried basil leaves after treatment with RF for 35 and 115 s were 1.13 ± 0.179 and 1.45 ± 0.86, respectively. The ΔE more than 1 and less than 2 indicates that the color difference can only be noticed by an experienced observer (Mokrzycki & Tatol, 2011). In accordance, Verma et al. (2021a) also observed no significant change in the color values between the untreated control and RF-treated dried basil leaves. From the quality analysis study, it is evident that RF treatment can significantly affect the water activity and moisture content (%) of dried basil leaves without altering the color and total phenolics of the dried basil leaves.

Volatiles are heat sensitive compounds that provide the distinct flavor and aroma of herbs and spices. The volatile compositions of herbs and spices can be changed during application of a thermal processing method because of losing some volatile compounds. Table 2 summarizes the total volatile composition of untreated and RF treated dried basil leaves. The head space Gas-Chromatographic analysis of the dried basil leaves indicated the presence of 14 volatile

compounds. No significant difference in the relative composition of the compounds was noticed between the untreated and RF treated basil leaves as shown in the chromatogram (Fig. 7). Among the various compounds detected on dried basil leaves, linalool (33.99 ± 0.70 %), methyl chavicol (31.80 ± 0.72 %), and 1,8 -cineole (21.16 ± 1.69 %) were found to be the major compounds constituting more than 86% of the total volatile composition in dried basil leaves. Similarly, Verma et al. (2021a) reported 1, 8- cineole, linalool, and methyl chavicol as the major compounds, in addition to E-methyl cinnamate and trans-alpha-bergamotene in untreated and RF treated basil leaves (65 s). However, the total composition of these major compounds reported under their study constituted only 69% as against 86% reported in the present study for the three major compounds. In addition, both the studies reported 1, 8 cineole as one of the major compounds, the present study recorded higher relative composition (22.07 ± 0.94 %) as compared to their study (6.38 ± 1.50 %) with shorter RF treatment. Interestingly, we observed that the volatile composition of certain compounds in the dried basil leaves increased after RF treatment. Comparing the volatile composition between the two RF treatment times, it was observed that shorter RF processing time i.e 35 s, increased the volatile composition of certain compounds prominently. On the other hand, the volatile composition of the few compounds decreased when treated to RF for a longer processing time of 115 s; however, the decrease was not significant ($P<0.05$). This variation in the volatile composition between the two processing times could be due to the disintegration of the volatile compounds at the longer processing time tested. The volatile composition of some of the compounds was higher compared to the control irrespective of the RF processing time. Díaz-Maroto et al. (2004) indicated that the collapse of the cuticle layer of basil leaves during drying can result in cell structure expansion and excitation of the volatile compounds into the environment. Verma et al. (2021a) observed no significant

change in the volatile composition of the compounds between the untreated and RF treated (65 s) dried basil leaves, except for camphor which showed about 5 to 10% drop in the total composition. In another study, (Calín-Sánchez et al., 2012) observed about 55% of loss in total volatile composition following hot air drying of sweet basil at 40°C. From the present study results, it is evident that the dried basil leaves could retain all the major volatile compounds after the RF treatment. Because the aroma of the basil leaves is mostly attributed to the major compounds present in them (Lee et al., 2005), observing no significant change in the volatile composition demonstrates that RF can ensure microbial safety without altering the product quality.

5.5 Conclusion

Temperature distribution indicated that the steam generated in the package improved the overall heating uniformity during the RF pasteurization. The horizontal placement of the sample container (electrode gap = 10.5 cm) showed rapid and uniform heating of dried basil leaves as compared to vertical placement (electrode gap = 16 cm). RF treatment for 105 s in vertical orientation reduced *Salmonella* and *E. faecium* population by 4.58 ± 0.14 and 2.59 ± 0.46 log respectively. *E. faecium* was more heat resistant than *Salmonella,* indicating its suitability as a surrogate in RF pasteurization of dried basil leaves. In the horizontal orientation, RF treatment reduced the bacterial populations below the detection level within 35 s. Corresponding treatment time to reduce the bacterial population below detection level for vertical orientation was 115 s. Quality evaluation studies indicated no significant change in color, total phenolics, antioxidant activity and volatile composition of dried basil leaves due to RF treatment when compared to the control. Incorporating a steam vent during in-package RF pasteurization of dried basil leaves can eliminate potential post-process cross-contamination, improve heating uniformity by building

steam pressure in a controlled manner, enhancing the pasteurization process without significantly impacting the quality.

5.6 References

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Table 5.1. Comparison of quality parameters of untreated and RF treated (115 s for vertically and 35 s for horizontally positioned bottle) dried basil leaves.

	Untreated	**RF for 115 s in Vertical orientation**	**RF for 35 s in Horizontal orientation**
Water activity	0.620 ± 0.003^{a}	0.500 ± 0.005^{b}	0.540 ± 0.009^{c}
Moisture content (%, wb)	10.9 ± 0.25^{a}	9.10 ± 0.05^{b}	9.56 ± 0.16^{b}
L*	53.23 ± 0.55^{a}	53.47 ± 0.25^{a}	53.5 ± 0.66^{a}
a*	2.47 ± 0.23^{a}	2.47 ± 0.15^{a}	2.40 ± 0.30^{a}
b*	13.43 ± 0.67^{a}	14.57 ± 0.57^{a}	13.97 ± 0.31^{a}
ΔE	0	1.45 ± 0.86^{a}	1.13 ± 0.17^{a}
Total phenolics (mg GAE/g)	5.46 ± 0.85^{a}	5.19 ± 0.79^{a}	4.50 ± 0.94^{a}

Within a row, numbers with the same letter superscript are not significantly different at $p < 0.05$

Table 5.2. Comparison of total volatile compounds (% area) in untreated and RF treated dried basil leaves.

Compound	Control	RF for 115 s in Vertical orientation	RF for 35 s in Horizontal orientation
beta-myrcene	1.11 ± 0.14 [a]	1.14 ±0.08 [a]	1.36 ± 0.27 [a]
alpha-Terpinen-7-al	1.37 ± 0.26 [a]	1.27 ± 0.07 [a]	1.50 ± 0.34 [a]
1,8-Cineole	21.16 ± 1.69 [a]	22.07 ± 0.94 [a]	22.41 ± 1.59 [a]
alpha-Terpineolene	0.48 ± 0.23 [a]	0.52 ± 0.14 [a]	0.73 ± 0.21 [a]
Linalool	33.99 ± 0.70 [a]	33.10 ± 0.72 [a]	32.20 ± 0.90 [a]
Camphor	1.18 ± 0.34 [a]	1.23 ± 0.50 [a]	1.40 ± 0.32 [a]
Terpinen-4-ol	1.32 ± 0.73 [a]	0.90 ± 0.27 [a]	1.32 ± 0.57 [a]
Methylchavicol	31.80 ± 0.72 [a]	32.20 ± 0.94 [a]	30.57 ± 0.87 [a]
Borneol acetate	0.28 ± 0.24 [a]	0.20 ± 0.07 [a]	0.33 ± 0.20 [a]
Eugenol	0.50 ± 0.40 [a]	0.36 ± 0.11 [a]	0.63 ± 0.38 [a]
E-Methyl cinnamate	0.86 ± 0.31 [a]	1.16 ± 0.41 [a]	1.33 ± 0.34 [a]
trans-alpha-Bergamotene	5.32 ± 0.42 [a]	4.93 ± 0.78 [a]	5.40 ± 0.73 [a]
gamma-Cadinene	0.63 ± 0.11 [a]	0.93± 0.20 [a]	0.82 ± 0.27 [a]

Within a row, numbers with the same letter superscript are not significantly different at $p < 0.05$

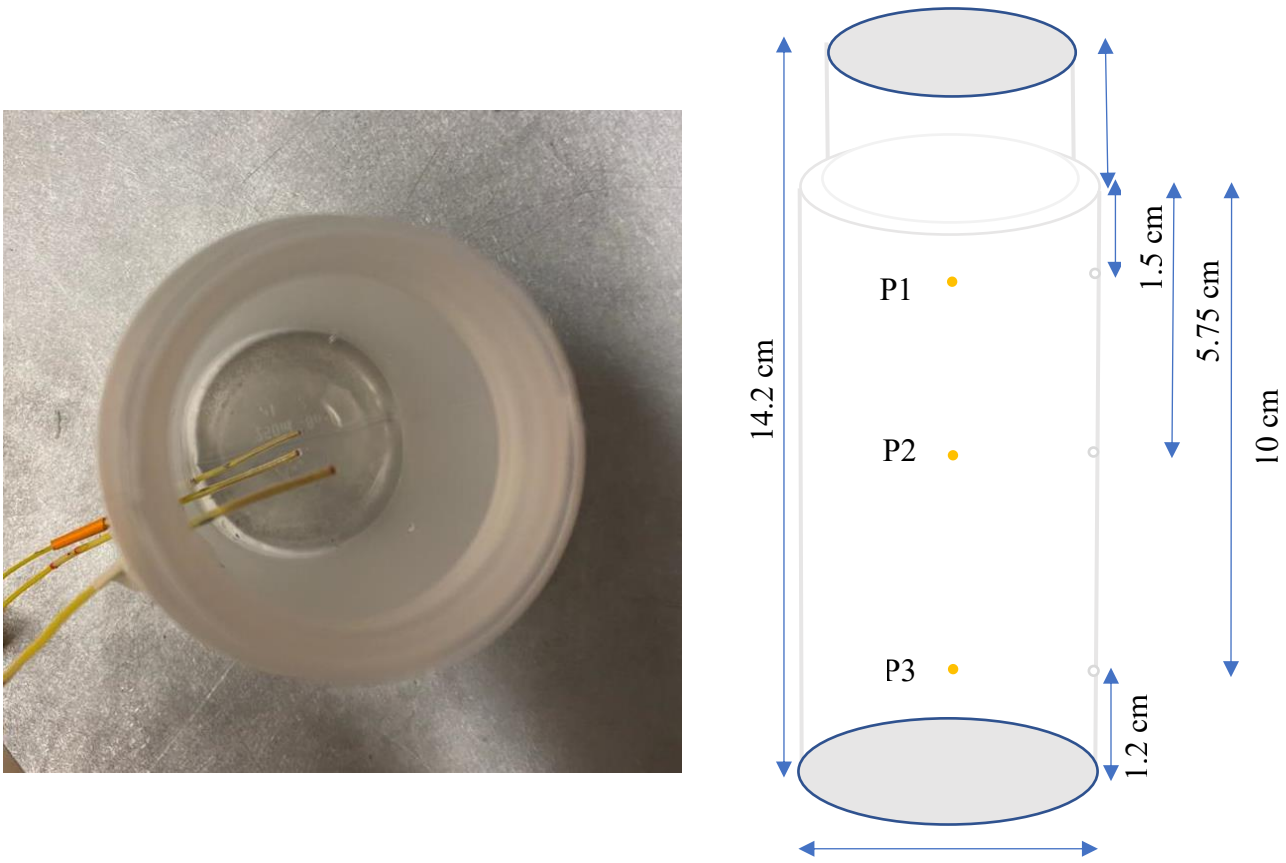


Fig 5.1. Location of three fiber optic sensors in the polypropylene bottle (P1: Top; P2: Middle; P3: Bottom).

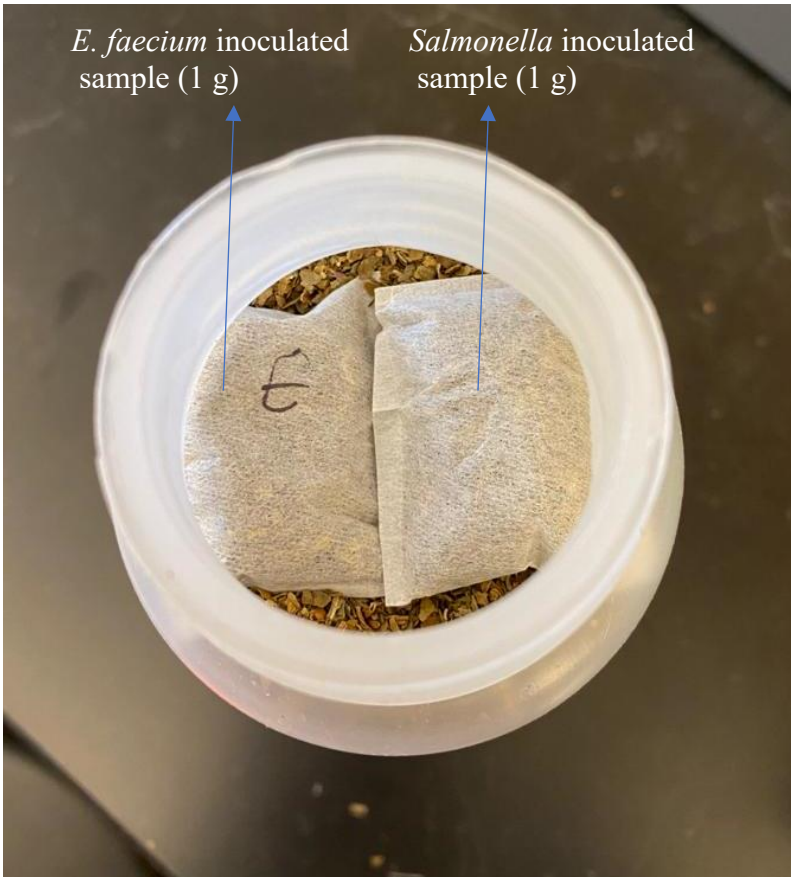
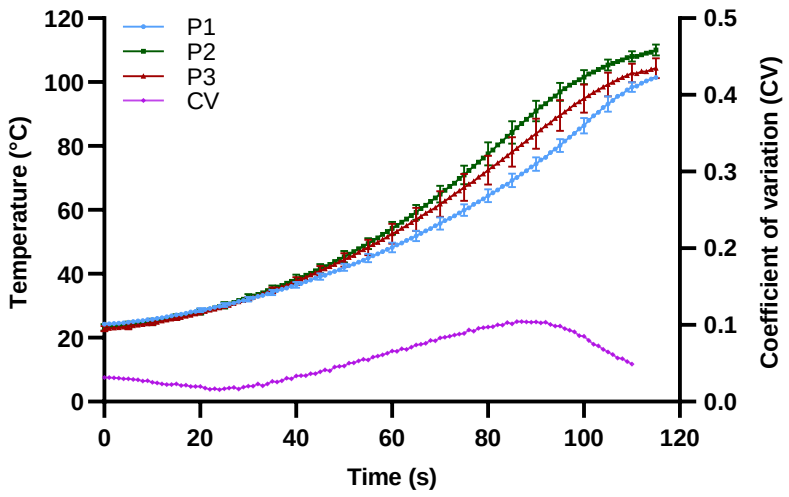


Fig 5.2 Paper bags packed with *Salmonella* and *E. faecium* inoculated dried basil leaves (1 g) and placed at the cold spot.

(a)



(b)

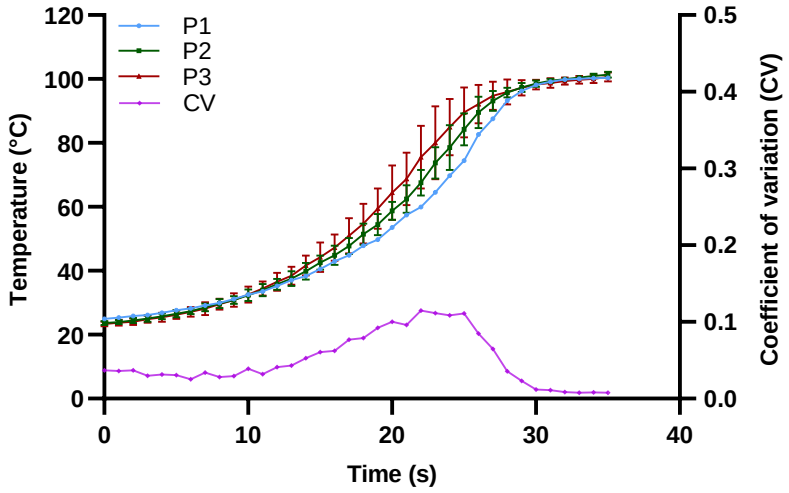


Fig 5.3. Time-temperature profile of dried basil leaves packed in polypropylene bottle during RF heating (a) vertical position (electrode gap = 16 cm) (b) horizontal position (electrode gap = 10.5 cm). Error bars indicate standard deviation of temperatures from the same location for five replications.

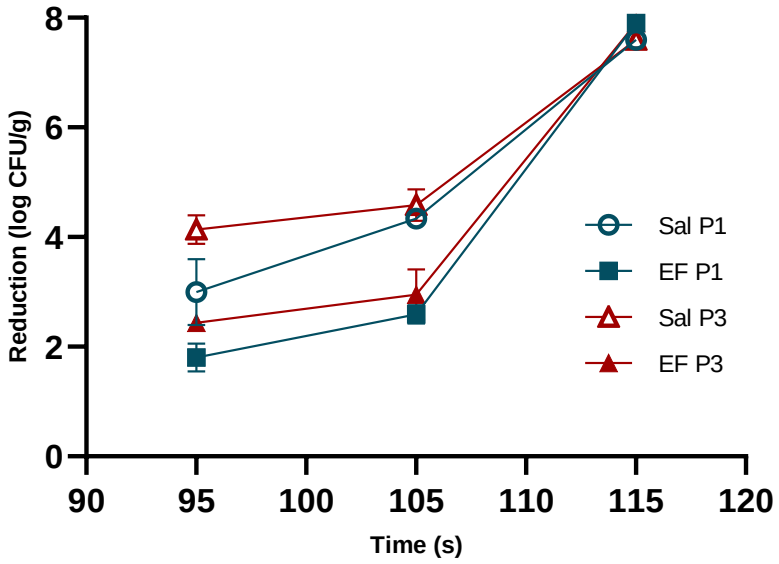


Fig 5.4. Inactivation of *Salmonella* and *E. faecium* NRRL B-2354 during RF heating at vertical position of the bottle for 115, 105, and 95 s with an electrode gap of 16 cm. Error bars indicate the standard deviation of microbial log reductions from three replicates. The population of both microorganisms was below the detection limit (<10 CFU/g) at 115 s of RF treatment, resulting in >6.5 log (CFU/g) reduction.

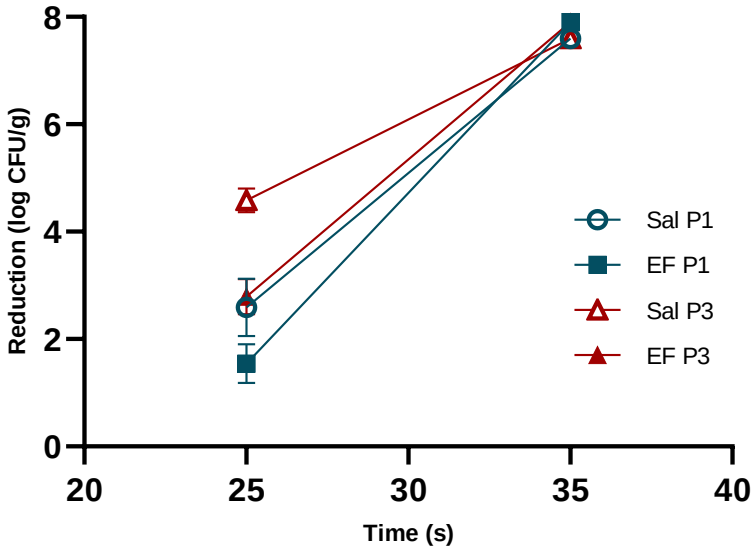


Fig 5.5. Inactivation of *Salmonella* and *E. faecium* NRRL B-2354 during RF heating at horizontal position of the bottle for 35 and 25 s with an electrode gap of 10.5 cm. Error bars indicate the standard deviation of microbial log reductions from three replicates. The population of both microorganisms was below the detection limit (<10 CFU/g) at 35 s of RF treatment, resulting in >6.5 log (CFU/g) reduction.

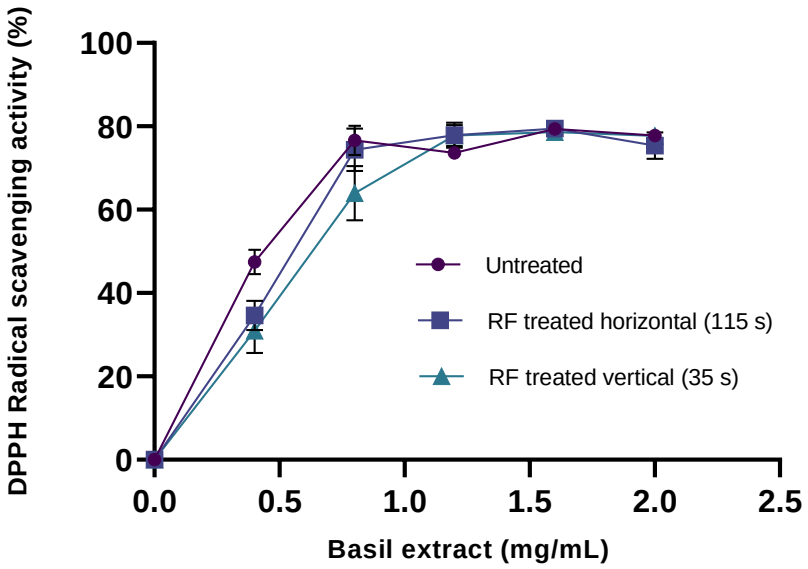


Fig 5.6. Antioxidant activity of untreated and RF treated dried basil leaves (115 s for vertically positioned bottle and 35 s for horizontally positioned bottle). Error bars indicate the standard deviation of antioxidant scavenging activity from three replicates.

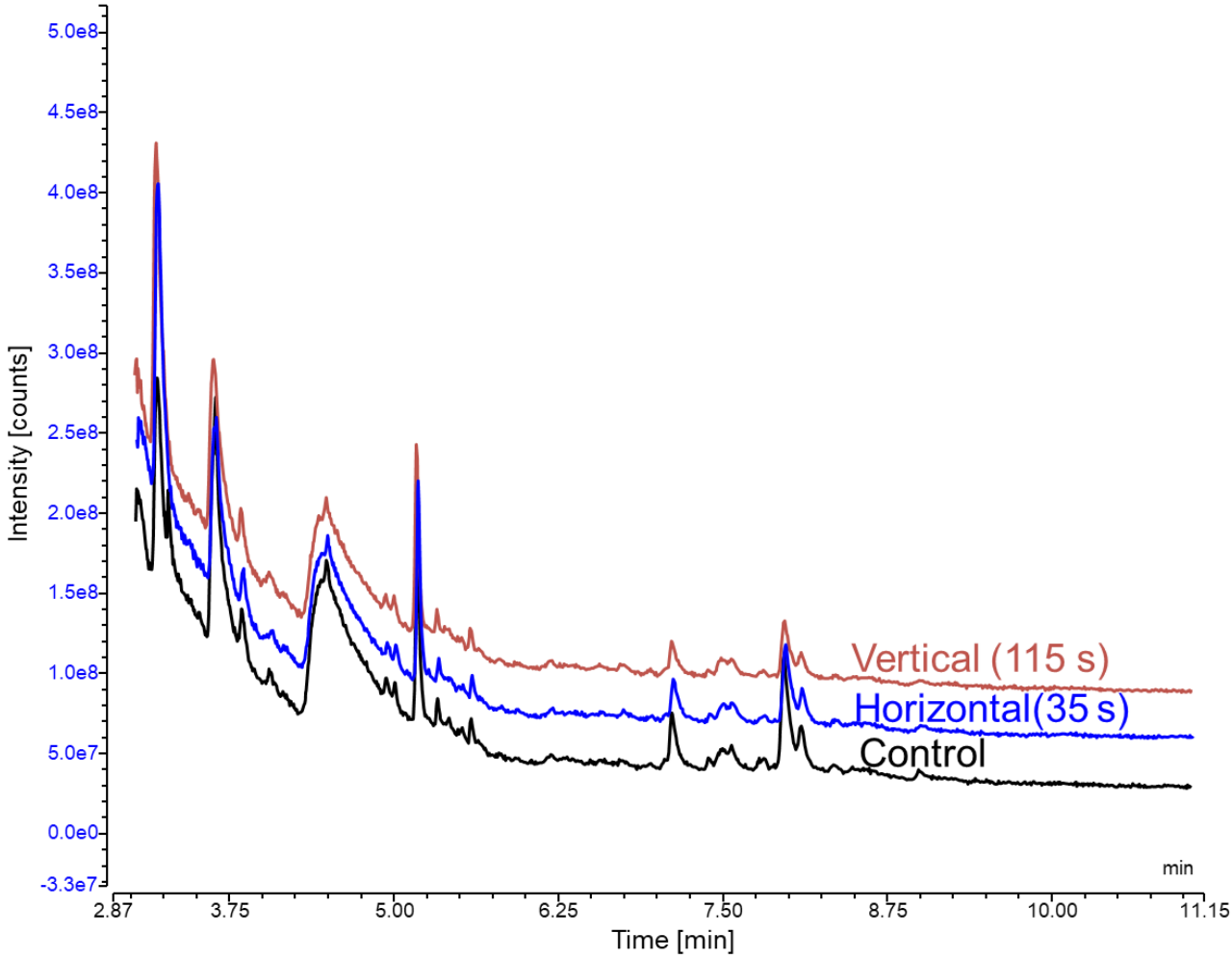


Fig 5.7. Chromatogram of volatile detected in control and RF treated dried basil leaves (115 s for vertical orientation and 35 s for horizontal orientation) by gas chromatography.

Chapter 6. Radiofrequency pasteurization of black peppercorns and dried basil leaves using in-package steaming

6.1 Abstract

Radiofrequency (RF) heating has been extensively studied for pasteurizing low moisture foods. Currently, bulk foods are treated with radiofrequency; potential cross-contamination may occur during packaging of pasteurized products. As an alternative, in-package RF processing was evaluated for *Salmonella* inactivation on black peppercorns and dried basil leaves and prevention of cross-contamination during storage post processing. The samples were RF treated in a steam vent package to enable accumulation and retention of steam during the treatment. This treatment achieved good heating uniformity which could be because of the circulation of steam within the package. One-way steam vent allowed the release of excess steam once a threshold pressure was achieved and later returned to its original position to seal the package, when the RF energy was removed. In-package RF steaming of black peppercorns and dried basil leaves for 135 s and 40 s, respectively resulted in more than 5 log reduction of *Salmonella*. The steam vent remained stable post treatment and properly sealed the package to protect the product from any external contamination. These results indicate that the use of steam vent could effectively pasteurize black peppercorns and dried basil leaves could be beneficial in preventing the potential cross-contamination post processing.

Keywords: *Salmonella*, heating uniformity, Radiofrequency, in-package steaming

6.2 Introduction

Dried spices and herbs have low water activity (a_w< 0.85) and were considered to be safe because they are inhospitable environments that prevent microbial growth (FAO, 2014). However, prior research has demonstrated that pathogens like *Salmonella* can persist in spices and herbs for long periods and grow when conditions are conducive (Keller et al., 2013). Moreover, spices and herbs are typically consumed as a seasoning without prior cooking, therefore, contaminated food is a huge public health concern (Gurtler et al., 2019). For instance, contaminated black pepper was responsible for multistate foodborne illness outbreaks due to the consumption of seasoned salami products (Gieraltowski et al., 2013).

Black pepper is the most commonly used spice, often referred to as the "king of spices", and accounts for 20% of the global spice trade (FAO, 2019). Dried basil leaves are a versatile herb used as a seasoning to enhance the flavor but also have medicinal properties. Fourteen ou*tbreaks* have been reported to be linked to the contamination of *spices* between 1973 and 2010 (Van Doren et al., 2013). These outbreaks infected 1946 people with *Salmonella* being the major cause of contamination. Further, the investigation revealed that 71% of the cases were caused due to the addition of contaminated spices to cooked food. Fresh basil has been associated with foodborne illness outbreaks due to *Salmonella* contamination in the US and caused more than a thousand infections. Zhang et al. (2017) reported that in a retail establishment in the USA, 0.19 % of basil samples (529) and 0.24% of black pepper (1264) samples were positive for the presence of *Salmonella*.

Steam, ethylene oxide, and irradiation have been recommended for the pasteurization of spices and herbs (ASTA, 2011). However, the commonly used wet steam treatment could invariably elevate the product moisture, which can reduce the shelf life of the final product and

necessitate a further drying step to return to the original moisture. Improper use of ethylene oxide gas has potential carcinogenic effects and is not recommended due to probable ethylene chlorohydrin residue formation (U.S. Environmental Protection Agency, 2009). Irradiation treatment has concerns with poor consumer acceptance and additional labeling requirements (Wason et al., 2021).

Traditional thermal processing technologies are often inefficient for processing spices and herbs due to the low thermal conductivity of food and high heat resistance of *Salmonella* requiring the treatments to be longer. Radiofrequency heating is an effective pasteurization technique which volumetrically heats the food product by causing molecular friction when exposed to a rapidly changing electromagnetic field at frequencies of 1-300 MHz (Huang et al., 2018). With RF treatment, the commodity can achieve rapid heating due to high penetration capacity compared to other thermal treatments (Jiao et al., 2014). Although radiofrequency pasteurization has been extensively used for a variety of products such as cumin seeds (Chen et al., 2019); red and black pepper (Jeong & Kang, 2014; Kim et al., 2012); dried basil leaves (Verma et al., 2021); black peppercorns (Wei et al., 2018), very few studies have investigated their effect on packaged foods. The majority of the studies were carried out with open containers enabling the release of steam generated during heating. In general, current RF treatment mainly pasteurizes the bulk products first before packing which increases the chances of potential cross-contamination from the outside environment during packing of spices. Evidently, Cross-contamination was identified as the top cause of foodborne illnesses and outbreaks (Soon et al., 2020). Moreover, when RF has been applied to bulk products, the loss of moisture reduces the water activity of the product and further increases the D-value of microorganisms resulting in

low pasteurization efficiency. Hence, subjecting the packaged produce to RF heating can enhance the pasteurization efficacy and prevent post-processing cross contamination.

Non-uniform heating is another drawback that limits the use of radiofrequency on a commercial scale. Due to non-uniformity, some of the product may get overheated, while the other part may not achieve enough heating, leading to microbial safety concerns in the product and quality deterioration (Piyasena et al., 2003; Tiwari et al., 2011; Jiao et al., 2014). Steam venting packages have been used in microwavable food products to improve the heating uniformity and safety of the meals. The packaging is designed to build steam in a sealed plastic pouch or container that has a self-venting release valve. Microwaving and simultaneously generated steam offer even distribution of heat and decreased cooking time improving food safety. For instance, microwave assisted steam generation could inactivate *Vibrio vulnificus* and *Vibrio parahaemolyticus* in oyster meat also retaining the sensory quality of the cooked meat (Espinoza, 2013). However, the steam venting packages that are typically used for microwavable food products do not seal after it is ruptured. This type of steam vent package is suitable for foods that are designed to be consumed immediately after heating.

A previous study on RF pasteurization of dried basil leaves in-package using a steam vent package provided more than 5 log reduction of *Salmonella* and *E. faecium* (Wason et al., 2022a). However, the authors manually fixed a venting nut on the plastic film over the food container which acted as a two-way vent, and thus could not guarantee the prevention of cross-contamination post RF heating. Recent technological advances in packaging allow for the integration of one-way steam vent in the package that can reliably seal after steam release. Huang et al. (2015, 2016, 2018) reported that wheat and soybean packed in plastic containers did

not have a significant impact on RF heating. Such packages can be used in RF heating applications and allow in-package pasteurization.

Therefore, the present research was aimed at evaluating i) the RF pasteurization using single vent steam packaging for effective *Salmonella* inactivation in black peppercorns and dried basil leaves, and ii) the impact of in-package steaming on the product quality.

6.3 Materials & Methods

6.3.1 Bacterial strains and sample inoculation

The *Salmonella enterica* strains used to produce the cocktail were Agona 447967, Mbandaka 698538, Montevideo 488275, Reading Moff 180418, and Tennessee K4643. All the bacterial strains were stored in glycerol solution (40%) at -80°C until further use. The strains were selected based on their past association with foodborne illness outbreaks. The inoculum was prepared as described in Wason et al. (2022b). In brief, frozen cultures were thawed and grown in TSBYE (tryptic soy broth supplemented with 0.6% (w/w) yeast extract (TSBYE; Difco, Sparks, MD) followed by streaking on TSAYE plates (tryptic soy agar supplemented with 0.6% (w/w) yeast extract (TSAYE; Difco, Sparks, MD)) to prepare working plates. Bacterial lawns were produced separately for each *Salmonella* strain by isolating the colony in TSBYE and spreading 0.1 mL of the overnight culture onto a TSAYE plate that was incubated at 37°C for 24±2 h. Each bacterial lawn was then harvested with 3 mL of BPW using a sterile L-shaped spreader and mixed in equal proportions in a centrifuge tube.

Black peppercorns and dried basil leaves from three production batches were procured from McCormick & Co., Inc. (Hunt Valley, MD) and refrigerated until further use as three replicates. The samples were placed at room temperature for at least 24 h before inoculation. The particle size of black peppercorns and dried basil leaves was analyzed using RO-TAP sieve

shaker (Rx-29, W.S. Tyler, OH, US) according to ASABE standards (ASABE, 2017). The protocol used was similar to the earlier published research by Wason et al. (2022a). The sieve size ranged from 0.3 mm to 2 mm for dried basil leaves and 0.85 mm to 5.6 mm for black peppercorns. The method described by Wason et al. (2022b) was followed for inoculating these food ingredients. Briefly, the *Salmonella* cocktail was sprayed over a thin layer of black peppercorns and dried basil leaves and manually mixed. Three biological replicates were each inoculated with fresh frozen bacterial stock. The natural water activity of the black peppercorns and dried basil leaves are 0.55 ± 0.01 and 0.53 ± 0.01 respectively. Based on preliminary trials, the inoculated sample was equilibrated to a higher water activity in a relative humidity chamber (Lau & Subbiah, 2020) for at least 5 days before being used for RF treatment to compensate for the moisture loss during RF heating. This time was considered essential for bacteria to acclimatize to sudden changes in the outside environment upon inoculation in a low water activity food and ensuring that the treatment would provide a conservative estimation of inactivation efficacy.

6.3.2 Radiofrequency treatment

The treatments were conducted in a steam vent package procured from Sirane Packaging Ltd (Telford, United Kingdom). The package is made of polystyrene film with a steam vent (red patch) integrated typically in the center on one of the sides as shown in Fig. 1. The steam vent was circular in shape with a diameter of 25 mm. According to the manufacturer, the steam vent allows the release of steam at temperatures closer to 100°C. Based on the preliminary trials, the electrode gap (10.5 cm) that achieved the maximum heating rate and better heating uniformity was selected for further studies. Tiwari et al. (2011) and Huang et al. (2018) reported that the corners and edges of the container tend to absorb more electric field due to fringe effects and

tend to be hotter compared to the center. Therefore, the temperature was recorded at the top center, corner, geometric center, and edge positions of the packaged product during RF heating as shown in Fig. 1. The temperature at these locations was measured using fiber optic probes (Neoptix, Inc., Quebec City, Quebec, Canada) with an accuracy of ±0.6 °C as shown in Fig. 1. The fiber optic probes were inserted into the plastic pouch through a hole punched using a pin. Duct tape was used to seal the hole and secure the fiber probe at the desired point. Pre-weighed black peppercorns (150 g) and dried basil leaves (60 g) were then filled into the package and positioned in the middle of the bottom electrode. The temperature data was recorded every second during the entire RF treatment. The location which achieved 100°C temperature at the slowest rate was identified as the cold spot.

The non-uniformity index (λ) was calculated to determine the uniformity in heat distribution within the package (Wang et al., 2005)

$$\lambda = \frac{\Delta\sigma}{\Delta\mu}$$

where $\Delta\sigma = \sqrt{\sigma^2 - \sigma_0^2}$ is the rise in standard deviation and $\Delta\mu = \mu - \mu_0$ is the rise in average temperature at various locations in the sample package during RF processing. Smaller λ values indicated better heating uniformity in the sample.

The microbial challenge study was performed using the inoculated pack method described by Liu et al. (2018). During RF heating, the paper bag with the inoculated sample and the remaining uninoculated sample was placed at the observed cold spot. Immediately after the treatment, the paper bag was removed from the package, placed in a sterile whirl pack bag, and immersed in an ice cold water bath for at least 5 min to terminate the thermal treatment. The *Salmonella* population was enumerated by plating it on m-TSAYE (TSAYE supplemented with 0.03 (w/v)

sodium thiosulfate (Fisher Scientific, Fair Lawn, NJ), and 0.05% (w/v) ammonium iron citrate (Sigma Aldrich, St. Louis, MO)) and incubating at 37°C for 24 h.

6.3.3 Quality analysis

The treatment conditions which reduced *Salmonella* by more than 5 log CFU/g was chosen to study the effect of in-package RF steaming on quality attributes (moisture content, water activity, and color) of black peppercorns and dried basil leaves. Uninoculated samples used for the quality analysis are equilibrated at the same water activity as the inoculated sample used for the microbial challenge study. Post treatment, the sample was instantly emptied into a Ziplock bag and allowed to cool down at ambient conditions before the analysis. The moisture content and water activity of the control and treated sample were measured using a halogen moisture analyzer (Model: HR73, Mettler Toledo, Greifensee, Switzerland) and dew point water activity meter (Model: 4TE, Meter Group, Pullman, WA). The color analysis was performed using a Konica Minolta colorimeter (model: BC-10, Osaka, Japan) as described by Wason et al. (2022a).

6.4 Results and Discussion

6.4.1 Time-temperature profile of black peppercorns and dried basil leaves

The temperature of black peppercorns and dried basil leaves increased from 22 to 100°C within a treatment time of 155 s and 40 s respectively (Fig. 2). The particle size of dried basil leaves and black peppercorns was 1.00 ± 0.07 mm and 4.25 ± 0.06 mm. The difference in come up time of two products could be attributed to variation in their chemical composition, particle size, shape (Kong et al., 2022; Boreddy & Subbiah, 2016; Venkatesh and Raghavan, 2004). The particle size of whole black peppercorns was larger than dried basil leaves which influences their

bulk density and in turn, impacts dielectric characteristics. According to Chen et al. (2020), one batch of cumin seeds with larger particle size exhibited lower RF inactivation than the other two batches subjected to the same treatment time. Similarly, Ozturk et al. (2018) evaluated the dielectric properties and RF heating rate of six different spices and found them to be significantly influenced by variations in moisture content, chemical composition, and bulk density.

As shown in Fig. 2A, the heating rates of black peppercorns at all locations were similar and followed a linear increase with increasing time. In contrast, Tiwari et al. (2011) observed that during RF heating, the samples at the corner of the container received more heating compared to the samples at the center of the open container. Dried basil leaves showed a similar phenomenon where corners heated more rapidly compared to other parts. Chen et al. (2017) stated that this phenomenon could be due to the electric field's fringe effect, which causes excessive heating on the container's borders. The steam vent package was designed in such a way that steam is built inside the plastic pouch during RF heating which enhances the heat transfer throughout the sample. When steam is generated, the steam vent package expanded until the internal pressure reached a threshold, followed by the release of excess steam through the one-way steam vent enabling the package to retain its original condition. After 30 s of RF heating, dried basil leaves at the corner reached 80 °C and the package started bulging as the steam was produced was observed by the bulging of the package. The generated steam assisted in improving heat distribution within the package minimizing the temperature difference at different locations as the heating continued (Fig. 2B).

Minimal temperature difference was observed among the four different locations of the packaged black peppercorns (after 155 s) and dried basil leaves (after 40 s) following RF treatment, with standard deviation of 1.13 °C and 2.69 °C. Previous studies by Verma et al.

(2021) and Wason et al. (2022a) also reported standard deviations for dried basil leaves treated with RF in different packaging materials. Verma et al. (2021) calculated the standard deviation from locations at three layers of dried basil leaves (125 g) packed in a laminated paper tray after RF treatment. Wason et al. (2022a) treated dried basil leaves (60 g) in a polypropylene bottle with temperature measurements at three locations along the length of the bottle. In both the previously mentioned studies, packaging containers were covered with a film equipped with a venting nut at the center to permit the escape of excess steam during RF heating. The heating time for dried basil leaves differed between the bottle and tray, with leaves reaching 100°C within 35 s in the bottle (Wason et al., 2022a), while almost double the time (65 s) was required for RF treatment in the paper tray (Verma et al., 2021) at the same electrode gap (10.5 cm). This difference in heating rate could be attributed to variations in sample size, mass per unit surface area (m/SA), and packaging material. To understand the effect of different treatments on the heating rate, mass per unit surface area (m/SA, g/cm^2) was calculated by dividing the sample mass by the surface area of packaging container exposed to the top electrode at the same electrode gap (10.5 cm). The m/SA values for dried basil leaves in a bottle (Wason et al., 2022a), steam vent package, and tray (Verma et al., 2021) are 0.23, 0.27, and 0.60 g/cm^2, respectively. When the cold spot temperature reached 100°C, the corresponding values for standard deviation among the different locations of the package following RF heating are 0.78, 2.33, and 2.80 °C. In general, a decrease in m/SA resulted in reduced standard deviation. The cylindrical container used for heating the dried basil leaves likely improved the heating uniformity. The treatment in the bottle exhibited the lowest standard deviation, possibly due to the focusing of electric fields from the curved surface of the bottle in horizontal orientation resulting in rapid and uniform heating (Tiwari et al., 2011). The heating non-uniformity index of dried basil leaves in the bottle

(Wason et al., 2022a) and steam vent package in this study were 0.013 ± 0.005 and 0.025 ± 0.019, respectively. In the case of black peppercorns treated in steam vent package, the heating non-uniformity index was estimated to be 0.064 ± 0.034. In contrast, Wei et al. (2018) reported a considerably higher λ of 0.092 ± 0.002 for black peppercorns treated in a container covered by a film with a venting nut in the center. The lower m/SA of black peppercorns treated in the steam vent package (0.66 g/cm^2) compared to the tray (1.92 g/cm^2 in Wei et al. 2018 study) could have contributed to improved heating uniformity. Earlier studies pertaining to RF treatment in open containers reported higher heating non-uniformity indexes of 0.097 ± 0.007 for chickpeas, 0.089 ± 0.010 for green peas and 0.079 ± 0.005 for lentils (Wang et al., 2010). It is important to note that these studies calculated the non-uniformity index based solely on the surface temperature of the sample from thermal images, which may not reflect the overall heating uniformity of the sample. In general, in-package steaming assisted in distributing heat throughout the sample Inside the package to achieve uniform heating. Many other approaches have been explored to enhance the uniformity of RF heating such as hot air assistance, immersion of soybean oil, and intermitted mixing (Wang et al., 2005; Wang et al., 2007; Dag et al., 2021; Mahmood et al., 2022). All the above-mentioned approaches are complicated and not feasible to be directly applicable to the current RF systems for implementation on the industrial scale. The geometric center of the package showed the lowest heating rate since it achieved 100°C after the longest heating time and was considered the cold spot for both products. The lower heating rate at the center of the product may be due to its proximity to the steam vent, which may have caused heat loss to the environment. These results are in agreement with previously published studies which indicated that the top center was the cold spot which was closer to a vented nut fixed on a film over the container (Wei et al., 2018, 2019; Verma et al., 2021, Chen et al., 2019). In addition,

Luechapattanaporn et al. (2004) and Villa-Rojas et al. (2017) also observed the top center to be the cold spot in their studies with mashed potatoes covered with an aluminum foil lid and treatment of wheat flour samples in a petri dish, respectively. When a small sample size of black peppercorns (a small glass beaker filled up to 1 cm) was heated with RF for 50 s, Kim et al. (2012) reported a final temperature of 60°C. In contrast, Wei et al. (2018) used a different RF system to heat a larger sample of black peppercorns (400 g) in a paper tray and reported an average temperature of around 38°C at the cold spot after 50 s of RF treatment. Furthermore, Verma et al. (2021) utilized the same RF system to heat dried basil leaves and they observed a higher average temperature of 70°C at the cold spot after 50 s. Wei et al. (2018) and Verma et al. (2021) treated black peppercorns and dried basil leaves, respectively, in a paper tray covered with a plastic film and a venting nut at the center.

Higher temperature was achieved in dried basil leaves (>100°C) and black peppercorns (45°C) when treated in a steam vent package for the same treatment time in our study indicating rapid heating due to steam retention. In contrast to the use of venting nut in earlier research (Wei et al., 2019; Verma et al., 2021), which are always open and may release steam as it is generated, steam was kept inside the package for a short period before being vented out in our study.

6.4.2 Microbial inactivation after RF treatment

The log reduction of *Salmonella* in spices and herbs was directly proportional to the RF treatment time (Fig. 3). RF heating of inoculated black peppercorns for 135 s and simultaneous steam generation within the package provided rapid inactivation of *Salmonella*, reducing the population below the limit of detection (2 log CFU/g). A shorter treatment time of 115 s reduced *Salmonella* by 2.7 log CFU/g. In the case of dried basil leaves, a treatment time of 40 s was sufficient to reduce *Salmonella* population below the limit of detection (>6 log reduction).

RF treatment for 50 s reduced *Salmonella* in black peppercorns by 3.18 log CFU/g (Kim et al., 2012). Wei et al. (2018) reported > 6 log reduction of *Salmonella enterica* in black peppercorns following 3 min of RF treatment while Verma et al. (2021) achieved the same reduction in dried basil leaves within 65 s. Lower treatment time required in our study could be attributed to the use of a steam vent which helped in retaining the steam inside the package for a little while before venting it out, as opposed to the use of a venting nut in their studies which is always open and may release steam as it is produced. A much longer treatment time of 8 min was reported by Tong et al. (2021) for >6 log reduction of *Salmonella* Typhimurium ATCC 14028 in black peppercorns. However, the quality of the treated peppercorns was not assessed. The use of a steam vent in our study helped to accumulate steam within the package which improved the heat distribution as well as *Salmonella* inactivation due to the moist environment. Boreddy et al. (2019) reported that RF heating of wheat flour in an open container required 6 min to achieve 100 °C which led to a significant reduction in its moisture content as compared to unpasteurized samples. Dag et al. (2022) treated milk powder packaged in an enclosed polypropylene plastic container until it reached 75°C followed by holding the sample at 75°C in an incubator. They reported that RF treatment alone reduced the *Salmonella* by 0.8 log CFU/g while holding the sample at 75°C for 90 min increased the *Salmonella* reduction to 4.3 log CFU/g. Steam production was not reported in the study. Moreover, longer treatment time required to decontaminate milk powder could be because of the protective effect of fat content on *Salmonella* inactivation and the absence of antimicrobials.

The United States imports a large amount of spices and herbs from developing countries. Traditionally, they are dried mainly under the sun outside on farms and are at high risk of carrying pathogenic microorganisms. Bourdoux et al. (2016) reviewed different drying methods

for spices and herbs and stated that drying technologies have not been assessed for log reduction but mainly for reducing the moisture content. However, they reported that microbial populations are reduced by treatments such as dielectric drying and low-pressure superheated steam. Alternatively, non-thermal technologies such as antimicrobial gases, pulsed ultraviolet, and cold plasma have been found effective to decontaminate black peppercorns but require longer treatment time and pose concerns of potential harmful byproduct formation and residues. Wei et al. (2021) reported that chlorine dioxide gas treatment (10 mg/L) for 5 h was needed for a 5-log reduction of *Salmonella* in black peppercorns at 80% RH. At a similar RH, Rane et al. (2020) observed a 4-log reduction of *Salmonella* in black peppercorns after exposure to 0.4 mg/L ClO_2 gas for 4 h. The studies exploring non-thermal decontamination of dried basil leaves are limited. Similarly, Verma et al. (2022) reported long treatment times (2.5 h) at 80% and 10 mg/L gas concentration for achieving about a 4-log reduction of *Salmonella* in dried basil leaves. Moreover, gases such as ozone and hydrogen peroxide were not found effective in inactivating *Salmonella* in black peppercorns even at long treatment times (Summers, 2022; Rane et al., 2020). Lower inactivation of 0.5 log CFU/g was observed on black peppercorns after ozone treatment at 17.83 mg O_3/g for 4 h Hydrogen peroxide vapor generated from 35% H_2O_2 solution provided a maximum of 2.83 log reduction after 1 h of treatment at 60 °C (Summers, 2022).

Similarly, pulsed-ultraviolet treatment was found to reduce only 1.9 log CFU/g was reduced in black peppercorn (Xie & Hung, 2020). However, none of the studies reported the effect of these treatments on the product quality, except for the studies by Summers (2022) involving the treatment of black peppercorns with hydrogen peroxide vapor. They observed no significant difference in color, piperine, total phenolics, antioxidant and volatile compounds in H_2O_2 treated black peppercorn samples when compared to the control. On the other hand,

radiofrequency pasteurization is rapid and has been shown to not affect the quality of black peppercorns (Wei et al, 2019) and dried basil leaves(Verma et al., 2021). Moreover, the shorter treatment time and better heating uniformity achieved by in-package steaming in this study further reduce the impact of heat on the quality of black peppercorns. During the study, the seal of the steam vent remained undisturbed for 30 days. However, an additional layer of seal could be placed over the vent as a precaution to prevent cross contamination during storage and distribution.

6.4.3 Quality analysis

The effect of RF processing on the product quality was assessed in terms of water activity, moisture content (MC, w.b.), and color values and compared with the control samples (Table 1).

The RF treatment for 155 s significantly ($P< 0.05$) decreased the water activity from 0.66 ± 0.00 to 0.57 ± 0.01 in black peppercorns and 0.62 ± 0.003 to 0.54 ±0.003 in dried basil leaves after 40 s. Similar to the difference in the water activity, the moisture % (w.b.) significantly reduced from 12.76 ± 0.18 to 10.71 ± 0.51 in treated black peppercorn samples and 10.9 ± 0.18 to 9.26 ± 0.13 in dried basil leaves. However, the final moisture content of treated black peppercorns was within the recommended value of 12% MC set by the American Spice Trade Association (2011). The initial moisture content of the samples was increased to compensate for the moisture loss of samples anticipated post-RF treatment. Similarly, many researchers have earlier adapted this technique to avoid the loss of quality of low moisture foods such as black peppercorns (Wei et al., 2018), cumin seeds (Chen et al., 2019), dried basil leaves (Verma et al., 2021; Wason et al., 2022a). Overall, the heat treatment is expected to reduce the moisture content and water activity in the spices and herbs. The study was designed such that the final

moisture content and water activity were close to its native values and within the approved ASTA standards (max 12 % MC). Wei et al. (2018) and Verma et al. (2021) treated black peppercorns and dried basil leaves respectively in a laminated paper tray covered with a film with a venting nut fixed in the middle of the film. They did not observe changes in color, piperine content, total phenolics, antioxidants, and volatiles after RF treatment. Because the treatment times used in their study were lower than our study, we do not expect quality deterioration. Dag et al. (2022) assessed the RF pasteurization of packaged milk powder. They reported that the release of moisture due to lactose crystallization during RF heating increased the a_w of the sample. Because the package was sealed, the water remained in the package and formed clumps in the milk powder.

Colorimetric analysis of the RF-treated black peppercorns and dried basil leaves revealed no significant differences in L*, a*, and b* values post-RF processing. The L* values for both the RF treated and untreated black peppercorns were within 40-41. Though the a* values, denoting the red-green color were low for the RF treated black peppercorn samples (0.40 ± 0.61) when compared to the control (0.70 ± 0.40), no significant difference was obtained between them. Similarly, a* values of dried basil leaves before (2.23 ± 0.15) and after (2.30 ± 0.10) treatment did not change significantly. With respect to the b* values i.e yellow-blue color, the estimated values for both RF treated and untreated black peppercorn samples and dried basil leaves were within the range of 2.14 – 2.85 and 13.18 – 14.98 respectively suggesting no significant impact of the RF treatment. While a small color change could be expected due to heat treatment, no significant change was evident, probably due to the reasonably shorter processing time used in this study. The color difference (ΔE) values for the black peppercorns and dried basil leaves treated with RF were estimated to be 1.62 and 1.55 respectively. This ΔE value is

greater than 1 and less than 2, indicating that the color difference could be picked up only by an experienced observer as earlier indicated by Mokrycki & Tato. (2011).

6.5 Conclusion

The time-temperature profile of black peppercorns and dried basil leaves subjected to RF heating showed that steam vent packaging could improve the heating uniformity of the samples as evidenced by a low non-uniformity index. RF heating of black peppercorns for 135 s and dried basil leaves for 40 s in a steam vent package reduced the *Salmonella* population to below the limit of detection. This integrated radiofrequency (RF) and in-package steaming technology has overcome two major hurdles of RF applications for low-moisture foods – increased heat resistance of *Salmonella* in dry environments and non-uniform heating. Steam vent retained the steam within the package which provided a humid environment for better *Salmonella* inactivation and achieved uniform distribution of heat. The water activity and moisture loss were observed for the RF treated black peppercorns, product color was not significantly impacted. Thus, RF pasteurization of packaged foods can considerably improve LMF safety without compromising the food quality. The effective inactivation observed in the steam vent package will be useful for the process scale-up. The spices and herbs in the steam vent package can be continuously processed on a conveyor belt and passed on through the supply chain reducing additional packaging steps and potential cross-contamination post processing. Further investigation is required on the effect of continuous processing on heating uniformity and *Salmonella* inactivation in spices and herbs packaged in a steam vent package. In addition, storage analysis must be performed to assess the efficacy of steam vent in preventing cross-contamination at different climatic conditions.

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Table 6.1. Comparison of quality parameters of untreated and RF treated LMFs.

	Black peppercorns		Dried basil leaves	
	Untreated	**Treated (155 s)**	**Untreated**	**Treated (40 s)**
Water activity	0.66 ± 0.00^{a}	0.57 ± 0.01^{b}	0.62 ± 0.003^{a}	0.54 ± 0.003^{b}
MC (%), wb	12.76 ± 0.18^{a}	10.71 ± 0.51^{b}	10.9 ± 0.18^{a}	9.26 ± 0.13^{b}
L*	40.63 ± 1.53^{a}	40.00 ± 0.36^{a}	53.57 ± 0.78^{a}	52.47 ± 0.87^{a}
a*	0.70 ± 0.40^{a}	0.40 ± 0.61^{a}	2.23 ± 0.15^{a}	2.30 ± 0.10^{a}
b*	2.50 ± 0.36^{a}	2.73 ± 0.12^{a}	13.77 ± 0.59^{a}	14.07 ± 0.91^{a}
ΔE	0	1.62 ± 0.79^{a}	0	1.55 ± 0.32^{a}

Within the same row for each product, values with the same superscripts are not significantly different at $p<0.05$.

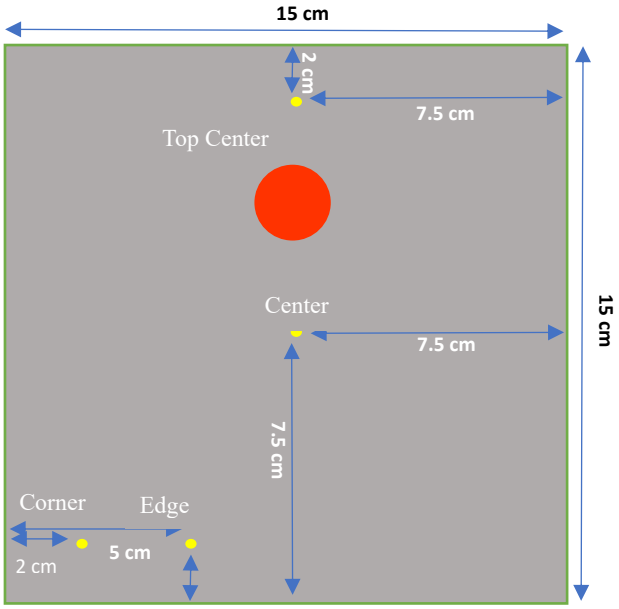

Fig 6.1. Top view (A) schematic and (B) photograph of black peppercorns and C) dried basil leaves packed in a plastic pouch instrumented with fiber optic probes (yellow dots and wires) and a steam vent (red dot).

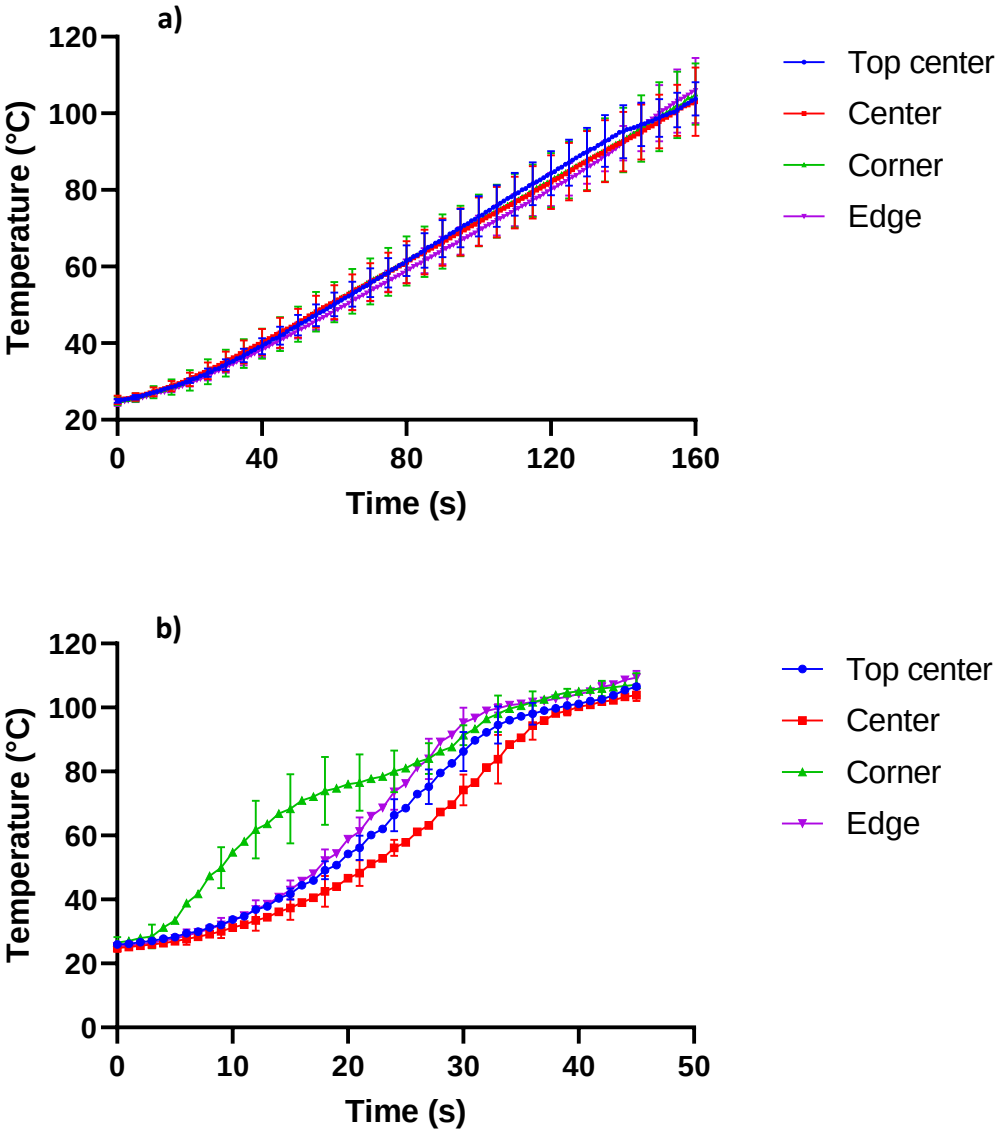

Fig 6.2. Time-temperature profile of a) black peppercorns and b) dried basil leaves packed in steam vent package during RF heating.

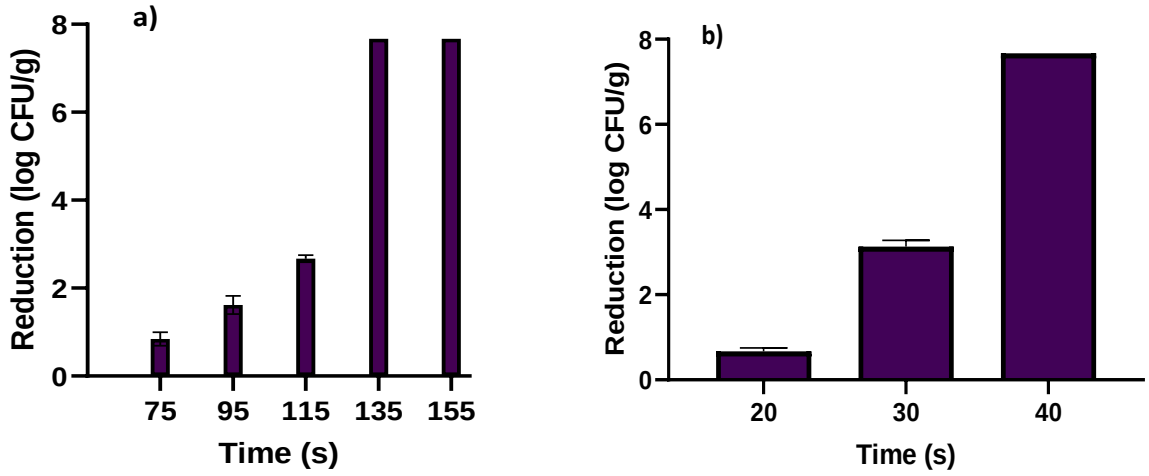

Fig 6.3. Inactivation of *Salmonella* in a) black peppercorns and b) dried basil leaves packed in steam vent package after RF heating at different intervals.

Chapter 7. Gaseous chlorine dioxide for inactivating *Salmonella enterica* and *Enterococcus faecium* NRRL B-2354 on chia seeds

7.1 Abstract

Consumer awareness on health benefits of chia seeds has led to the increased consumption including in its raw form. As chia seeds are preferably soaked in water overnight before consumption, prior contamination with *Salmonella* can enhance the microbial load during soaking as the pathogens are provided with a high nutrition environment. In the present study, the antimicrobial efficacy of chlorine dioxide (ClO_2) gas was evaluated for decontaminating *Salmonella enterica* in chia seeds and to determine if *E. faecium* can be a suitable surrogate for *Salmonella enterica* in validation studies. Chia seed samples were inoculated either with a cocktail of five *S. enterica* serovars or with *E. faecium*. The efficacy of ClO_2 gas was evaluated at different concentration (5.0, 7.5 and 10.0 mg/L) and relative humidity (60, 70 and 80%) conditions exposed for 1-5 h. Reduction of both the bacterial populations was significantly influenced by ClO_2 gas concentration, RH, and exposure time. At a gas concentration of 10 mg/L and 80% RH over a 5 h exposure period, *Salmonella* and *E. faecium* populations were reduced by 3.7 ±0.2 and 3.2 ±0.3 log CFU/g, respectively. Lower log reduction of *E. faecium* at treatment conditions achieving higher reduction (>2.5 log CFU/g) suggested its suitability as a surrogate for *Salmonella* during validation studies in chia seeds. Color, fatty acid composition, and germination capacity of treated chia seeds were not significantly impacted except for peroxide value which increased post gaseous treatment. The ClO_2 byproducts in treated chia seeds were significantly higher than control; however, the chlorite concentration was below the maximum permissible levels in drinking water and therefore can be rendered safe.

Keywords: *Salmonella*, Chia seeds, Surrogate, Quality, Non-thermal inactivation

7.2 Introduction

Chia seeds are abundant with essential amino acids, omega-3/omega-6 fatty acids, flavonoids, dietary fibers, total phenolics and antioxidants (Reyes-Caudillo et al., 2008; Grancieri et al., 2019; Tuncil & Celik, 2019). Due to its high nutritive value, chia seeds are becoming increasingly popular among health-conscious consumers. Germinated chia seeds possess enhanced protein quality, total phenolics, and antioxidant activity. However, during germination, the presence of moisture, nutrients, and optimum temperature makes it ideal for microbial growth. Sprouted chia seed powder was linked with foodborne illness outbreak in 2013 and 2014 due to *Salmonella* contamination (Harvey et al., 2017). Investigation into the outbreak revealed that the chia seeds used to produce powder were not processed before sprouting (Tamber et al., 2016).

Commonly, after harvesting, chia seeds are spread in the open ground to dry under the sun and then the flower heads are crushed to loosen the seeds. During this process, the seeds are prone to environmental contaminants posing safety risks throughout the post-harvest supply chain. Unhygienic practices during storage and packaging can render the chia seeds susceptible to *Salmonella* contamination. In addition, *Salmonella* can survive for a long period in low moisture foods (Keller et al., 2018; Fong and Wang, 2016). Chia seeds are commonly consumed with yogurt or water without prior cooking. Therefore, contaminated seeds are a huge public health concern. Thermal inactivation technologies are not feasible for chia seeds because of its tendency to gel in the presence of moisture. Therefore, the decontamination method for chia seeds must be a non-thermal technique that can maintain seed viability without sprouting or gelling during treatment. Keller et al. (2018) reported that soaking chia seeds in sterile water and drying them at 60°C reduced *Salmonella* population by 5 logs, while no reduction was observed

at lower temperature 25°C which is typically preferred by manufacturers to maintain the quality. The authors suggested that *Salmonella* reduction could only be possible when soaked seeds are still at high a_w and drying would not be effective when the a_w reaches below 0.30. Therefore, a decontamination step for chia seeds prior to sprouting is necessary due to increase in microbial population during sprouting and limited efficacy of drying. Hylton et al. (2019) reported that treatment of chia seeds (100 g) with 4 mL of diluted peracetic acid reduced *Salmonella* by 4 logs. However, the treated chia seeds were required to be dried at 70°C to bring them back to their original moisture content. Chia seeds treated with high-intensity pulsed light for 15 s resulted in a 4 log reduction (Reyes-Jurado et al., 2019). High intensity pulsed UV light may not be suitable for large-scale treatment due to shadowing effect and potential gelling due to condensation at longer treatment times.

Gaseous chlorine dioxide (ClO_2) is an antimicrobial gas with high oxidizing potential offering bactericidal properties. ClO_2 gas has been used to improve the microbial safety of drinking water for the past two decades (Benarde et al., 1965; Jonnalagadda & Nadupalli, 2014). ClO_2 has been approved by United States Environmental Protection Agency (EPA) and Food and Drug Administration (FDA) to be used as an antimicrobial agent in water to process poultry and wash fruits and vegetables under 21 CFR 173.300 (FDA, 2016). One of the main advantages of using ClO_2 in its gaseous form is its high diffusion and penetration ability and less residue formation compared to aqueous ClO_2 (Gordon and Rosenblatt, 2005). The bactericidal property of ClO_2 gas is reported in several high moisture foods such as lettuce, apples, cantaloupe, navel oranges, baby-cut carrots, tomatoes, blueberries strawberries, cabbage, spinach, and raspberries (Alicea et al., 2018, Chai et al., 2020, Mahmoud & Linton, 2008, Park & Kang, 2017, Tan et al., 2021, Trinetta et al., 2011). However, studies pertaining to the efficacy of ClO_2 gas in low

moisture foods are limited (Wason et al., 2021). Rane et al. (2020) observed that ClO_2 treatment at a gas concentration of 0.40 mg/g reduced *Salmonella* by 2.3 log CFU/g in black peppercorns (45% RH) after 4 h and 2.6 log CFU/g in almonds after 6 h of treatment. They also reported that increasing the relative humidity (RH) to 80% during treatment improved the *Salmonella* reduction in black peppercorns. In addition, several studies have concluded that processing parameters such as gas concentration, RH, and exposure time influence the efficiency of ClO_2 gas (Han et al., 2001; Park and Kang, 2015). *Salmonella* inactivation was found to be higher at higher relative humidity and gas concentration during ClO_2 gas treatment in dried basil leaves (Verma et al., 2022); black peppercorns, and cumin seeds (Wei et al., 2021a). So far, no studies have been published on ClO_2 inactivation for decontamination of chia seeds.

According to Food Safety Modernization Act (FDA, 2015), it is mandatory for the food industries to validate the lethality step to ensure safety of processed foods. However, the process validation cannot be performed with *Salmonella,* due to potential risk of contaminating the processing facility. *Enterococcus faecium* NRRL B-2354 has been reported to be a suitable surrogate for *Salmonella* during thermal processing (Wason et al., 2022 a, b; Ozturk et al., 2020; Lau et al., 2021; Chen et al., 2021) and non-thermal processing (Wei et al., 2021a, b; Verma et al., 2022; Rane et al., 2021) of low moisture foods. However, the processing method and the product matrix may influence the suitability of surrogate. Study on surrogate suitability of *E. faecium* for *Salmonella* during ClO_2 gas treatment of chia seeds is limited in the literature.

ClO_2 gas readily undergoes oxidation, and can breakdown into chlorate and chlorite ions, which further get converted to chloride ions (Gomez-Lopez et al. 2009). The analysis of these ion species is crucial to warrant the product safety and the commercial adoption of gaseous ClO_2

technology. While there are standards for residual and byproducts of CLO_2 for drinking water (Federal Register 1998), they are not available for treated food products.

The present study investigates 1) the effects of ClO_2 gas concentration, relative humidity, and exposure time on the inactivation of *Salmonella* in chia seeds, 2) suitability of *E. faecium* as a potential surrogate for *Salmonella* and 3) evaluate the impact of ClO_2 gas treatment on the quality and byproduct formation in chia seeds.

7.3 Materials and Methods

7.3.1 Chia seeds

Chia seed samples (Organic Chia Seeds, BetterBody Foods, Lindon, UT) were procured from online retailers and stored at ambient conditions until further use. The chia seeds (mixture of white and black seeds) belonging to three different production lots were used as replicates. Particle size of chia seeds were analyzed using RO-TAP sieve shaker (Rx-29, W.S. Tyler, OH, US) according to the ASABE standards (ASABE, 2017) as described in Wason et al (2022a). The sieve size ranged from 0.6 mm to 1.7 mm.

7.3.2 Bacterial strains and inoculum preparation

The bacterial strains used were five serotypes of *Salmonella enterica*, namely, *Salmonella enterica* serovars Montevideo 488275, *S.* Mbandaka 698538, *S.* Agona 447967, *S.* Tennessee K4643, and *S.* Reading Moff 180418. The inactivation of *Enterococcus faecium* NRRL B-2354 was compared with *Salmonella* for its potential use as a surrogate. These strains were chosen based on its past association with foodborne illness outbreaks in low moisture foods. The inoculum was prepared according to the method described by Verma et al. (2021).

7.3.3 Inoculation of Sample

Chia seeds (300 g) in a Ziplock bag were inoculated inside a biosafety cabinet by spraying 6 mL of the inoculum either containing *Salmonella* cocktail or *E. faecium* NRRL B- 2354. To prevent clump formation, the spraying of the inoculum was performed 10 cm apart from the samples. Once spraying was done, the samples were shaken gently for 5 min and placed on a sterile aluminum tray and was held in a relative humidity (RH) chamber (Lau & Subbiah, 2020) to equilibrate the sample to its native water activity of 0.53 (Lau et al., 2021). Stability and homogeneity tests were conducted for two weeks to determine the impact of inoculation protocol on the bacterial distribution in the sample, as described in Verma et al. (2021).

7.3.4 Chlorine dioxide gas treatment

After bacterial population had stabilized in chia seeds, samples were treated with gaseous ClO_2 treatments as described by Verma et al. (2022). The ClO_2 gas was generated by Minidox-M system (ClorDisys Solutions, Inc.) which maintains the desired gas concentration and RH in the treatment chamber (0.73 m x 0.44 m x 0.68 m).

Inoculated chia seeds (2 g) packed in heat sealed paper bags were treated with ClO_2 gas at different combinations of relative humidity (60, 70, and 80%) and gas concentration (5.0, 7.5, and 10.0 mg/L) exposed for five time points ranging from 60-300 min. During the treatment, the ClO_2 gas concentration inside the chamber was recorded continuously using in-built gas sensor. The wide range of RH, gas concentration and time points were selected based on preliminary trials to determine the optimal combination resulted in the most effective decontamination of *Salmonella* without causing any significant visual quality changes. The corresponding values for 5.0, 7.5, and 10.0 mg/L are 1810 ppm, 2715 and 3620 ppm as provided by the manufacturer (ClorDisys Solutions, Inc. where 1 mg/L of ClO_2 is equivalent to 362 ppm). Immediately after

the treatment, the chamber was aerated for 5 min using an activated carbon scrubber to completely remove ClO_2 gas before the chamber door was unlocked. All the treatment conditions (3 gas concentrations x 3 RH x 5 exposure times) were replicated thrice using different batches of inoculated chia seeds. Post gaseous treatment, the samples were diluted with neutralizing buffer (containing 160 mg of sodium thiosulfate solution and 42.5 mg of monopotassium phosphate in 1 L deionized water) in the 1:30 ratio (Lau et al., 2021) to neutralize the ClO_2 residues in the sample (Garcia et al., 2022; Park et al., 2018). Microbial enumeration was performed as mentioned in section 2.3.

1.4.1 Microbial inactivation models

The survival data at each RH-gas concentration was fitted to the log linear model:

Log-linear model: $$\log_{10}\left(\frac{N}{N_0}\right) = -\frac{t}{D} \quad (1)$$

where N_0 (CFU/g) and N (CFU/g) are the microbial counts before and after the gas treatment, respectively; D is the decimal reduction time in mins (time required to achieve 1 log bacterial reduction at specific RH and gas concentration); and t is the gas exposure time (min). The goodness of fit for the log-linear model was evaluated by estimating adjusted coefficient of determination (adjusted R^2) and root mean square error (RMSE) values. The primary models were fit for *Salmonella* and *E. faecium* species using GraphPad Prism version 8.0.2 (GraphPad Software, San Diego, CA, USA)

Further, modified Bigelow model was developed to assess the impact of ClO_2 gas concentration and RH on the D-values of *Salmonella* and *E. faecium*.

Modified Bigelow model:

$$D(C, RH) = D_{ref} * 10^{\frac{C_{ref}-C}{z_C}} * 10^{\frac{RH_{ref}-RH}{z_{RH}}} \quad (2)$$

Where D_{ref} is the time taken for 1 log reduction in bacterial count at C_{ref} and RH_{ref}; $C_{ref}$and RH_{ref} (the fitted parameters); z_C and z_{RH} are defined as the increase in gas concentration and relative humidity required to reduce the D-value by 10-fold, respectively. Ordinary Least Square minimization with nlinfit was performed using MATLAB 2018 (MathWorks Inc., MA) to estimate the parameters for the modified Bigelow-type model. The effect of the process parameters such as gas concentration, RH and treatment time on *Salmonella* inactivation in chia seeds was estimated using second order response surface model:

$$\log(N/No) = ß_0 + ß_1.C + ß_2.RH + ß_3.t + ß_{12}.C.RH + ß_{13}.C.t + ß_{23}.RH.t + ß_{11}.C^2 + ß_{22}.RH^2 + ß_{33}.t^2 \quad (3)$$

where C is the gas concentration (mg/L); RH is the relative humidity (%). $ß_0$ is the intercept; $ß_1$, $ß_2$ and $ß_3$ are the linear regression coefficients for gas concentration, RH and time, respectively; $ß_{11}$, $ß_{22}$ and $ß_{33}$ are the quadratic regression coefficients for gas concentration, RH and time, respectively; $ß_{12}$, $ß_{13}$ and $ß_{23}$ are the linear regression coefficients for the interaction effects. The parameters of the model were estimated using response surface method functions in the open-source statistical software, R (Lenth, 2009).

7.3.6 Quality analysis

The quality analysis was performed for the uninoculated chia seed samples treated to conditions with highest ClO_2 gas concentrations and the longest treatment time tested in this study. Immediately after the treatment, the samples were mixed in a Ziplock bag manually and the moisture content and water activity were measured using a halogen moisture analyzer

(Model: HR73, Mettler Toledo, Greifensee, Switzerland), and dew point water activity meter (Model: 4 TE, Meter Group, Pullman, WA) respectively. Color values (L*, a*, b*) were determined using a colorimeter (Konica Minolta, model: BC-10, Osaka, Japan) at five random locations on the samples spread on a Petri dish, as described in Wason et al. (2022b).

a. Fatty acid composition and peroxide value

Lipid Extraction: The lipid extraction from control and treated chia seed samples were performed by following Folch et al. (1957). Five grams of ground chia seeds were extracted in 150 mL of methanol and chloroform solution (1:2) for 2 hours. The non-lipid substances from this extract was removed by adding 50 mL water. The solids were filtered out and the solvent was passed through sodium sulfate anhydrous. Solvent was then dried in a rotovap to obtain the lipid extract.

Fatty acid composition analysis: The extract was then analyzed for fatty acids according to the method described in AOAC method 996.06 (AOAC, 2000). A portion of ground sample containing about 100 to 200 mg of lipids was mixed with 2 mL ethanol, 100 mg of pyrogallol (antioxidant), and an internal standard solution of glycerol triundecanoate in chloroform. To this sample, 10 mL of 8.3 M hydrochloric acid was added and digested at 75 °C for 40 minutes. Lipids were extracted in a solution containing 25 mL diethyl ether and 25 mL hexane. The solvents were dried off and the lipids were derivatized with 12% boron trifloride in methanol for 30 minutes at 95°C. The samples were mixed in a solution containing 5 mL water, 1 g sodium sulfate anhydrous and 1 mL hexane. The organic phase was collected and injected into an Agilent 7820A GC system with a flame ionization detector fitted with Supelco SP-2560 column (100 m x 0.25 mm x 0.2 mm). The temperature for the injection port was set at 225°C and the detector temperature was maintained at 250°C with the oven temperature set to 100°C held for 4 minutes,

rising it to 240°C at a rate of 3°C per minute with a holding time of 20 minutes at 240°C. Sample peaks were identified and compared with an external standard.

Peroxide value: The peroxide analysis was carried out according to the method described by Li et al. (2001). The lipid sample (0.5 g) was transferred in triplicate to test tubes and dissolved in 3 mL of chloroform, acetic acid (2:3) followed by 50 µL of saturated potassium iodide in water. For the separation of phases, 3 mL of water was mixed with the samples followed by centrifugation (GS-6R Beckman) at 2800 RPM for 10 minutes. An aliquot of 1 mL from upper phase was added to a new test tube; to which 100 µL of starch indicator solution was added to record the absorbance at 563 nm using a Cole Parmer S2100+ Spectrophotometer, Vernon Hills, IL. A standard curve was prepared from elemental iodine dissolved in water and combined with 100 µL of the starch indicator.

b. Total Phenols

The total phenolic acid content in treated chia seeds was analyzed following Folin-Ciocalteu method (Singleton & Rossi, 1965). The treated sample (1 g) was extracted twice with 5 mL of 1.2 N hydrochloric acid in water: methanol (1:1) for 2 h. Organic solvent phases were combined and purified by centrifugation and were passed through a 0.2 µM filter. 100 µL of the sample was diluted in 4.5 mL water and 100 µL of 2 N Folin-Ciocalteu's reagent (Sigma-Aldrich) was added to the sample solution. The pH of the solution was adjusted by adding 300 µL of 2% sodium carbonate. The mixture was stored at room temperature under dark conditions for 2 h to record the absorbance at 760 nm using a Cole Parmer S2100+ Spectrophotometer with ethanol (0.2 mL) as blank. Sample absorbances were compared to an external standard curve of gallic acid. The estimation was performed thrice, and the results were expressed as gallic acid equivalent (GAE).

c. Germination capacity

The germination capacity of control and treated chia seeds was determined by using Geneve et al. (2017) method on the same time. Fifty chia seeds were spread on a damp blotting paper in a Petri dish to allow germination under ambient temperature and RH. The Petri dish was exposed to sunlight for 8 h and dark for 16 h each day. The germinated seeds were counted every day for a week until no change in germination was observed. Germination capacity was calculated as given below:

$$\text{Germination capacity} = \frac{\text{Number of germinated seeds}}{\text{Total number of seeds}} \text{ x } 100 \quad (4)$$

d. Scanning electron microscopy (SEM) analysis

SEM analysis was performed as per Wang et al. (2020) method with slight modifications. The inoculated chia seeds were mounted on aluminum specimen stubs and were placed in 12 well plates. Ethanol (75%) was poured slowly from the sides without disturbing the cell surface allowing the cells to fix at room temperature. Chia seeds were fixed for 15 min at room temperature and the stubs were dried in a 42°C vacuum oven overnight. The chia seeds were then sputter-coated with a thin layer of chromium using a sputter coater (Desk V, Denton Vacuum, Moorestown, NJ). A field-emission scanning electron microscope (S-4700, Hitachi, Tokyo, Japan) was used for imaging at 5 kV at magnifications ranging from 5.0 to 10.0 K X.

e. Byproduct analysis

Chia seeds (50 mg) were hydrated with distilled deionized water (DDW) in the ratio of 1:40 in a microcentrifuge tube for 2 h. The tube was centrifuged for 50 min at 8300 rpm to obtain the supernatant. The extract was then passed through a 1cc C18 cartridge preconditioned with methanol and double distilled water and analyzed immediately. An ion chromatography

system (IC5000) was used to determine the byproducts: chlorite, chlorate and chloride following the ASTM method (ASTM, 1988). The standards for chloride, chlorite and chlorate in DDW was prepared in six different concentrations (0.20, 0.50, 1.00, 3.00, 5.00 and 10.00 mg/L). A solution made from mixing 8 mL of 500 mM Na_2CO_3 and 6 mL of 500 mM $NaHCO_3$ in 2 L DDW was used to elute IC-pack column (Dionex, Sunnyvale, California). The concentration of each byproduct was estimated by comparing with standard curve. The detection limits of chlorite, chlorate, and chloride were 0.5, 0.3 and 2 µg/g, respectively. All the tests were performed in triplicates and the results were expressed as reported as mg/g of chia seed samples. Untreated chia seeds served as controls for comparing the byproduct in ClO_2 treated samples.

7.4 Results and discussion

The native water activity and geometric particle diameter of chia seeds were 0.53 ± 0.03 and 1.09 ± 0.02 mm, respectively. The chia seeds were tested for the presence of background microbiota by estimating the total aerobic bacteria. The total aerobic bacterial count was below 2 log CFU/g, which was much lower than inoculated *Salmonella* population. Therefore, it was regarded not to influence the antimicrobial efficacy of ClO_2 against *Salmonella* in chia seeds.

7.4.1 Stability and homogeneity tests

The water activity of chia seeds following inoculation was 0.72 ± 0.03 and was equilibrated to 0.53 thereafter. *Salmonella* needs > 0.92 a_w to grow in the product. When *Salmonella* is exposed to a low moisture environment, it undergoes stress and becomes sensitive to the inactivation treatments as evident from results reported by Jeong & Kang (2014) and Wei et al. (2019). Jeong & Kang (2014) reported higher inactivation rate in ground black pepper when samples were treated immediately after inoculation as compared to the results reported by Wei et

al. (2019) who provided equilibration time of 5 days after inoculation prior to thermal treatment. As shown in Fig. 1., there was 0.5 log CFU/g initial reduction in *Salmonella* and *E. faecium* population during the first day. After day 1, *Salmonella* (7.67 ± 0.03 log CFU/g) and *E. faecium* (7.76 ± 0.17 log CFU/g) continued to be stable for 14 more days. Accordingly, further experiments were conducted after equilibrating the inoculated chia seeds for 3 days. Standard deviation below 0.3 log CFU/g between the sub samples from the same batch indicated that the bacterial inoculation was homogenous in the sample.

7.4.2 Antimicrobial efficacy of gaseous ClO_2

The treatment involves a pre-condition phase, a conditioning phase, a charging phase, an exposure phase, and an aeration phase. During pre-conditioning phase, a target relative humidity was achieved by passing humid air into the chamber. The conditioning phase involved holding the target RH for a desired amount of time. ClO_2 gas was generated in the charging phase by allowing the chlorine gas to pass through sodium chlorite cartridges to a set level before being introduced into the chamber. Gas concentration and RH were held constant all the way through the exposure phase as shown in Fig. 2.

The log reduction of *Salmonella* and *E. faecium* in chia seeds after gaseous treatment at three different gas concentrations of 5.0, 7.5 and 10 mg/L and RH of 60, 70 and 80 % are shown in Fig. 3. A rise in gas concentration from 5 to 10 mg/L at 80% RH exposed for 300 min increased *Salmonella* reduction from 2.6 to 3.7 log CFU/g. Similarly, *Salmonella* inactivation enhanced as the RH increased during the treatment. After 300 min of exposure to 5 mg/L gas concentration, the log reduction of *Salmonella* in chia seeds were 0.9, 1.4 and 1.9 at 60, 70 and 80% RH respectively. Similarly, increase in RH enhanced *Salmonella* inactivation were reported in black peppercorns and cumin seeds (Wei et al., 2021a)

ClO_2 has been extensively researched for microbial decontamination in many fresh produce. For example, treatment of apples to 4 mg/L ClO_2 gas concentration exposed for 10 min reduced *Listeria monocytogenes* population by 3 logs (Du et al., 2002). Increasing the exposure to 30 mins gave 5 log reduction of *E. coli* O157:H7 and *L. monocytogenes* on strawberries (Han et al., 2004). Mahmoud & Linton (2008) reported more than 5 log reduction of *Salmonella* on lettuce following ClO_2 treatment at 5 mg/L for 19 mins. As expected, the treatment time required for desired log reduction in case of high moisture foods is much lower than low moisture foods such as chia seeds reported in this study, indicating the role of moisture content and water activity in ClO_2 inactivation.

Studies pertaining to ClO_2 decontamination of low moisture foods are limited. Earlier researchers have shown that the efficacy of ClO_2 gas on low moisture foods is dependent on various factors including commodity, gas concentration, relative humidity and exposure times (Verma et al., 2022; Wei et al., 2021a; Lim et al., 2021; Rane et al., 2020; Wang et al., 2019). Treating almonds with ClO_2 for 1 h at 50°C with peak gas concentration between 354 – 510 ppm reduced *Salmonella* Enteritidis by more than 5 logs (Lim et al., 2021). However, increasing the exposure time to 8 h reduced the population to below the detection level (1.7 log CFU/3 almonds). Golden et al. (2019) observed 2.2 log CFU/g *Salmonella* reduction on black peppercorns following ClO_2 treatment at 0.4 mg /g exposed for 24 h. On the other hand, Rane et al. (2020) observed the exposure of black peppercorns to ClO_2 at 0.4 mg ClO_2/g concentration achieved 3.7 log CFU/g and 2.3 log CFU/g reduction of *Salmonella* within 240 min at 80% and 45% RH, respectively. Wang et al. (2019) reported an increase in *Salmonella* reduction on almonds from 1.0 to 1.5 log CFU/g with increasing gas concentration from 0.26 to 4.64 mg/L exposed for 4 h. In the above-mentioned studies, the ClO_2 gas was generated using mixture of

dry precursors and the gas concentration could not be held constant during the treatment. At peak ClO_2 gas concentration, the product color and other physiochemical composition of the almonds may be affected. Thus, treatment at controlled process parameters could provide reliable data to the food industry for the application of ClO_2 gas to improve the safety of chia seeds.

The inactivation kinetics provides beneficial data to the food industry for estimating processing conditions for pasteurization of a specific food product. The log reduction data was fit to log-linear model and D-values were calculated as shown in Table 1. The high adjusted R^2 values indicate the suitability of using log linear model to fit this data except for D-values of *E. faecium* at 5 mg/L which had adj R^2 values between 0.75-0.79. As the RH (%) and gas concentration was increased during the ClO_2 treatment, D-values for both bacteria reduced. For instance, D-value of *Salmonella* reduced from 225 to 139 min when chia seeds were treated at 5 mg/L ClO_2 gas concentration and the RH was increased from 60 to 80%. Further, increase in gas concentration from 5 to 10 mg/L at 80% RH reduced the D-value of *Salmonella* from 139 to 82 min. Similarly, Verma et al. (2022) reported a decrease in D-value of *Salmonella* in dried basil leaves from 68.5 to 46.5 min with increase in gas concentration from 5 to 15 mg/L at 70% RH. However, the range of D-value at the same gas concentration achieved for chia seeds in the present study was very high compared to dried basil leaves, which had higher antimicrobials than chia seeds. The D-value of chia seeds during ClO_2 treatment at 70% RH and 5 mg/L was 173 min which is more than double of 68.5 min reported for dried basil leaves. The corresponding D-value reported for black peppercorns and cumin seeds were 144 min and 149 min (Wei et al., 2021a). One of the probable reasons for achieving decreased antimicrobial activity in chia seeds is owing to the action of antioxidants such as tocopherols in neutralizing ClO_2 and/or as a result of ClO_2 reaction with unsaturated lipids (Ghanbari et al., 1982). Vandekinderen et al. (2009)

suggested that the antimicrobial efficiency of ClO_2 depends on the presence of proteins, starch, fats and the presence of organic matter (Fukuyama et al., 1986). Vandekinderen et al. (2009) studied the influence of varying composition of agar inoculated with *Leuconostoc mesenteroides* on the microbial inactivation efficacy of ClO_2. The authors concluded that ClO_2 is not effective for the decontamination of foods rich in proteins and fats and is effective for carbohydrate rich foods (Vandekinderen et al., 2009).

7.4.3 Effects of gas concentration and RH

Modified Bigelow model and response surface models were developed to determine the effect of process parameters on the inactivation kinetics of *Salmonella* by ClO_2 gas. Only significant terms ($p<0.05$) were included in the developed response surface model for *Salmonella* and *E. faecium* as shown below:

Salmonella: $\log(N/No) = 5.3309 - 0.29934*C - 0.12825*RH - 0.011258*t + 0.0038753*C*RH + 0.00052270*C*t + 0.00024055*RH*t + 0.00078531*RH^2 - 0.0000080202*t^2$

Adjusted R^2=0.93; RMSE=0.23 log CFU/g; AIC_c=0.92

E. faecium: $\log(N/No) = 8.8846 - 0.67730*C - 0.19681*RH - 0.0029254*t + 0.00043644*C*t + 0.00016138*RH*t + 0.043203*C^2 + 0.0014669*RH^2 - 0.000019169*t^2$

Adjusted R^2=0.92; RMSE=0.20 log CFU/g; AIC_c=-33.01

The gas concentration, RH, and exposure period had a significant ($P<0.05$) effect on the log reduction of *Salmonella* and *E. faecium*. Inactivation efficacy increased when gas concentration, RH, and exposure time were increased. Several studies have reported an increase

in the antimicrobial efficacy of ClO_2 at higher RH. For instance, Park and Kang (2015) indicated that ClO_2 treatment of spinach leaves at 50 ppm by volume for 20 min at 70% RH achieved 2.5 log reduction of *Salmonella* Typhimurium which increased to more than 5.6 log reduction at higher RH of 90%. Similarly, treatment of almonds (MC= 4%) for 3 h reduced *Salmonella* by 1.2 log CFU/g, which increased to 1.8 log CFU/g at a higher moisture content of almonds (MC= 7%) (Rane et al., 2021). Shirasaki et al. (2016) reported that ClO_2 treatment (1 mL/m^3 sodium chlorite solution) completely inhibited *Staphylococcus aureus* and *Escherichia coli* on porous beads at 80% humidity, while only 1-2 log reduction could be achieved at ambient RH of 30-40%.

The modified Bigelow model developed for the D-values of *Salmonella* and *E. faecium* are shown below:

Salmonella: $D(C, RH) = 180.40 * 10^{\frac{7.5-C}{34.97}} * 10^{\frac{70-RH}{96.49}}$

Adjusted R^2= 0.79; RMSE= 23.77 min; AIC_c=177.74

E. faecium: $D(\mathrm{C}, \mathrm{RH}) = 256.16 * 10^{\frac{7.5-C}{22.62}} * 10^{\frac{70-RH}{92.95}}$

Adjusted R^2=0.68; RMSE=32.33 min; AIC_c=194.34

The performance of modified Bigelow models was compared with response surface models in estimating the log reduction at different treatment conditions. The developed modified Bigelow model was used to estimate D-values at different gas concentration and relative humidity combinations. The estimated D-values were then used to calculate log reduction at different gas exposure time. Based on RMSE value, response surface model (RMSE = 0.25 log

CFU/g) predicted the log reduction of *Salmonella* and *E. faecium* better as compared to modified Bigelow model (RMSE = 0.57 log CFU/g) at all tested conditions. Because RSM model performed significantly better than Modified Bigelow model and are recommend being used by the industry, only RSM results are shown in Fig. 3. for better visualization. Modified Bigelow model was reported to predict the thermal inactivation of *Salmonella* better than the response surface model in low moisture foods such as wheat flour (Smith et al., 2016), dried basil leaves (Verma et al., 2021), and ground black pepper (Wason et al., 2022a). On the other hand, response surface model has been widely recommended for evaluating the effect of process parameters on bacterial inactivation during both thermal inactivation (Bianchini et al., 2012; Verma et al., 2018) and non-thermal inactivation (Wei et al., 2021b; Chen et al., 2021; Verma et al., 2022).

Response surface model developed in this study could be beneficial for chia seed industry to identify treatment conditions for ClO_2 inactivation. Therefore, contour surface plots were developed based on response surface model to predict *Salmonella* inactivation as shown in Fig. 4. The contour lines were linear at shorter treatments and became curvilinear as treatment time increased, thus indicating a significant interaction effect between gas concentration and exposure time. A vertical line (high slope) indicates that gas concentration is not effective at all. At lower RH and lower treatment time, the slope was very high indicating that the effect of concentration is less. However, at higher concentration, the slope decreased indicating that the gas concentration is effective at higher exposure time. The decrease in slope becomes more predominant at higher RH and higher exposure time showing that the concentration is much more effective at those conditions. The contour plots provide a good visualization tool to estimate the process parameters required to achieve target log reduction. However, the models should not be employed for predicting inactivation outside the range of tested treatment

conditions. FDA recommends a 5-log reduction of *Salmonella* in some low moisture foods such as peanuts and pistachios (FDA, 2009 a, b). Various gas concentration-RH combinations tested in the present study did not result in the desired 5 log reduction of *Salmonella* in chia seeds. Treatment of inoculated chia seeds with 10 mg/L ClO_2 for 300 min at RH of 80% was the most desirable among the tested conditions achieving 3.7 log reduction of *Salmonella.* Few studies explored the efficacy of mild heating followed by ClO_2 in improving the *Salmonella* inactivation (Wang et al., 2019; Golden et al., 2019). Rane et al. (2020) reported that heating almonds at 65°C post ClO_2 treatment increased the *Salmonella* inactivation by about 1.8 log CFU/g. Therefore, further studies must be conducted to ensure the suitability of mild heating following ClO_2 treatment in enhancing the inactivation without compromising the quality of food product.

7.4.4 *E. faecium* as a suitable surrogate

With the implementation of the Food Safety Modernization Act in 2011, it is essential for the food industry to validate their processing intervention to control the identified hazard in food. However, there is a potential risk of contaminating the food facility during validation studies with pathogenic *Salmonella*. For thermal processing of low moisture foods such as the chicken meat powder (Rachon et al., 2016), oat flour (Verma et al., 2018), savory seasoning (Rachon et al., 2016) and wheat flour (Smith et al., 2016), *E. faecium* NRRL B-2354 is reported to be an appropriate surrogate for *Salmonella* for the thermal processing of many low moisture foods such as chicken meat powder, savory seasoning (Rachon et al., 2016); wheat flour (Smith et al., 2016); Oat flour (Verma et al., 2018). However, the studies evaluating the surrogate suitability of *E. faecium* for *Salmonella* in the validation of gaseous technologies are limited. Chen et al. (2021) reported that *E. faecium* showed higher resistance to ethylene oxide gas inactivation as compared to *Salmonella* in cumin seeds at all tested conditions. Similarly, in the case of EtO

treated black peppercorns, *E. faecium* showed lower inactivation than *Salmonella* (Wei et al., 2021b). On the contrary, Newkirk (2016) observed that *E. faecium* reduction was not consistently lower than *Salmonella* in EtO treated cumin seeds questioning the suitability of *E. faecium* as a surrogate for *Salmonella* in cumin seeds. As shown in Fig. 5., it is evident that *E. faecium* was more tolerant to ClO_2 compared with *Salmonella* with lower log reductions observed during the study in the treatment conditions that achieved > 2.5 log reduction of *Salmonella*. Because the industry would be interested in processing samples at conditions achieving higher inactivation, *E. faecium* could still be used for process validation in chia seeds. Similarly, Wei et al. (2021a) also concluded *E. faecium* to have lower log reduction compared to *Salmonella* in ClO_2 inactivation studies with cumin seeds.

7.4.5 Quality analysis

a. Water activity and moisture content

The quality of chia seeds treated at 10 mg/L gas concentration for 300 min with various relative humidity conditions is provided in Table 2. ClO_2 treatment of chia seeds at different relative humidity showed a significant increase ($P<0.05$) in a_w at 70 % RH (0.58 ± 0.01) and 80 % RH (0.60 ± 0.01) compared to control/untreated sample (0.53 ± 0.01). Similarly, the moisture content values for the ClO_2 treated chia seeds significantly increased after gas treatment at 70% RH and 80% RH. These results concur with the findings by Wang et al. (2019) who reported an increase in the weight of almonds by 2-3% after ClO_2 treatment at high humidity. Although the moisture content increased during high RH treatments, it was not high enough to cause gelling or affect the visual appearance of chia seeds. Therefore, ClO_2 treatments at high RH conditions could be applied to improve the microbial safety of chia seeds without negatively impacting their texture.

b. Color value

After exposure to ClO_2 gas at 10 mg/L for 300 mins, the L*- lightness and b*- yellow-blue color values increased significantly from 49.43 to 52.27 and from 4.97 to 7.60, respectively. However, no significant change in L* and b* values were observed between the different RH conditions. In addition, the a* values representing red-green color did not vary between treated and control samples. This was more obvious as the color of chia seeds is mostly attributed to L* values representing the light-colored seeds. Rane et al. (2020) observed no visual change in color between control and ClO_2 treated almond samples. However, Wang et al. (2019) observed an increase in L* values in almonds treated with varying ClO_2 concentrations at 45, 50, 55 and 60 ° C exposed for 4 h. The estimated ΔE values for the chia seeds treated to ClO_2 gas at 10 mg/L concentration for 5 h at three different relative humidities (60, 70, and 80 % RH) ranged between 3.56 – 4.34 indicating the color difference in treated chia seeds could be perceived by the human eye (Mokrzycki & Tatol, 2011). Further analysis will be required to assess the consumer acceptability of ClO_2 treated chia seeds.

c. Fatty acid composition and peroxide value

ClO_2 is known to be a powerful oxidizer and it can affect the fatty acid, peroxide value, and total phenolic content of treated foods. Hence, quality analysis was performed for chia seeds treated with ClO_2 gas at the highest tested concentration (10 mg/L) for the maximum exposure period (5 h) tested in this study. It was observed that the total fat, omega-3, and omega-6 fatty acid composition in ClO_2 treated chia seeds did not significantly differ as compared to the control samples (Table 3.). Lau et al. (2021) also reported no significant difference between control samples and samples thermally treated at 80°C, 85°C, and 90°C to achieve similar *Salmonella* inactivation (4 log reduction) as reported in this study.

The peroxide value corresponds to the measure of lipid products as a result of primary oxidation. We observed a notable increase (more than 6 times) in the peroxide values i.e. from 8.34 to 58.2 mM Equiv I_2 per kg in the treated samples as against the control chia seeds. This result correlated with the high oxidation potential of ClO_2 gas on organic matter (Ganiev et al. 2005). The peroxide value of the oil-water emulsion significantly increased after ClO_2 treatment (Vandekinderen et al., 2009); since fat gives reactive sites to inhibit inactivation effect of ClO_2 gas. Treatment of chia seeds with thermal treatment at 85°C also showed significantly higher peroxide values in comparison to controls (Lau et al., 2021). Chia seeds contain high levels of unsaturated fatty acids, which makes them susceptible to oxidation. After ClO_2 treatment, the peroxide value of chia seeds is significantly higher, it is an indication that there has been a significant level of lipid peroxidation. Chia seeds contain high levels of unsaturated fatty acids, which makes them susceptible to oxidation. Treatment at higher relative humidity or subsequent post-treatment with mild heating could be investigated to reduce the exposure time of ClO_2 which could potentially minimize the oxidation. In addition, shelf-life studies and sensory analysis will be required to assess the consumer acceptability of ClO_2 treated chia seeds.

d. Total phenols

The phenolic compounds are known to possess antioxidant properties which can inhibit the oxidative action of ClO_2 gas. However, no noticeable change was evident in the concentration of phenolic acids between the control and treated samples irrespective of the RH condition tested. The total phenols for the control and treated samples ranged between 0.34 and 0.39 mg GAE/g.

e. Germination ability

Chia seeds are consumed as sprouts or sprouted seeds are used for the preparation of protein rich powders. Therefore, it is essential to study the effect of the ClO_2 gas on the germination of chia seeds. ClO_2 treatment of chia seeds at 10 mg/L for 5 h showed no effect on the germination capacity of chia seeds. Table 2. shows that about 66 - 76% of chia seeds germinated after ClO_2 treatment, while 70% of control seeds germinated. One of the batches consistently showed lower germination compared to other batches in all control and treated samples. When that batch was removed, the average percent germination ranged from 83 to 87%. Similarly, Trinetta et al. (2011) reported that treatment of tomato seeds to 10 mg/L ClO_2 for 3 min at 75% RH was very effective on *Salmonella* reducing the population by 5.3 log CFU/g and did not affect the germination of treated seeds. However, the treatment time reported by Trinetta et al. (2011) was reasonably low compared to the present study. In other study, combined thermal treatment with ClO_2 at 3.5 mg/L concentration exposed for 4 h gave 3 log CFU/g reduction of *Salmonella* Montevideo without affecting the germination of treated mung bean seeds (Annous and Burke, 2015). Han et al. (2018) reported that 12 h treatment of ClO_2 at 50 or 100 ppm did not affect the seed viability in rice but affected the seed viability of wheat. These studies indicate that the ClO_2 treatment could be applied for bacterial reduction in chia seeds without affecting the seed viability.

f. SEM analysis of *Salmonella* in chia seeds

From Fig. 6, it is evident that *Salmonella* cells were able to attach on the surface of chia seeds and form biofilms. The biofilm formation is a serious concern in food processing industries as the biofilm produced by *Salmonella* is more resistant to the inactivation strategies. In control samples, the biofilm dominated in the SEM images and few *Salmonella* cells could be observed

(highlighted by small circles in Fig. 6A). In ClO_2 treated samples (Fig. 6B), the biofilm is less predominant, cells can be readily seen in the SEM image (Fig. 6B) and highlighted areas (medium circles) indicates some damage to the cells. At higher magnification (Fig. 6C), some cell damage can be visualized (highlighted by large circles). Wei et al. (2021a) reported loss of membrane integrity and damaged cell surface in ClO_2 treated black peppercorn surface. The higher damage in black peppercorns as well as higher log reduction reported in their study compared to chia seeds could be due to the presence of antimicrobial compounds and lesser fat content compared to chia seeds.

g. ClO_2 byproduct analysis

The byproduct analysis of chia seeds treated with ClO_2 at 10 mg/L for 300 min exposure is given in Table 4. Because ClO_2 gas readily undergoes oxidation, it can break down into chlorate and chlorite ions, which further gets converted to chloride ions (Gomez-Lopez et al. 2009), and the byproduct analysis was performed for these compounds. Interestingly, it was observed that the untreated chia seeds had mild traces of chloride (0.027 mg/g) and chlorite (0.003 mg/g) residues. This could be due to the probable use of chlorinated water during farming of chia plants. Mastrocicco et al. (2017) reported that the chloride level ranged from 85 to 3462 mg/L in groundwater tested multiple times between November 2010 to June 2011. Garcia-Villanova et al. (2020) reported the presence of chlorite (0.005 mg/L) and chlorate (0.119 mg/L) in drinking water due to the use of hypochlorite as a disinfectant. With respect to the chlorate residues, there was a significant increase in values between the untreated (below the detection limit) and ClO_2 treated chia seeds (0.058 - 0.099 mg/g) irrespective of the RH condition tested. Similarly, for chloride residues, the obtained values were significantly different between the control (0.027 mg/g) and ClO_2 treated samples (0.394 - 0.594 mg/g). Because the antimicrobial activity of ClO_2

is attributed to its oxidation potential, an increase in chlorite, chlorate and chloride ions in treated samples can be correlated with the increase in microbial log reduction observed in chia seeds at higher relative humidity. For instance, as the RH increased from 60% to 80% during ClO_2 treatment, the chlorate and chloride residues increased by almost 2 and 1.5 times, respectively. On the other hand, the chlorite residues between the control and ClO_2 treated chia seeds were not significantly different due to high experimental variability between the replicates. The chlorite concentrations at higher RH (70% and 80%) were lower than that at 60% RH. Because of the significant increase in chloride concentration in the treated sample, the range of chromatogram significantly increased. The chlorite peaks were close to the detection limit and therefore, were difficult to analyze. This high experimental error could be due to complexity of chia seeds matrix which gels at high moisture content and centrifugation might not have extracted all compounds consistently.

Studies by Trinetta et al. (2011) on alfalfa sprouts treated with 5.0 mg/L ClO_2 gas exposed for 20 min at 90% to 95% RH showed significantly higher levels of chloride (5.31 mg/g of fruit), chlorate (18.01 mg/g of fruit) and chlorite (1.3 mg/g fruit) residues. Comparing the results between our studies with Trinetta et al. (2011), the byproduct residues recorded in the present study are comparatively lower. However, the variation in the product matrix such as the moisture content, fat composition etc. could have attributed to the difference, indicating the importance of evaluating different product matrix in developing gaseous ClO_2 treatments. Furthermore, the obtained chlorite ion value for ClO_2 treated chia seeds are much lower than the maximum contaminant level (MCL) of 1.0 mg/L in public drinking water recommended by the National Public Drinking Water Regulations (NPDWR): disinfectants and disinfection

byproducts (Federal Register, 1998). Therefore, ClO_2 treatment of chia seeds at the tested conditions can be considered safe, even though there are no standards currently available.

7.5 Conclusions

The process parameters such as relative humidity and gas concentration impacted the antimicrobial efficacy of ClO_2 gas against *Salmonella* in chia seeds. A maximum log reduction of 3.7 CFU/g of *Salmonella* was achieved at the most severe treatment condition tested i.e., 10 mg/L concentration and 80% RH for 300 min. Among the two models, the response surface model better predicted the microbial reduction in chia seeds and could be used by the industry to identify the process parameters to achieve desired log reduction of *Salmonella*. Lower log reduction of *E. faecium* indicated its suitability as a surrogate for *Salmonella* during in-plant validation at treatment conditions which achieved higher log reductions (>2.5 log CFU/g) in chia seeds. Because the industry would be interested in processing samples at conditions achieving higher inactivation, *E. faecium* could still be used for process validation in chia seeds. Due to the gelling property of chia seeds at higher humidity, ClO_2 gas is an effective waterless technology for *Salmonella* decontamination in chia seeds. In addition to antimicrobial and oxidizing potential, high diffusion, and penetration ability of ClO_2 gas offers potential for bulk treatment of chia seeds. The color, fatty acid composition, and germination capacity of treated chia seeds was not significantly impacted upon ClO_2 treatment, except for peroxide value which increased post treatment. Chia seeds contain high levels of unsaturated fatty acids, which makes them susceptible to oxidation. Further combination treatments with mild heating or high relative humidity could be studied to reduce the exposure time of ClO_2 which could potentially minimize the impact on peroxide value. In addition, shelf-life studies and sensory analysis will be required to assess the consumer acceptability of ClO_2 treated chia seeds. The ClO_2 byproducts (chlorite)

in treated chia seeds were below the maximum permissible levels in drinking water and therefore it can be considered as safe even though no standards are available for treated foods. The outcome of the present study suggests that though > 3 log reduction of *Salmonella* was observed in chia seeds at longer exposure times; target 5 log reduction could not be achieved possibly due to protective effect of higher fat content and lower a_w on microbial inactivation. Hence, further research is warranted to evaluate the effect of mild heating and storage on the improvement in antimicrobial efficacy of ClO_2.

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Table 7.1. Inactivation kinetics of inoculated *Salmonella* in chia seeds during ClO_2 gas treatment at different gas concentrations and RH.

Bacteria	Gas concentration (mg/L)	RH (%)	D-value (min) (CI)	Adjusted R^2	RMSE (log CFU/g)
Salmonella	5.0	60	225.1 (201.9 to 254.3)	0.96	0.10
		70	173.3 (148.0 to 209.1)	0.90	0.20
		80	138.8 (114.4 to 176.4)	0.85	0.31
	7.5	60	205.1 (171.9 to 254.1)	0.88	0.19
		70	155.7 (129.4 to 195.2)	0.86	0.27
		80	101.3 (89.6 to 116.4)	0.94	0.26
	10.0	60	184.2 (154.4 to 228.3)	0.88	0.22
		70	129.3 (111.1 to 154.9)	0.91	0.26
		80	82.6 (75.3 to 91.6)	0.97	0.24
E. faecium	5.0	60	263.1 (203.8 to 371.1)	0.75	0.23
		70	217.5 (171.1 to 298.2)	0.78	0.26
		80	142.0 (112.5 to 192.5)	0.79	0.38
	7.5	60	238.0 (198.8 to 296.6)	0.87	0.17
		70	204.8 (165.9 to 267.8)	0.83	0.24
		80	153.1 (126.5 to 193.9)	0.86	0.28
	10.0	60	193.2 (158.0 to 248.6)	0.84	0.24
		70	132.9 (116.6 to 154.4)	0.93	0.22
		80	107.7 (90.3 to 133.5)	0.88	0.37

Table 7.2. Color, water activity, moisture content and percent germination of chia seeds treated with ClO_2 gas at 10 mg/L concentration for 5 h at different relative humidity.

	Color value			ΔE	Water activity	Moisture content (%)	Germination (%)
	L*	a*	b*				
Untreated	49.43 ± 1.01^{b}	1.23 ± 1.10^{a}	4.97 ± 0.51^{b}		0.53 ± 0.01^{c}	7.09 ± 0.17^{c}	70.0 ± 29.9^{a}
60% RH	51.87 ± 0.93^{a}	2.43 ± 0.31^{a}	7.00 ± 0.30^{a}	3.56 ± 0.49^{a}	0.54 ± 0.01^{c}	7.39 ± 0.36^{bc}	76.0 ± 24.0^{a}
70% RH	52.3 ± 0.87^{a}	2.60 ± 0.21^{a}	7.47 ± 0.21^{a}	4.34 ± 1.44^{a}	0.58 ± 0.01^{b}	7.82 ± 0.03^{ab}	66.0 ± 34.9^{a}
80% RH	52.27 ± 1.16^{a}	2.40 ± 0.10^{a}	7.60 ± 0.72^{a}	4.15 ± 2.16^{a}	0.60 ± 0.01^{a}	8.29 ± 0.18^{a}	68.7 ± 26.4^{a}

Within a column, values with differing superscript alphabets are significantly different ($p < 0.05$).

Table 7.3. Fatty acids, peroxide value and total phenolics of chia seeds treated with ClO2 gas at 10 mg/L concentration for 5 h at different relative humidity.

	Total Fat (g per 100 g)	Omega-3 (g per 100 g)	Omega-6 (g per 100 g)	Peroxide Value (mM Equiv I_2 per kg)	Total phenolics (mg GAE/g)
Untreated	28.20 ± 0.52[a]	17.16 ± 0.84[a]	4.67 ± 0.19[a]	8.34 ± 10.45[b]	0.39 ± 0.14[a]
60% RH	28.00 ± 0.60[a]	17.84 ± 1.43[a]	4.57 ± 0.33[a]	53.96 ± 11.52[a]	0.33 ± 0.11[a]
70% RH	28.13 ± 1.80[a]	17.00 ± 1.62[a]	4.47 ± 0.34[a]	58.37 ± 11.10[a]	0.33 ± 0.20[a]
80% RH	28.75 ± 2.45[a]	17.95 ± 1.33[a]	4.80 ± 0.48[a]	58.19 ± 5.43[a]	0.34 ± 0.10[a]

Within a column, values with differing superscript alphabets are significantly different ($p < 0.05$).

Table 7.4. Chlorine dioxide byproducts analysis of chia seeds treated with ClO_2 gas at 10 mg/L concentration for 5 h at varied relative humidity.

	Chlorate (mg/g)	Chlorite (mg/g)	Chloride (mg/g)
Untreated	BDL	0.0030 ± 0.0030^{ab}	0.027 ± 0.011^{c}
60% RH	0.058 ± 0.011^{b}	0.0060 ± 0.0010^{a}	0.394 ± 0.073^{b}
70% RH	0.068 ± 0.017^{ab}	0.0006 ± 0.0002^{b}	0.480 ± 0.049^{ab}
80% RH	0.099 ± 0.019^{a}	0.0009 ± 0.0004^{b}	0.594 ± 0.040^{a}

Within a column, values with differing superscript alphabets are significantly different ($p < 0.05$).

BDL- below detection limit. Detection limits for chlorate, chlorite, and chloride were 0.0003, 0.0005, and 0.002 mg/g.

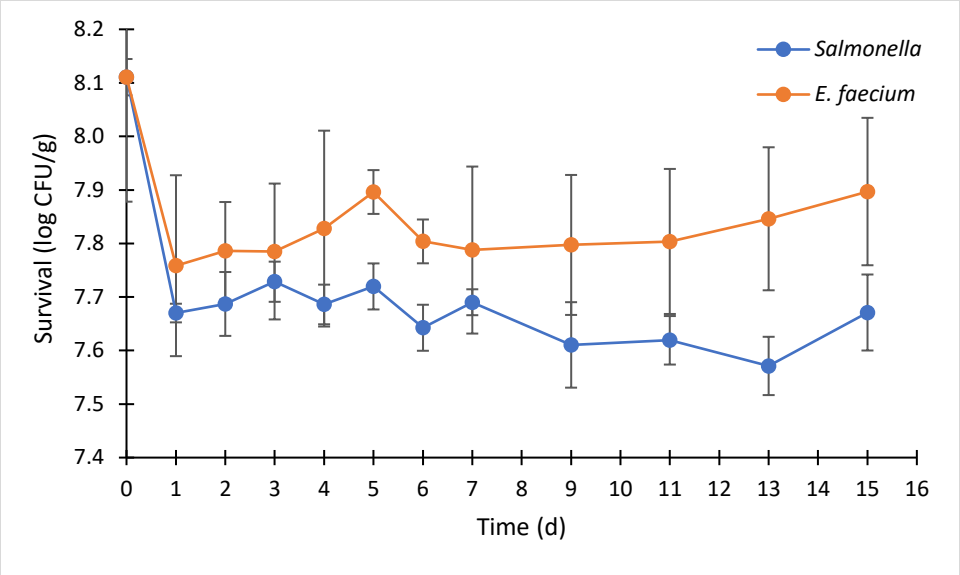


Fig 7.1. Stability and homogeneity test of *Salmonella* and *E. faecium* NRRL B-2354 in chia seeds for 14 days equilibrated at a_w = 0.53. Error bar represents ± 1 standard deviation.

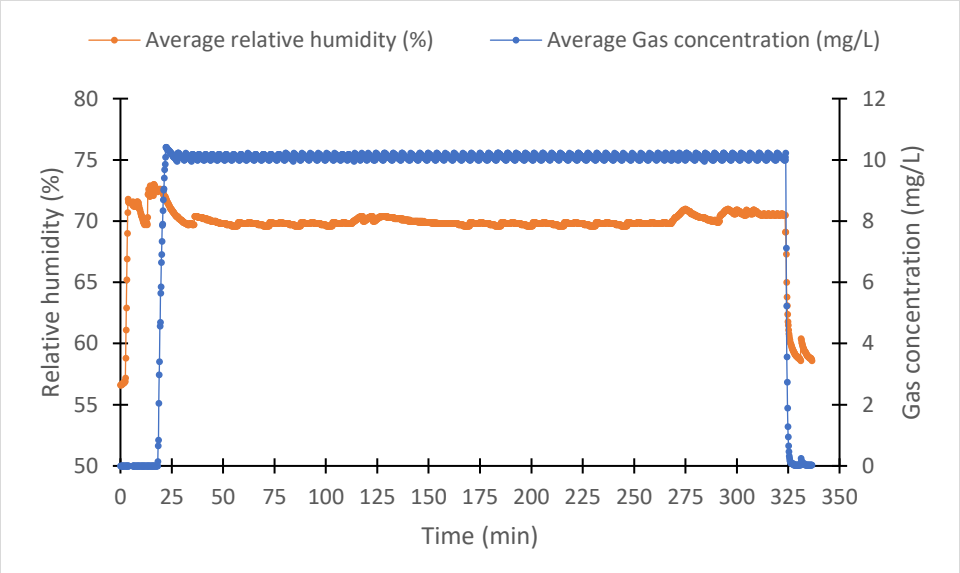


Fig 7.2. Relative humidity (%) and gas concentration (mg/L) during 5-h ClO_2 treatment set at 70% RH and 10 mg/L at ambient temperature. The points represent mean value and standard deviation of three replicates ranged from 0.2-2.0% for RH and 0.1-0.3 mg/L for gas concentration.

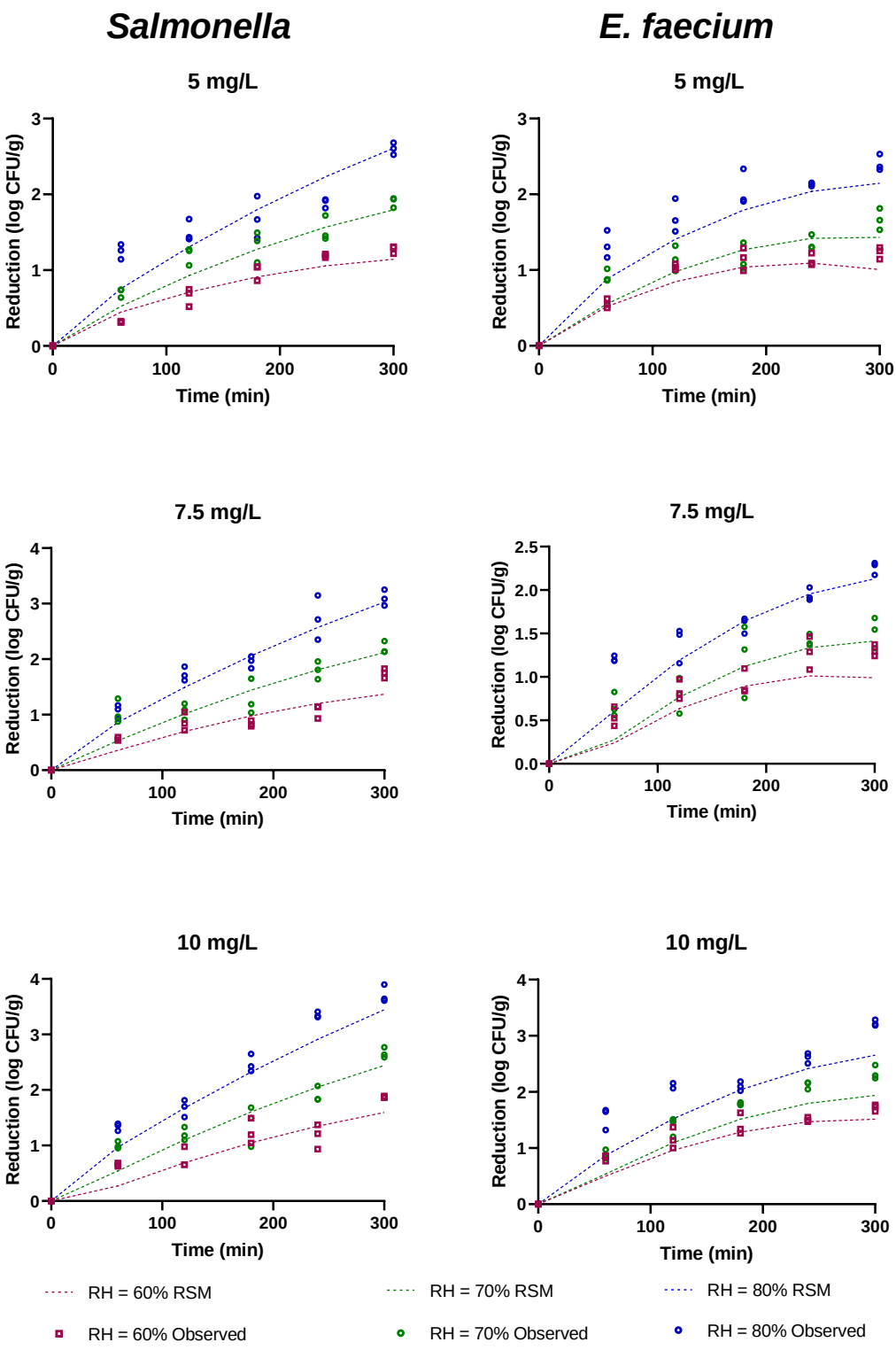
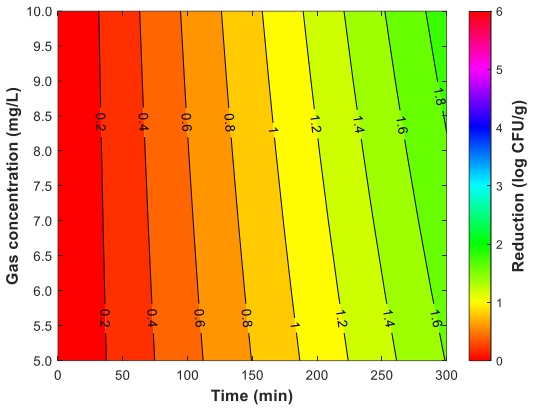
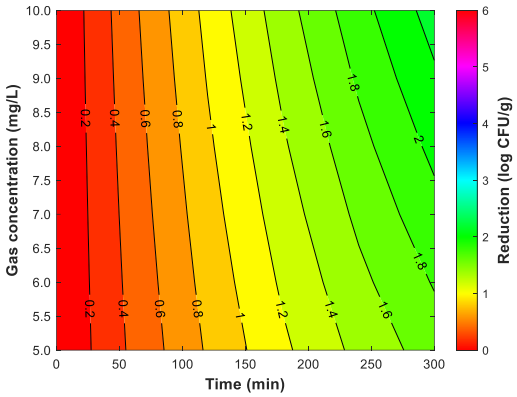


Fig 7.3. Predictions of log reduction of *Salmonella* and *E. faecium* NRRL B-2354 in chia seeds at different gas concentration and relative humidity using response surface model.

RH = 60 %



RH = 70 %



RH = 80 %

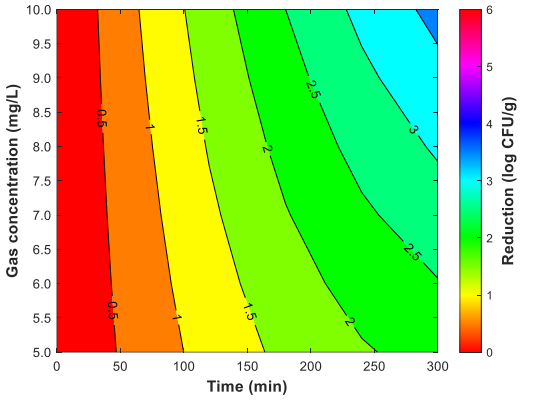


Fig 7.4. Contour surface plot developed using response surface model showing the log reduction of *Salmonella* in chia seeds during ClO_2 treatment.

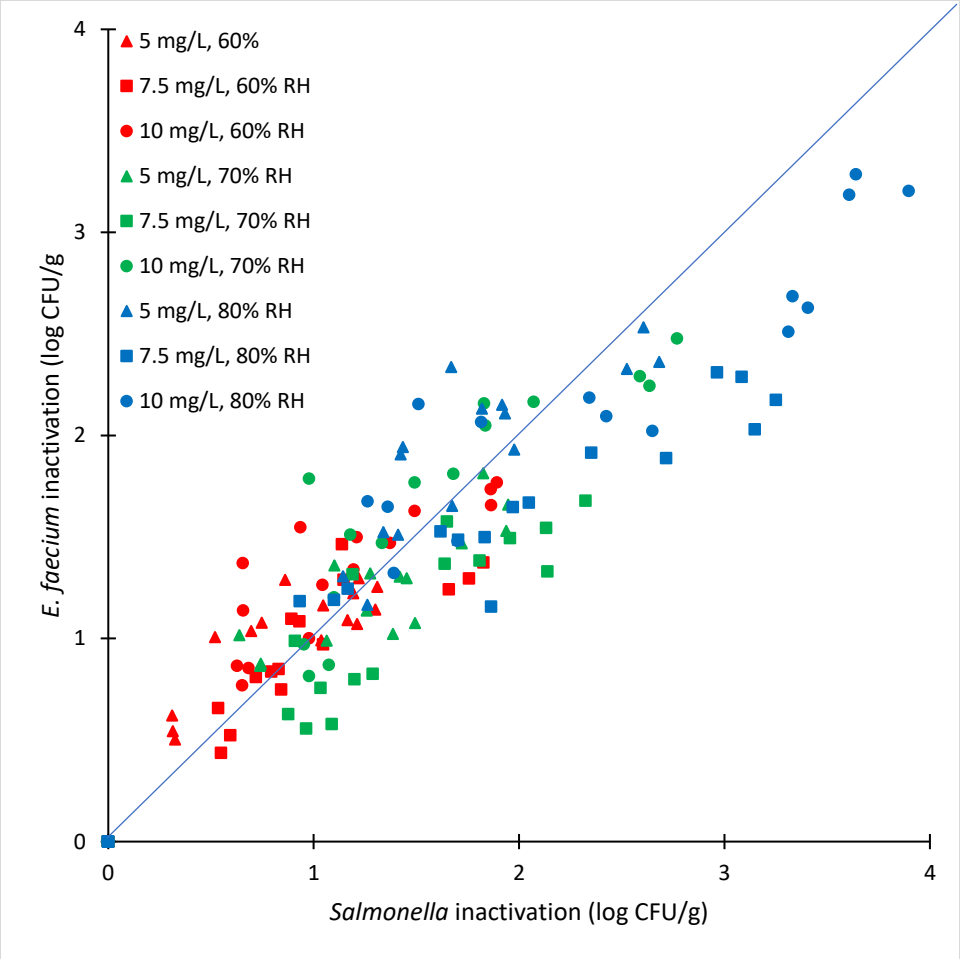


Fig 7.5. Comparison of observed log reduction of *Salmonella* and *E. faecium* NRRL B-2354 during ClO2 gas treatment of chia seeds. Each point represents an individual treatment.

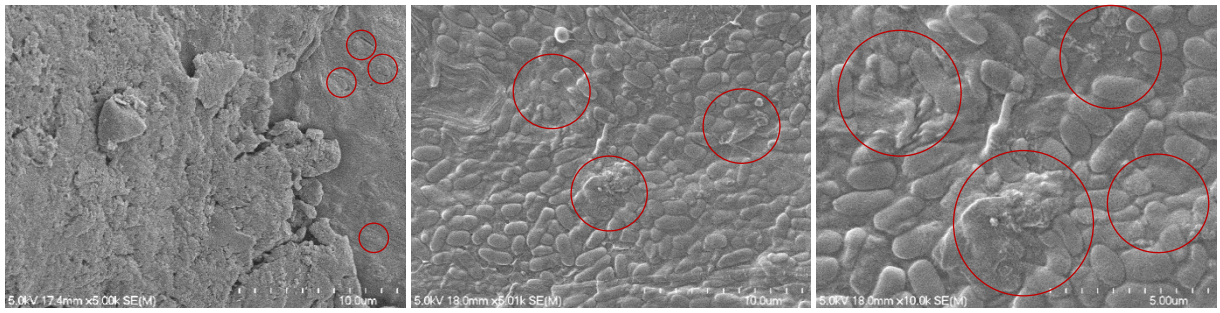


Fig 7.6. Scanning electron microscopic images of *Salmonella* inoculated chia seeds, A. Surface of untreated chia seeds (magnification - 5 K); B. Surface of ClO_2 treated chia seed sample (magnification - 5 K); C. Surface of ClO_2 treated chia seed sample (magnification – 10 K).

Chapter 8. Mild heating and ambient storage following gaseous chlorine dioxide treatment of chia seeds enhanced inactivation of *Salmonella* spp.

8.1 Abstract

Chlorine dioxide (ClO_2) gas has been recognized as a potent antimicrobial agent effective against various microorganisms. Chia seeds are prone to microbial contamination, and thermal treatments are not feasible due to potential quality issues. The study is aimed at investigating the efficacy of chlorine dioxide (ClO_2) gas followed by mild heat for the inactivation of *Salmonella enterica* in chia seeds**.** Chia seeds were inoculated with a cocktail of five *Salmonella enterica* serovars and equilibrated at a target a_w of 0.53. The gaseous treatment was performed for 2 h at two conditions – 5 mg/L gas concentration at 80% RH and 3 mg/L at 90% RH. The ClO_2 treated samples were subjected to mild heating at 40 and 60 °C for 1 or 2 hours and further analyzed for *Salmonella* reduction during one month of ambient storage. Gaseous treatment of chia seeds at 90% RH achieved significantly ($p<0.05$) higher log reduction (2.82 log CFU/g) of *Salmonella* than 80% RH (1.80 log CFU/g); additional log reductions of 1.32 and 1.14, respectively, were achieved by mild heat treatment at 60 °C for 2 hours immediately after treatment. Storage study revealed that *Salmonella* population continued to decrease over time. ClO_2 treatment at 80% and 90% RH without mild heating achieved a cumulative log reduction of 2.87 and 3.81 after 7 days of storage. ClO_2 treatment (3 mg/L at 90% RH) followed by mild heating (60 °C for 2 h) and 7 days of storage achieved close to 5 log reduction. The quality of chia seeds was minimally affected by ClO_2 treatment, both alone and in combination with mild heating, as indicated by the insignificant changes in color values, fatty acids, and phenolic content, germination ability, and byproduct residues. This study demonstrates the effectiveness

of post ClO_2 mild heating and subsequent storage to enhance the microbiological safety of chia seeds.

Keywords: low-moisture food ingredients; antimicrobial efficacy; hurdle concept; synergistic effect; non-thermal technologies

8.2 Introduction

Chlorine dioxide (ClO_2) gas is a potent oxidizing agent with a wide range of antimicrobial activity making it effective against bacteria, viruses, and fungi (Kim et al., 2011). As per 21 CFR 173.300, ClO_2 is permitted for use as an antibacterial agent in foods (FDA, 1998). ClO_2 is a gas at room temperature and has a molecular size much smaller than bacteria, therefore gas can easily diffuse and inactivate bacteria hiding in nooks and crannies of the food product (Trinetta et al., 2012; Shirasaki et al., 2016). The antibacterial effects of ClO_2 gas were observed on ceiling, walls, and floor (Shirasaki et al., 2016. These results indicate that gaseous ClO_2 is highly diffusible and permeable, thereby gaining access to the small spaces that are hard to disinfect with aqueous agents. Compared to other disinfectants, such as aqueous chlorine and hydrogen peroxide, ClO_2 gas is less likely to form harmful byproducts and has a faster reaction time, making it a desirable option for food processing (Sofos & Geornaras, 2010).

Chia seeds, a popular superfood, have gained immense popularity over the last few years due to their high nutrient content and various health benefits. Germination of chia seeds has been shown to enhance the nutritional composition including protein content by 13%, fiber by 46%, tryptophan by 93%, total phenolics by 300% and flavonoids by 197%. In addition, Vitamin C was only detected in germinated seeds. However, chia seeds may be contaminated with harmful microorganisms such as *Salmonella*, *Escherichia coli*, and other bacteria which pose a significant

risk to human health. Germination provides ideal conditions for microbial growth due to the presence of moisture, nutrients, and optimum temperature. *Salmonella* contamination in sprouted chia seed powder was linked to an epidemic of foodborne disease in 2013 and 2014 (Harvey et al., 2017). Therefore, it is crucial to decontaminate chia seeds to ensure their safety before consumption. Thermal decontamination is not feasible due to the potential gelling of chia seeds due to steam production and condensation. Annous and Burke (2015) reported that although heat treatment of mung bean seeds at 60 °C for 2 h reduced *Salmonella* below the detection limit, treated seeds were not viable. Moreover, heat treatment of chia seeds could produce acrylamide due to its high fat content (Mesias et al., 2023). These drawbacks of thermal treatment encourage the utilization of non-thermal treatment such as gaseous technologies. Wason & Subbiah (2023) reported that ClO_2 treatment was effective in inactivating *Salmonella* in chia seeds; however, the desired 5 log reduction was not achieved even at the most severe treatment conditions tested of high gas concentration (10 mg/L), RH (80%) at a long exposure time of 5 hours. Wei et al. (2021) and Verma et al. (2022) reported 5 log reduction in black peppercorns and dried basil leaves after ClO_2 treatment at 15 mg/L gas concentration. At such a high gas concentration exposed for long treatment time, food products may be bleached; however, they did not perform any quality analysis following ClO_2 treatment.

Currently, ClO_2 has been found effective mostly for the decontamination of high-moisture foods such as carrots (Guan et al., 2019); Cantaloupe (Alicea et al., 2018); tomatoes, blueberries, baby carrots (Chai et al., 2019; Tan et al., 2021); apple (Han and Linton, 2002); oranges, strawberries, lettuce, alfalfa sprouts, tomatoes (Trinetta et al., 2010). Wason & Subbiah (2023) indicated that the microbial inactivation using ClO_2 gas in high-moisture foods was more rapid than in low-moisture foods. In the case of low-moisture foods, *Salmonella* reduction required

longer exposure time (Verma et al., 2022; Wei et al., 2021; Rane et al., 2020; Golden et al., 2019). For instance, 0.4 mg/g ClO_2 reduced *Salmonella* in black peppercorns by 2.2 log CFU/g after 24 h (Golden et al., 2019) and 2.3 log CFU/g after 240 min at 45% RH (Rane et al., 2020). Combination treatments with mild heating and storage were found to significantly improve the efficacy of ClO_2 inactivation. Wang et al. (2019) demonstrated that increasing the temperature during ClO_2 treatment from 22 to 50 °C increased the *Salmonella* reduction from 1.46 log CFU/g to >4 log CFU/g in almonds within 4 h. In addition, the assistance of mild heat in improving microbial inactivation efficacy of ClO_2 was reported in mung bean seeds (Annous and Burke, 2015), radish seeds (Bang et al., 2011), and almond kernels (Rane et al., 2020; Wang et al., 2019). After fumigation, industries typically store food products for a few weeks in the open to allow the dissipation of gas. Golden et al. (2019) reported *Salmonella* reduction in treated low-moisture foods during storage post ClO_2 gas treatment. The presence of residual ClO_2 gas during storage might lead to bacterial inactivation in addition to gaseous treatment (Wei et al., 2023). Therefore, storage time is yet another factor that must be investigated for its role in enhancing bacterial reduction in treated foods.

The majority of the investigations on the antimicrobial efficacy of ClO_2 on low-moisture foods have not assessed the quality of treated food. During the treatment, ClO_2 can degrade to chlorate, chlorite and chloride ions. Although there is no federal regulation on the maximum permitted level of ClO_2 by products in foods, the National Primary Drinking Water regulations (NPDWR) regulates the level of chlorite ion as 1 mg/L and residual ClO_2 gas as 0.8 mg/L in drinking water (Federal Register, 1998). Apart from the residues and byproducts in treated foods, ClO_2 can significantly affect the quality of foods. Wason & Subbiah (2023) reported that

treatment with ClO_2 at 10 mg/L at 80% RH for 5 h induced lipid oxidation in chia seeds and significantly increased the peroxide value.

The goal of this study is to investigate the synergistic effect of mild heating and ambient storage following a milder ClO_2 treatment (lower concentration and a shorter treatment time) so that the quality impacts of chia seeds are minimized while achieving a 4-5 log reduction. The specific objectives of the present study were to a) investigate the efficacy of ClO_2 gas followed by mild heating to inactivate *Salmonella* in chia seeds, b) assess the effect of ambient storage after ClO_2 treatment on *Salmonella* reduction, and c) assess the impact of ClO_2 treatment assisted with mild heating and ambient storage on the byproduct formation and nutritional quality of chia seeds. The results of this research can provide valuable information for the food industry to use this method for reducing the risk of foodborne illnesses caused by *Salmonella* contamination in chia seeds.

8.3 Materials and Methods

8.3.1Chia seed samples

Chia seeds from Organic Chia Seeds, Better Body Foods, Lindon, UT were acquired from online vendors and kept at room temperature until usage. Three distinct batches (Batches 1, 2 and 3) were assigned to the samples from different manufacturing lots.

8.3.2 Bacterial strains and sample inoculation

The bacterial inoculum was produced from five serotypes of *Salmonella enterica*, including serovars Montevideo 488275, S. Mbandaka 698538, S. Agona 447967, S. Tennessee K4643, and S. Reading Moff 180418. These serotypes were selected because they have been associated with

foodborne illness outbreaks in low-moisture foods. The five serotypes were mixed in equal proportion to prepare a *Salmonella* cocktail as described in Wason et al. (2021a).

Salmonella cocktail (6 mL) was sprayed over a thin layer of chia seeds (300 g). To ensure homogenous distribution of the bacterial population, the sample was mixed thoroughly by shaking them in a Ziplock bag. Wason & Subbiah (2023) stated that storage of chia seeds in a relative humidity (RH) chamber (Lau & Subbiah, 2020) at 53%RH after inoculation for at least 4 days provided a stable *Salmonella* population. Similarly in the present study, the chia seeds were stored at 53% RH to stabilize the *Salmonella* population before gaseous treatment.

8.3.3 Chlorine dioxide gas treatment

ClO_2 gas treatment was conducted in an enclosed chamber using Minidox-M system (ClorDisys Solutions, Inc.) according to the method described in Verma et al. (2022). Inoculated chia seeds (2 g) packed in heat-sealed paper bags were treated with ClO_2 gas at two treatment combinations as mentioned in section 2.4. The treatment conditions were selected based on an earlier report by Wason & Subbiah (2023) who reported a higher significant effect of RH on microbial inactivation than the gas concentration. Higher RH conditions of 80% and 90% were selected to utilize lower gas concentrations for efficient microbial inactivation and minimizing the gaseous effects on the quality and byproduct formation in chia seeds. The treatment conditions provided around 2 log reduction of *Salmonella* were used to evaluate further microbial reduction during mild heating and ambient storage.

8.3.4 Experimental design

A split-split plot experimental design was applied to assess the effects of ClO_2 treatment, mild heating temperature-time combination, and storage time on the *Salmonella* inactivation in

chia seeds. *Salmonella* inoculated chia seeds packed in paper tea bags were either treated with ClO_2 at 5 mg/L and 80%RH for 2 h (ClO_2 treatment A) or 3 mg/L and 90%RH for 2 h (ClO_2 treatment B). ClO_2 treatment was the whole plot with three levels, treatment A, B, or no treatment (control). The sub plot represents the combination of mild heating temperature and holding time with 5 levels. The temperature and holding time combination used in this study were ambient temperature (23 °C), 40 °C for either 1 or 2 h, and 60 °C for either 1 or 2 h. For each subplot, the sub-subplot represented the eight storage periods consisting of 8 levels (8 time points over a month). Post mild heating, one sample from each mild heating temperature-time combination was enumerated and regarded as Day 0 population. The remaining samples in individual paper bags were stored at ambient conditions and enumerated on days 1, 3, 5, 7, 14, 21 and 28. After gaseous treatment, the samples were mixed with a neutralizing buffer (NB) in a 1:30 ratio. The NB was composed of 42.5 mg of monopotassium phosphate and 0.16 g of sodium thiosulfate dissolved in 1 L of deionized water. This was done to neutralize any remaining ClO_2 residues in the samples, as described by Garcia et al. (2022). *Salmonella* was enumerated by plating on m-TSAYE (TSAYE supplemented with 0.03 (w/v) sodium thiosulfate (Fisher Scientific, Fair Lawn, NJ), and 0.05% (w/v) ammonium iron citrate (Sigma Aldrich, St. Louis, MO) and incubating at 37 °C for 24 h.

8.3.5 Statistical analysis

The inactivation data of *Salmonella* treated with different ClO_2 treatments followed by mild heating and storage under ambient conditions were fit to a mixed model. The experiment was replicated three times using different batches of chia seeds. After ClO_2 treatment, sample bags were split into five groups and were treated with five different temperature-time combinations followed by storage under ambient conditions for up to a month. The mixed model

was chosen to address the potential variability in log reduction within the same ClO_2 treatment run or between different treatments. Fixed effects included ClO_2 treatment (ClO_2 trt), mild heating temperature-time (mild heating), ambient storage (storage time, d), as well as their two-way and three-way interaction effects. The random effects included batch nested within ClO_2 treatment, batch*mild heating nested within ClO_2 trt, and batch*storage time nested within ClO_2 trt. The analysis was performed using proc mixed in SAS.

8.3.6 Quality analysis

The effect of ClO_2 treatment followed by mild heating on the quality of chia seeds was evaluated at conditions leading to best log reduction. Moreover, ClO_2 byproducts were evaluated throughout the storage period on day1, 3, 7 and 28 after ClO_2 treatment to assess the stability of byproducts in chia seeds. The uninoculated samples were treated and analyzed for quality. Immediately after the treatment, the samples were mixed in a Ziplock bag and the water activity and moisture content were measured using a dew point water activity meter (Model: 4 TE, Meter Group, Pullman, WA) and halogen moisture analyzer (Model: HR73, Mettler Toledo, Greifensee, Switzerland), respectively.

The method deployed for quality analysis was similar to earlier research reported by Wason & Subbiah (2023) in detail. In brief, the color of the ClO_2 treated chia seeds was measured using a colorimeter (Konica Minolta, model: BC-10, Osaka, Japan) at five random locations on the samples spread on a Petri dish.

In brief, lipids were extracted from the treated chia seed samples using the method by Folch et al. (1957). Ground chia seeds were mixed with a methanol and chloroform solution (1:2) for 2 hours. The extract was then treated with water, filtered, and dried to obtain the lipid

extract. The extracted lipids were subjected to fatty acid composition analysis using the procedure described in AOAC method 996.06. Approximately 100 to 200 mg of the lipid-containing ground sample was mixed with 2 ml of ethanol, 100 mg of pyrogallol as an antioxidant, and an internal standard solution of glycerol triundecanoate in chloroform. The samples were further mixed with 10 ml of 8.3 M hydrochloric acid and digested at 75 °C for 40 minutes. Lipids were extracted using diethyl ether and hexane. After drying the solvents, the lipids were derivatized with 12% boron trifluoride in methanol at 95 °C for 30 minutes. The samples were then processed with water, sodium sulfate anhydrous, and hexane before being injected into an Agilent 7820A GC system with a flame ion detector. The fatty acid peaks were identified by comparison with an external standard.

The peroxide value of the lipid samples was determined according to the method described by Li et al. (2001). Triplicate samples of 0.5 g of lipid were dissolved in a mixture of chloroform and acetic acid (2:3) along with 50 µl of saturated potassium iodide in water. The samples were then mixed with water, and after centrifugation, the upper phase was transferred to a fresh test tube. A starch indicator solution (1% (W/V) Mercury Free, RICCA, Fisher 8050-16) was added, and the absorbance of the samples was measured at 563 nm. A standard curve was prepared using elemental iodine dissolved in water and combined with the starch indicator.

The total phenolic acid content in treated chia seeds was analyzed according to Folin-Ciocalteu method (Singleton & Rossi, 1965). One gram of the treated sample was extracted twice with a mixture of 1.2 N hydrochloric acid in water and methanol (1:1) for 2 hours. The organic solvent phases were combined, purified by centrifugation, and filtered through a 0.2 µm filter. A portion of the sample extract was then diluted with water and mixed with Folin-Ciocalteu's reagent. After adjusting the pH, the mixture was stored in the dark for 2 hours at

room temperature. The absorbance of the samples was measured at 760 nm, and the phenolic content was expressed as gallic acid equivalents.

The germination capacity of the treated chia seeds was determined using the method described by Geneve et al. (2017). Fifty chia seeds were placed on a damp blotting paper in a petri dish and exposed to alternating periods of sunlight (8 hours) and darkness (16 hours) each day under ambient temperature and relative humidity conditions. The number of germinated seeds was recorded daily for a week until no further increase was observed. Germination capacity was calculated as below.

$$\text{Germination capacity} = \frac{Number\ of\ germinated\ seeds}{Total\ number\ of\ seeds}\ x\ 100$$

To analyze the byproducts, chia seeds (50 mg) were hydrated with 1.95 mL of double distilled water (DDI) in a microcentrifuge tube for 2 hours. After centrifugation, the supernatant was collected and passed through a C18 cartridge. The byproducts (chlorite, chlorate, and chloride) were determined using an ion chromatography system following EPA Method 300. Standard curves were prepared, and the concentration of each byproduct was calculated by comparing the sample response. Detection limits for chlorite, chlorate, and chloride were 0.500, 0.300, and 2.00 µg/g, respectively. Results were reported as mg/g of chia seed samples.

8.4 Results and Discussion

8.4.1 Gaseous ClO_2 inactivation

Gaseous ClO_2 treatment of chia seeds for 2 h at 80%RH and 5 mg/L gas concentration resulted in a 1.80 ± 0.11 log reduction of *Salmonella*. Treatment at 90% RH and the same gas concentration and exposure time reduced *Salmonella* below the detection limit (data not

included). These results highlight the significant impact of RH on the antimicrobial efficacy of ClO_2 on chia seeds. To assess the effect of post-treatment mild heating and storage time on microbial inactivation, a lower gas concentration of 3 mg/L was used at 90% RH, resulting in 2.82 ± 0.16 log CFU/g reduction. Higher RH even at lower gas concentrations for the same exposure time led to greater microbial inactivation. Wang et al. (2019) sprayed with water on the chamber walls to achieve more than 90% RH during ClO_2 treatment of almonds and reported 1.46 ±0.57 log CFU/g reduction after 4 h exposure at 4.64 ±0.49 mg/L ClO_2. On the other hand, Rane et al. (2020) dipped almonds in water for a specific time before ClO_2 treatment at 0.40 mg ClO_2/g almond and reported a 2.6 ±0.6 log reduction in *Salmonella* after 6 h of exposure. Tan et al. (2021) further demonstrated that dipping almonds in water was more effective in microbial inactivation due to the presence of moisture on the surface of almonds. In both the previous studies, ClO_2 gas was generated using dry media precursors which increase the gas concentration until it reaches the peak and then slowly drops down. This dramatic change in gas concentration and uncontrolled relative humidity makes it difficult to analyze the efficacy of gaseous treatment in reducing bacterial population and could not ensure consistency and reproducibility. In this study, the use of a Minidox-M system allowed for the precise control of gas concentration and RH levels throughout the treatment, resulting in a reliable estimation of process conditions for effective log reduction at a particular gas concentration and RH. Using the same gas generation system, Wei et al. (2021) and Verma et al. (2022) reported a 5-log *Salmonella* reduction in black peppercorn and dried basil leaves at 15 mg/L concentration and 80%RH within 5 h of treatment. Chlorine dioxide gas is highly soluble in water and reacts to form chlorite and chlorate ions which are also very reactive. Additionally, the increased sensitivity of pathogens to inactivation at high humidity levels provides evidence that high RH is required for ClO_2 inactivation.

Previous studies on ClO_2 inactivation of high-moisture foods further demonstrate the significance of moisture in rapidly reducing Shiga toxin-producing *Escherichia coli* (STEC), *Listeria monocytogenes*, and *Salmonella* inoculated on tomatoes, blueberries, baby-cut carrots (Tan et al., 2021). The authors further reported that ClO_2 treatment at 1 mg/L at 70% RH resulted in >5 log reduction on high moisture foods while a higher RH of > 85% was required for efficient microbial inactivation in black peppercorns and almonds. Similarly, Rane et al. (2020) observed that increasing the RH from ambient (45-50%) to 80% significantly increased the *Salmonella* reduction from 2.3 ± 0.6 to 3.7 ± 0.2 log CFU/g during ClO_2 treatment at 0.40 mg ClO_2/g peppercorn for 4 h exposure. The use of high RH and low gas concentration is an effective way to utilize ClO_2 for pathogen inactivation. Wei et al. (2021) reported that the D-value of *Salmonella* at 80% RH and 10 mg/L was 69.6 min while, at 70% RH at 15 mg/L was 89.2 min. Similarly, Verma et al. (2022) found that D-value of *Salmonella* at 80% RH and 10 mg/L was significantly lower than that at 70% RH and 15 mg/L. Overall, ClO_2 treatment was more effective at 90% RH than at 80% RH for *Salmonella* inactivation on chia seeds.

8.4.2 Effect of mild heating post ClO_2 treatment

The application of mild heating post ClO_2 treatment resulted in a significant increase in the log reduction of *Salmonella* compared to ClO_2 treatment alone. Table 2. presents the improvements in log reduction achieved after mild heating at either 40 or 60 °C for 1or 2 h after ClO_2 treatment for 2 h at 5 at 80% and 3 mg/L at 90% RH, respectively. ClO_2 treatment alone at 80% RH and 5 mg/L concentration for 2 h resulted in a reduction of *Salmonella* by 1.80 log CFU/g. However, the reduction in *Salmonella* increased significantly when mild heating was applied after ClO_2 treatment, with the highest reduction (3.12 log CFU/g) achieved after mild heating at 60 °C for 2 h. Similarly, at 90% RH and 3 mg/L ClO_2 concentration, the ClO_2

treatment followed by mild heating resulted in a higher reduction of *Salmonella* compared to ClO_2 treatment alone. The highest cumulative microbial inactivation (3.96 log CFU/g) was achieved at ClO_2 treatment at higher RH followed by mild heating at 60 °C for 2 hours. These findings are consistent with previous studies that reported that mild heating can enhance the effectiveness of ClO_2 treatment in reducing pathogenic bacteria (Rane et al., 2020, 2021; Park et al., 2018; Lee et al., 2018). For instance, Rane et al. (2020) reported that heating almonds at 65 °C for 24 h post ClO_2 treatment at 0.40 mg ClO_2/g almond for 6 h increased the log reduction of *Salmonella* from 2.6 to 4.3 log CFU/g; Shiga toxin *E. coli* from 2.6 to 4.4 log CFU/g; *Listeria monocytogenes* from 2.3 to 3.5 log CFU/g.

Mild heating alone at 40 °C or 60 °C for 1 or 2 h did not significantly affect the *Salmonella* survival on chia seeds (Fig. 1). The maximum reduction in *Salmonella* observed was 0.4 log CFU/g. Therefore, it can be inferred that the synergistic effect of mild heating after ClO_2 treatment was responsible for microbial inactivation in chia seeds. In contrast, Park et al. (2018) reported that dry heat alone at a much higher temperature of 80 °C for 5 h resulted in a 3.23 log reduction of *S.* Typhimurium in alfalfa seeds and 2.27 log reduction in radish seeds. The sequential treatment of ClO_2 at 150 ppm for 1 h and heating provided 5.29 and 4.11 log reduction of *S.* Typhimurium in alfalfa and radish seeds. Similar observations were made in either studies that evaluated the combined or sequential of mild heating, without assessing the effect of mild heating alone (Lee et al., 2018; Wang et al., 2019; Rane et al., 2020, 2021).

The results also demonstrate that mild heating temperature (40 °C and 60 °C) significantly ($p<0.05$, Table 2.) affected the antimicrobial effect of ClO_2 on *Salmonella*. For instance, at 5 mg/L at 80% RH followed by mild heating at 40 °C, the cumulative log reduction ranged from 2.44 to 2.63, while at 60 °C, it ranged from 2.72 to 3.12. Similarly, after 3 mg/L at

90% RH, the cumulative log reduction ranged from 3.16 to 3.26, while at 60 °C, it ranged from 3.45 to 3.96. This suggests that the combination of ClO_2 treatment and mild heating can have a synergistic effect on pathogen reduction. These results are in agreement with Wang et al. (2019) that demonstrated the increased efficacy of gaseous ClO_2 with higher treatment temperatures. Gas concentrations of more than 4 mg/L and ambient temperature only reduced populations of *Salmonella* by 1.46 log CFU/g on almonds. At 55 and 60 °C, 1 mg/L ClO_2 exposed for 4 h led to 4 log reduction of *Salmonella* (Wang et al., 2019). It is important to note that Wang et al. (2019) treated almonds simultaneously with ClO_2 and heat whereas in this study mild heating of the sample was done after ClO_2 exposure. Mild heating can enhance the permeability of gas in bacterial cell membranes and increase the efficacy of antimicrobial agents. When mild heating follows ClO_2 treatment, oxidative potential of ClO_2 could injure the bacterial cells and disrupt their metabolic processes, making them more susceptible to mild heat. The holding time had a significant effect only when chia seeds were heated at 60 °C; one or two hours of holding time did not significantly influence the microbial inactivation at 40 °C. Another study by Rane et al. (2021) reported a small increase in log reduction from 1.1 to 1.5 when almonds were treated simultaneously with ClO_2 (around 0.3-0.5 mg/L) and mild heating at 40 °C compared to ClO_2 alone. Lower temperatures might not be effective in improving the antimicrobial efficacy of ClO_2. In contrast, Annous & Burke (2015) reported dry heat treatment (55 – 70 °C) for 8 h resulted in > 3 log reduction of *Salmonella* on mung bean seeds.

The improvement in *Salmonella* log reduction by mild heating did not consistently depend on the ClO_2 treatment conditions. For instance, mild heating at 40 °C for 2 h after ClO_2 at 3 mg/L at 90% RH resulted in a modest increase of 0.33 log CFU/g in *Salmonella* reduction compared to mild heating alone. In contrast, mild heating at 40 °C for 2 hours after ClO_2 5 mg/L

at 80% led to a larger increase of 0.64 log CFU/g in log reduction when compared to mild heating alone. However, mild heating at 60 °C for 2 h showed a more substantial improvement, with a log reduction increase by 1.32 and 1.14 log CFU/g after ClO_2 A and ClO_2 B, respectively. Generally, the highest log reduction values were observed at 60 °C for 2 h for both treatment groups, suggesting that a higher temperature may be particularly effective in reducing *Salmonella* on chia seeds. Specifically, mild heating at 60 °C for 2 h provided 3.12 ±0.23 and 3.96 ±0.07 cumulative log reduction after ClO_2 treatment at 80 and 90% RH, respectively. Overall, the results of this study suggest that the combination of ClO_2 treatment with mild heating can be an effective strategy for reducing pathogenic bacteria on chia seeds.

8.4.3 Effect of storage time and sequential treatment on the *Salmonella* inactivation

A mixed model analysis (Table 2.) was conducted to assess the effect of various parameters on *Salmonella* inactivation on chia seeds. In this section, the ClO_2 treatment A refers to treatment at 5 mg/L, 80% RH, 2h exposure and ClO_2 treatment B refers to treatment at 3 mg/L, 90% RH, 2h exposure. The results revealed that ClO_2 treatment, combination of mild heating temperature and holding time, and ambient storage significantly ($p<0.05$) impacted *Salmonella* reduction. Moreover, the process parameters had significant interaction effects on microbial inactivation. Fig 2. illustrates the change in log reduction after mild heating and subsequent ambient storage for 28 days. The results of the study indicate that subsequent storage after ClO_2 treatment and mild heating can lead to an increased log reduction of *Salmonella* on chia seeds. This finding suggests that microbial inactivation during storage could be due to the continued antimicrobial effects of residual ClO_2 and its byproducts. For ClO_2 trt A, *Salmonella* reduction at various treatment combinations ranged cumulatively from 1.80 to 3.12 log CFU/g on day 0 and gradually increased until day 7 and then plateaued. By day 7 of ambient storage,

mild heat-treated samples rapidly reached the asymptote value which was higher than samples not subjected to mild heating; *Salmonella* reduction cumulatively ranged from 2.87 to 3.63 log CFU/g. ClO_2 B resulted in higher log reduction values compared to ClO_2 A across all storage durations and temperatures. On day 0, the *Salmonella* reduction ranged from 2.82 to 3.96 log CFU/g. Over time, the log reduction increased, and the samples mild heat treated at 60 °C for 2 h rapidly reached significantly higher asymptote value (4.7 log CFU/g) while chia seeds treated at 40 °C for 2 h gradually reduced *Salmonella* until day 7.

In general, ambient storage for 7 days increased the *Salmonella* inactivation by 1 log CFU/g. Similarly, Wei et al. (2023) found that ambient storage for 7 days post ClO_2 treatment significantly improved the *Salmonella* reduction by about 1 log CFU/g on black peppercorns and cumin seeds at 5 and 10 mg/L, 80% RH for 300 min. At the same RH (80%), 10 mg/L for 150 min and 15 mg/L for 125 min, *Salmonella* on dried basil leaves were reduced by 1.4 and 1.3 log CFU/g after 7 days of ambient storage. The ClO_2 gas treatment used by Wei et al. (2023) involved higher gas concentrations and longer exposure times when compared to the current study. It is important to consider that the specific experimental conditions, such as ClO_2 concentration and duration of ambient storage, as well as storage conditions might impact the *Salmonella* reduction. Therefore, it is crucial to optimize these parameters for specific products and processes to achieve the maximum reduction of *Salmonella* while maintaining the quality and safety of chia seeds.

Overall, *Salmonella* reduction reached asymptote after a week of storage, therefore contrast analysis was run for the reduction data for day 7. Table 3. presents the pairwise comparison of process parameters on day 7 for optimizing the conditions to achieve maximum log reduction. The results suggest that there was a significant ($p<0.0001$) difference between

different types of ClO_2 treatment, with ClO_2 B resulting in higher log reduction values. The effect of mild heating is also significant at $p<0.0001$. The pairwise comparison estimates show that mild heat-treated samples exhibited higher log reduction compared to ambient samples. Furthermore, there were significant differences in *Salmonella* inactivation between mild heating at 40 and 60 °C ($p<0.0001$) and between 1 and 2 h ($p<0.0281$). It can be observed that the effect of holding time had a smaller effect than the temperature during mild heating.

The interaction effect between ClO_2 treatment and mild heating (c*h) was found to be non-significant ($p = 0.1097$), indicating that enhanced *Salmonella* reduction due to mild heating was not significantly affected by the ClO_2 processing parameters and vice-versa. Overall, the results suggest that ClO_2 treatment and mild heating had significant individual effects on *Salmonella* reduction on chia seeds. ClO_2 treatment was particularly effective, with ClO_2 treatment B showing higher log reduction values than ClO_2 treatment A. Mild heating at higher temperatures (60 °C) and for longer durations (2 h) enhanced log reduction. Lim et al. (2021) reported that increasing the treatment temperature from 22 to 60 °C resulted in a substantial increase in *Salmonella* reduction from 1.46 to >4 log CFU/g within 4 hours. Furthermore, at 60 °C, the combination treatment achieved more than 5 log reduction in less than one hour, regardless of the gas concentration used (Lim et al., 2021). These findings highlight the synergistic effect of mild heating and ClO_2 treatment. Another study by Golden et al. (2019) reported a significant effect of storage time after ClO_2 exposure (100-500 mg ClO_2/kg spice), with up to 2.5 log reduction of *Salmonella* on black peppercorns, cumin seeds, and sesame seeds after 30 days of storage.

8.4.5 Quality of chia seeds post ClO_2 treatment

a. Water activity and moisture content

The impact of ClO_2 treatment, both alone and in combination with mild heating, on the water activity and moisture content of chia seeds was investigated. Table 4-6 presents the results of the quality analysis conducted on chia seeds treated with ClO_2 at concentrations of 3 and 5 mg/L, exposed to 80% and 90% RH for 2 h, along with the combination treatment involving mild heating for 2 h.

The water activity in chia seeds samples was significantly higher ($p< 0.05$) in ClO_2 treated samples as compared to the control samples. The water activity increased from 0.46 ± 0.03 to 0.56 ± 0.002 after treating chia seeds with ClO_2 treatment at 3 mg/L concentration for 2 h under 90% RH. These results are in agreement with Wason & Subbiah (2023) who observed a significant increase in water activity in chia seeds treated to ClO_2 at 10 mg/L concentration at 80% RH (0.60) than at 70% RH (0.58). On the other hand, subjecting ClO_2 treated samples (80% RH 3 mg/L for 2 h) to mild heating at 60 °C for 2 h resulted in a substantial decrease in water activity well below the control samples from 0.53 to 0.18. Similarly, the moisture content of the chia seeds significantly decreased when chia seeds exposed to ClO_2 at 5 mg/L at 80% RH were subjected to mild heating. However, the moisture content of chia seeds did not show a significant increase when treated with ClO_2 alone as compared to the control. With no significant increase in the moisture content of chia seeds at higher relative humidities, it is anticipated that there would be no formation of gelling or a visual change in the quality of seeds.

b. Color value

The colorimetric analysis of ClO_2 treated chia seeds showed that ClO_2 treatment did not significantly influence ($p > 0.05$) the quality of chia seeds in most of the cases (Table 4). The L* values which represent the lightness of chia seeds showed a significant increase ($p<0.05$) following treatment to ClO_2 at 90% RH at 3 mg/L concentration followed by mild heating at 60 °C for 2 h (51.4 ± 0.17) as compared to 49.43 ± 1.01 observed in control chia seeds. However, the a* values indicating red to greenish color did not show any significant difference ($p < 0.05$) among the treatments and ranged from 1.23 – 1.73. The b* value representing the yellow to blue color, was significantly higher ($p < 0.05$) in chia seeds treated with ClO_2 at 5 mg/L concentration under 80% RH for 2 h (6.03 ± 0.06) compared to the control samples (4.97 ± 0.51). Similar findings were reported by Wason & Subbiah (2023), who observed significant increases in L* and b* values in chia seeds treated with ClO_2. However, their treatment included a higher concentration of 10 mg/L compared to 3 and 5 mg/L concentrations used in the present study. In contrast, Lee et al. (2018) found that subsequent drying at 55 °C for 6 h after ClO_2 treatment for 6 h reduced the a* and b* value in chili peppers. Rane et al. (2020) observed no change in the visual appearance of almonds subjected to ClO_2 treatment at 17.83 µg O_3/g almond followed by heating at 65 °C. However, the study did not include any quality analysis except for visual appearance which may not indicate the actual variations anticipated in the food matrix following exposure to a gas with high oxidation potential.

The ΔE values, which indicate the color difference, were analyzed to assess the perceptibility of the color changes. The ΔE values for the treatment involving 90% RH at 3 mg/L with mild heating and 80% RH at 5 mg/L concentration for 2 hours were greater than 2 and less than 3.5, suggesting that an inexperienced observer could notice the difference. On the other hand, the ΔE

values for 80% RH at 5 mg/L with mild heating and 90% RH for 3 mg/L for 2 h exposure was greater than 1 but less than 2, indicating their color change could only be picked up by an experienced observer (Mokrzycki & Tatol, 2011). Similarly, Wason & Subbiah (2023) estimated a ΔE value of 3.56- 4.34 in ClO_2 treatment with chia seeds at 10 mg/L concentration for 5 h at different relative humidities (60, 70 and 80% RH). Their results further indicated that the color difference can be perceived by human eye because the treatment conditions were severe compared to those used in the present study.

c. Fatty acids, peroxide and phenolic content

The nutritional analysis of chia seeds treated to ClO_2 treatment either with or without mild heating are presented in Table 5. The total fat and omega 6 fatty acid profiles in chia seeds remained unaltered irrespective of the treatment condition used. However, a significant decrease in omega 3 fatty acid content was observed in treatment involving ClO_2 treatment at 5 mg/L under 80% RH for 2 h (10.15 ± 7.60 g/100g) compared to control (17.16 ± 0.84 g/100g). No significant differences were observed under other treatment conditions, indicating that RH level and mild heating at 60 °C for 2 h following ClO_2 treatment did not show a positive correlation with omega-3 fatty acid content. Interestingly, studies by Wason & Subbiah (2023) showed that the ClO_2 treatment even at 10 mg/L concentration exposed for 5 h did not significantly influence the total fat, omega-3 and omega-6 values in treated chia seeds.

The peroxide value for chia seeds significantly increased ($p< 0.05$) by more than 10 folds after exposure to ClO_2 treatment, ranging from 87.60 ± 6.29 to 112.61 ± 11.56 mM Equiv I2 per kg compared to the untreated samples (8.34 ± 10.45). The increase in peroxide value can be attributed to the lipid oxidation products in chia seeds following primary oxidation during ClO_2 treatment. Another non-thermal inactivation technology, UV-C treatment did not significantly

impact the lipid peroxidation and volatiles in whole milk (Pendyala et al., 2022a,b; Vashisht et al., 2022). Wason & Subbiah (2023) also observed a significant increase in peroxide values in chia seeds after ClO_2 treatment at 10 mg/L concentration under 70% RH. The high oxidation potential of ClO_2 on food matrices (Ganiev et al., 2005) explains the differences observed in the treated chia seeds.

The total phenolics in chia seeds after ClO_2 treatment at 5 mg/L concentration at 80% RH exposed for 2 h was significantly higher (0.548 GAE/g) than the control samples (0.392 GAE/g). However, this change was not significant among other treatments. Overall, the quality of chia seeds was minimally affected upon treatment to ClO_2 at the tested condition with or without mild heating at 60 °C for 2 h.

d. Germination ability

The ClO_2 gas treatment as well as sequential mild heating at 60 °C did not significantly affect the germination% of chia seeds (Table 4.). The germination % of control chia seed was 70.0 ± 29.9 while for treated chia seeds, it ranged from 62.0 ± 29.5 to 74.0 ± 35.2. However, when radish seeds were subjected to ClO_2 treatment followed by a higher temperature of 80 °C for 5 h, the germination rate was significantly reduced as compared to the control (Park et al., 2018). The authors further reported that the sequential treatment did not significantly affect the germination ability of alfalfa seeds. Therefore, the effects of ClO_2 treatment followed by mild heating are specific to each product and should be evaluated individually.

e. Residue analysis

The residue analysis of ClO_2 treated chia seeds samples included the estimation of byproducts such as chlorite and chlorate as the oxidation of ClO_2 can lead to the formation of

chlorate and chlorite ions which can further form chloride ions (Gomez-Lopez et al., 2009). The analysis performed for samples treated to ClO_2 at 3 and 5 mg/L at 80 and 90% RH respectively with and without mild heating is given in Table 6. The residues were estimated on 1, 3, 7 and 28 days of storage period to understand the dissipation of byproduct residues over time. The results revealed that the chlorate residues in chia seed samples were significantly higher (0.03 ± 0.003 mg/kg) for ClO_2 treatment at 5 mg/L concentration exposed for 2 h, with or without mild heating compared to control chia seeds which were below the detection limit after 1 day of storage period. However, no noticeable change in chlorate residue was observed among treatments after 3, 7, and 28 days of storage.

The chloride residues in the ClO_2 treated chia seeds after 1 day of storage significantly differed ($p < 0.05$) for treatments performed at 5 mg/L concentration exposed for 2 h at 80% RH both with and without mild heating, compared to the control. However, the chloride residues did not show significant differences within the treatments over 1, 3, 7 and 28 days of storage period. The chlorite residues in chia seeds were below the detection limit of 0.0005 mg/Kg in chia seeds after 1 day of storage in all the treatments except for ClO_2 treatment at 3 mg/L concentration at 90% RH followed by mild heating at 60 °C for 2 h which recorded 0.006 ±0.0001 mg/Kg. On the other hand, the maximum residue (0.010 ± 0.00 mg/Kg) was observed in the same treatment after 3 days of storage of chia seeds. It was observed that the chlorite residues were significant among storage periods and were consistently higher on day 3. However, the residue values were well below the maximum residue limit of 1 mg/L for chlorite ion and 0.8 mg/L for residual ClO_2 gas set by the National Primary Drinking Water regulations (NPDWR).

8.5 Conclusions

The results of this study demonstrate that ClO_2 gas treatment followed by mild heating can effectively reduce *Salmonella* on chia seeds. The combination of ClO_2 gas treatment and post-treatment mild heating led to significant *Salmonella* reductions (>1 log CFU/g addition reduction), offering a promising alternative to thermal decontamination methods. Storage of ClO_2 treated chia seeds under ambient conditions further enhanced the microbial reduction over time, additional 0.8 – 1 log reduction. The best treatment condition was found to be ClO_2 treatment at 90% RH, 3 mg/L for 2 h followed by mild heating at 60 °C for 2 h and subsequent 7 days of ambient storage which provide close to 5 log reduction on chia seeds. The findings indicate that ClO_2 treatment, especially at higher concentrations and relative humidity levels, can induce lipid oxidation and affect the peroxide value of chia seeds. However, the formation of ClO_2 byproducts in the treated seeds was within acceptable limits. Overall, this research provides valuable insights into the efficacy and quality aspects of ClO_2 gas treatment for decontaminating chia seeds, which can inform the food industry in implementing effective strategies to ensure the safety and quality of chia seed products.

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Table 8.1. *Salmonella* reduction (log CFU/g) after ClO_2 treatment, post mild heating and subsequent storage for 7 days.

ClO_2 treatment	**Mild heating temperature (°C), time (h)**	**ClO_2 treatment**	**ClO_2 +mild heating**	**ClO_2 +mild heating + storage (7 d)**
Trt A. 80% RH, 5 mg/L, 2 h	RT (23 °C)	1.80 ±0.11[a]		2.87 ± 0.15[a]
	40 °C (1 h)		2.63 ±0.20[b]	3.02 ±0.24[ab]
	40 °C (2 h)		2.44b ±0.24[b]	3.20 ±0.27[b]
	60 °C (1 h)		2.72 ±0.10[b]	3.49 ±0.26[c]
	60 °C (2 h)		3.12 ±0.23[d]	3.63 ±0.33c
Trt B. 90% RH, 3 mg/L, 2 h	RT (23 °C)	2.82 ±0.16[a]		3.81 ±0.22[a]
	40 °C (1 h)		3.16 ±0.35b	3.99 ±0.21ab
	40 °C (2 h)		3.26 ±0.17b	4.11 ±0.28b
	60 °C (1 h)		3.45 ±0.11c	4.50 ±0.06c
	60 °C (2 h)		3.96 ±0.07d	4.70 ±0.16c

Values (mean ± SD) in the same column that are followed by the same lowercase letter for Trt A. or B. are significantly different ($P < 0.05$).

Table 8.2. Mixed model analysis of factors influencing pathogen reduction in ClO_2 treated Chia seeds subjected to mild heating and subsequent storage time".

Source	dF	F Ratio	Prob > F
ClO_2 trt	1	868.87	<.0001*
Mild heating	4	62.39	<.0001*
Storage time	7	57.96	<.0001*
ClO_2 trt*Mild heating	4	7.00	<.0001*
ClO_2 trt*Storage time	7	10.54	<.0001*
Mild heating*Storage time	28	3.32	<.0001*
ClO_2 trt*Mild heating*Storage time	28	2.81	<.0001*

Table 8.3. ANOVA table and significant contrasts for log reduction on day 7 of ambient storage after ClO2 treatment.

Effect	DF Num	DF Den	F Value	Pr > F
C	2	6	620.94	<.0001
H	4	24	32.58	<.0001
C*H	8	24	1.89	0.1097

Label	Estimate	Standard error	DF	t Value	Pr > |t|
C1 (ClO_2) = chl trt vs. control	3.58	0.10	6	34.30	<.0001
C2 (ClO_2) = ClO_2 A. vs. ClO_2 B.	-0.98	0.12	6	-8.10	0.0002
H - Ambient (am) vs. treated (trt)	-0.38	0 05	24	-6.89	<.0001
H - 40 °C vs. 60 °C	-0.43	0.05	24	-8.80	<.0001
H - 1 h vs 2 h	-0.11	0.05	24	-2.34	0.0281
C1 x H-am.vs.trt	-0.33	0.12	24	-2.83	0.0093
C1 x H-40 °C vs.60 °C	-0.2106	0.10	24	-2.03	0.0531

C - ClO_2 treatment
H - mild heating temperature- time combination

Table 8.4. Effect of ClO_2 treatment on the quality of chia seeds.

	L*	a*	b*	ΔE	a_w	MC (%)	Germination (%)
Control	49.43 ± 1.01^{b}	1.23 ± 1.10^{a}	4.97 ± 0.51^{b}		0.53 ± 0.001^{b}	7.26 ± 0.07^{a}	70.0 ± 29.9^{a}
Trt A. 80%RH, 5 mg/L gas, 2 h exposure	50.73 ± 0.57^{ab}	1.73 ± 0.31^{a}	6.03 ± 0.06^{a}	2.20 ± 0.80^{a}	0.55 ± 0.004^{a}	7.76 ± 0.40^{a}	70.0 ± 32.9^{a}
Trt A followed by heating at 60 °C, 2 h	50.33 ± 0.60^{ab}	1.63 ± 0.29^{a}	5.77 ± 0.38^{ab}	1.95 ± 0.46^{a}	0.18 ± 0.007^{c}	4.43 ± 0.26^{b}	74.0 ± 35.2^{a}
Trt B. 90%RH, 3 mg/L gas, 2 h exposure	50.47ab ± 0.60^{ab}	1.37 ± 0.06a	5.44 ± 0.24^{ab}	1.72 ± 1.15^{a}	0.56 ± 0.002^{a}	7.83 ± 0.28^{a}	66.7 ± 23.2^{a}
Trt B followed by heating at 60 °C, 2 h	51.4 ± 0.17^{a}	1.60 ± 0.40^{a}	5.80 ± 0.26^{ab}	2.49 ± 0.92^{a}	0.21 ± 0.007^{c}	4.79 ± 0.08^{b}	62.0 ± 29.5^{a}

Values (mean ± SD) in the same column that are followed by the same lowercase letter are significantly different ($P < 0.05$).

Table 8.5. Fatty acid composition, peroxide value and total phenolics in ClO_2 treated chia seeds.

	Total Fat (g per 100g)	Omega-3 (g per 100g)	Omega-6 (g per 100g)	Peroxide Value (mM Equiv I2 per kg)	Total phenolic content (mg Gallic acid /g)
Control	28.20 ±0.52^{a}	17.16 ±0.84^{a}	4.67 ±0.19^{a}	8.34 ±10.45^{c}	0.40 ±0.13^{b}
Trt A. 80%RH, 5 mg/L gas, 2 h exposure	28.10 ±1.42^{a}	10.15 ±7.60^{b}	4.72 ±0.25^{a}	93.01 ±7.43^{b}	0.42 ±0.08^{ab}
Trt A followed by heating at 60 °C, 2 h	28.15 ±1.84^{a}	15.14 ±1.05 ^{a}	4.91 ±0.46^{a}	87.60 ±6.29^{b}	0.55 ±0.11^{b}
Trt B. 90%RH, 3 mg/L gas, 2 h exposure	29.96 ±3.34^{a}	17.57 ±2.22 ^{a}	5.42 ±0.98^{a}	112.61 ±11.56^{a}	0.32 ±0.14^{b}
Trt B followed by heating at 60 °C, 2 h	29.13 ±3.03^{a}	16.89 ±1.94^{a}	5.09 ±0.60^{a}	101.62 ±14.43^{ab}	0.45 ±0.07^{ab}

Values (mean ± SD) in the same column that are followed by the same lowercase letter are significantly different ($P < 0.05$).

Table 8.6. Chlorine dioxide byproducts after ClO_2 treatment at varying conditions followed by mild heating during storage over a month.

ClO_2 byproducts	Storage (d)	Untreated	Trt A. 80%RH, 5 mg/L gas, 2 h exposure	Trt A followed by heating at 60 °C, 2 h	Trt B. 90%RH, 3 mg/L gas, 2 h exposure	Trt B followed by heating at 60 °C, 2 h
Chlorate (mg/g)	Day 1	0.00^{b}	0.03 ± 0.003^{aA}	0.031 ± 0.019^{aA}	0.02 ± 0.00^{abA}	0.02 ± 0.003^{abA}
	Day 3		0.03 ± 0.009^{aA}	0.030 ± 0.003^{aA}	0.02 ± 0.00^{aA}	0.03 ± 0.00^{aA}
	Day 7		0.03 ± 0.008^{aA}	0.045 ± 0.039^{aA}	0.04 ± 0.02^{aA}	0.02 ± 0.008^{aA}
	Day 28		0.03 ± 0.007^{aA}	0.02 ± 0.009^{aA}	0.02 ± 0.005^{aA}	0.02 ± 0.006^{aA}
Chloride (mg/g)	Day 1	0.026 ± 0.011^{b}	$0.22aA \pm 0.04^{aA}$	0.204 ± 0.11^{aA}	0.17 ± 0.019^{abA}	0.14 ± 0.02^{abA}
	Day 3		$0.15aA \pm 0.05^{aA}$	0.143 ± 0.01^{aA}	0.14 ± 0.03^{aA}	0.17 ± 0.02^{aA}
	Day 7		$0.19aA \pm 0.07^{aA}$	0.272 ± 0.20^{aA}	0.24 ± 0.08^{aA}	0.17 ± 0.04^{aA}
	Day 28		$0.19aA \pm 0.04^{aA}$	0.14 ± 0.03^{aA}	0.16 ± 0.05^{aA}	0.16 ± 0.04^{aA}
Chlorite (mg/g)	Day 1	0.0039 ± 0.0035^{a}	0.0001 ± 0.0002^{aB}	0.0003 ± 0.0002^{aD}	ND^{aC}	0.006 ± 0.0001^{aB}
	Day 3		0.005 ± 0.0005^{bA}	0.006 ± 0.00018^{aA}	0.004 ± 0.0002^{bA}	0.010 ± 0.000^{aA}
	Day 7		0.0005 ± 0.0003^{bB}	0.0009 ± 0.0002^{aC}	0.0005 ± 0.0001^{bB}	0.0010 ± 0.0010^{aB}
	Day 28		0.0004 ± 0.0003^{cB}	0.0019 ± 0.0002^{aB}	0.0005 ± 0.0005^{cB}	0.001 ± 0.0002^{bB}

BDL: below detection limit. Detection limits for chlorate, chloride and chlorite are 0.0003, 0.002, 0.0005 mg/L.
Values (mean ± SD) in the same row that are followed by the same lowercase letter are significantly different ($P < 0.05$).
Values (mean ± SD) in the same column that are followed by the same uppercase letter are significantly different ($P < 0.05$).

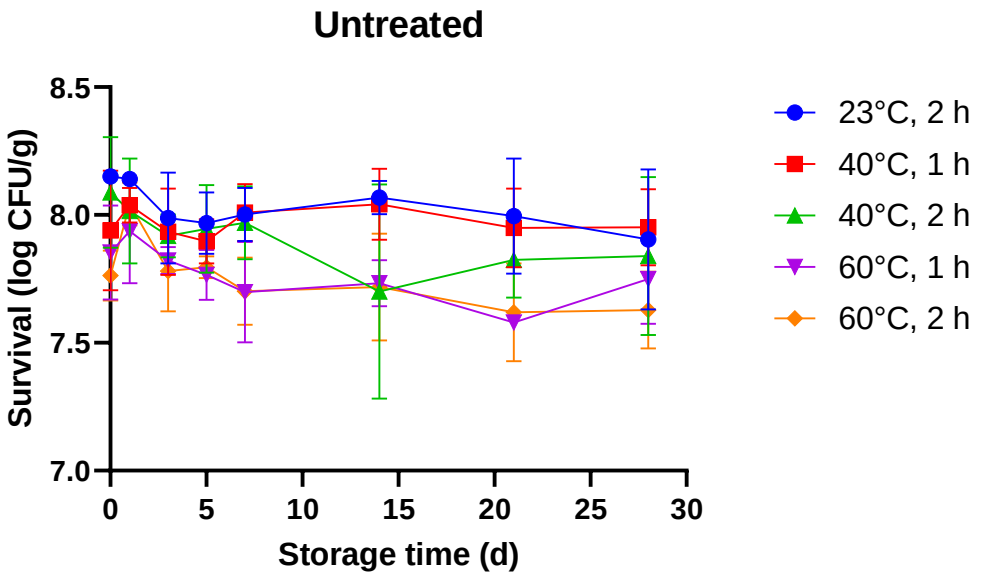

Fig 8.1. Effect of mild heating alone on Salmonella survival on control chia seed.

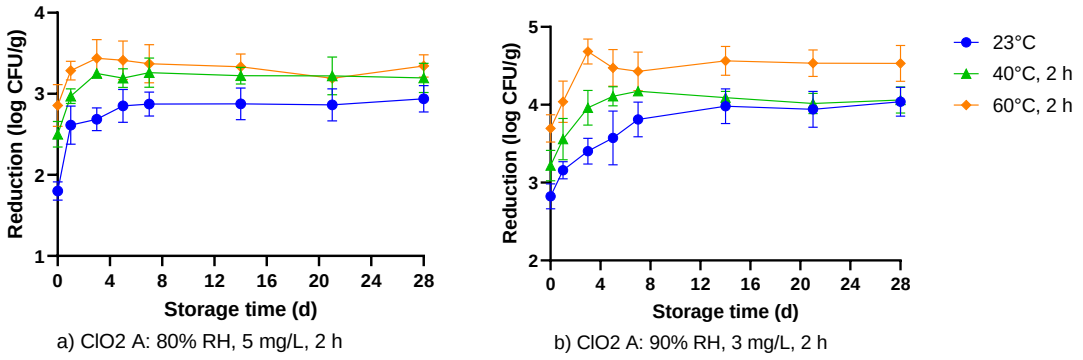

Fig 8.2. *Salmonella* reduction after mild heating during subsequent storage at ambient conditions a) ClO_2 A: 80% RH, 5 mg/L, 2 h; b) ClO_2 A: 90% RH, 3 mg/L, 2 h.

Chapter 9. Effect of temperature and relative humidity on ethylene oxide inactivation of *Salmonella* and *Enterococcus faecium* NRRL B-2354 on chia seeds

9.1 Abstract

Ethylene oxide (EtO) sterilization is commonly used by the food industry to inactivate pathogens in spices, but its efficacy on edible seeds, a high-risk food commodity, remains unexplored. This study aimed to evaluate the antimicrobial efficacy of EtO gas on chia seeds and assess the impact of processing parameters on microbial reduction, byproduct formation, and seed quality. Further, *Enterococcus faecium* NRRL B-2354 was evaluated for its suitability as a surrogate for *Salmonella*. Chia seeds were inoculated with a cocktail of five strains of *Salmonella enterica* or *E. faecium*. The inoculated sample was treated with EtO at different combinations of relative humidity (30, 40, and 50%) and temperature (46, 53, and 60 °C) for exposure times ranging from 2 to 120 min. The results showed that EtO treatment effectively reduced the bacterial populations on chia seeds, with the inactivation trend following a non-linear pattern that was described well by the Weibull model. Response surface model indicated that relative humidity (RH) and exposure time had significant (P <0.05) main and interaction effects on the bacterial inactivation, while temperature had smaller effects on bacterial inactivation; higher RH, temperature, and longer exposure time resulted in greater log reductions of *Salmonella* and *E. faecium*. The treatment time required for a 5-log reduction of *Salmonella* was less than 10 minutes at 50% RH and 53-60 °C. *E. faecium* was found to be a reliable surrogate for *Salmonella* during EtO treatment. Fatty acid composition, protein content, color, and germination ability of chia seeds were not impacted significantly (P >0.05) by EtO fumigation. These findings suggest that EtO gas can effectively inactivate *Salmonella* on chia seeds while preserving their viability and quality.

Keywords: non-thermal, low moisture food safety, antimicrobial, gaseous technology, quality

9.2 Introduction

Edible seeds are an immense source of nutrition such as protein, fiber, healthy fats, and vitamins. They can be shelf stable for years due to their low water activity, below 0.70 (Beuchat et al., 2013). However, edible seeds and their products were implicated in foodborne illness outbreaks and recalls (Acuff et al., 2023; Olaimat et al., 2020; Wason et al., 2021). Between 2012 and 2020, contaminated nuts and seeds were found to be the source for more than half of all outbreaks and recalls tied to these low-moisture foods. More than 80% of the outbreaks associated with low-moisture foods were linked to *Salmonella* contamination (Acuff et al., 2023). Foodborne outbreaks related to edible seed contamination were reported in alfalfa seeds (Taormina et al., 1999), in chia seeds sprouted powder (Harvey et al., 2017), fenugreek sprouts (King et al., 2012), sesame seed products such as tahini and halva (Unicomb et al., 2005), alfalfa sprouts (Proctor et al., 2001), mung bean sprouts (PHAC, 2006) and in clover sprout seeds (Brooks et al., 2001). Although the majority of outbreaks are linked to sprouted seeds, contamination most likely occurs in the seeds in the agricultural fields (FDA, 2017; NACMCF, 1999). During sprouting, seeds are soaked in water which provides a conducive environment for microbial growth. Therefore, a small number of *Salmonella* cells could proliferate to large numbers when seeds are sprouted (Sarno et al., 2021; Bintsis, 2018).

Chia seeds are one of the edible seeds that have gained immense popularity owing to their high nutritional composition (Motyka et al., 2023; Kulczynski et al., 2019). *Salmonella* contamination of chia seeds resulted in a foodborne outbreak that infected 94 individuals from multiple states in the US and Canada during 2013-2014. Investigation on the foodborne outbreak with chia seeds sprouted powder indicated seeds that did not undergo any processing were the

source of contamination. Therefore, decontamination of chia seeds is imperative to minimize the risk of foodborne illness related to the uptake of infected raw chia seeds. Chia seeds have been considered microbiologically safe due to their low a_w and high fat content which can protect the pathogenic microorganisms from thermal inactivation (Santillana Farakos et al., 2013).

Thermal processing is widely used for decontaminating low-moisture foods and ingredients. Kottapalli et al. (2020) reported that roasting sunflower seeds at 135 °C for 45 min gave >7 log reduction of *Salmonella*. Based on microbial risk assessment, the treated sunflower seeds must not pose any food safety risks (Kottpalli et al. 2020). However, the quality of the sunflower seeds was not analyzed. Due to the high fat content in the majority of edible seeds, thermal treatment can lead to fat oxidation resulting in the production of off-flavors and other by-products. Mesias et al. (2023) reported acrylamide formation in chia seeds treated at 160 °C for 15 min. Moreover, heat energizes the water molecules present in chia seeds leading to the absorption of water by the soluble fibers. This phenomenon leads to fibers swelling and forming a gel-like structure. The gelling can alter the texture of chia seeds and affect consumer acceptability. Therefore, non-thermal interventions need to be investigated for decontaminating chia seeds.

Ethylene oxide (EtO) fumigation is a dry process broadly used as an inactivation method by the food industries for mitigating *Salmonella* and *E.coli* in spices (Schweiggert et al., 2007). In the United States, approximately 50% of spices undergo EtO treatment every year, as reported by American Spice Trade Association (ASTA, 2017). Previous studies have explored the use of EtO for decontaminating black peppercorns (Wei et al., 2021a; Newkirk, 2016); cumin seeds (Chen et al., 2020). Newkirk (2016) observed significant variability in *Salmonella* inactivation among two separate processing facilities that treated black peppercorns with EtO. The

effectiveness of EtO decontamination is influenced by the processing parameters such as relative humidity (Gilbert et al., 1964; Mendes et al., 2007; Chen et al., 2020; Wei et al., 2021a), temperature (Heider et al., 2002; Chen et al., 2020), and treatment time (Chen et al., 2020; Wei et al., 2021a). To ensure sufficient pathogen inactivation and prevent issues related to EtO residues and byproducts in treated foods, the Environmental Protection Agency (EPA, 2023) has established guidelines for processors to adhere to label requirements for EtO fumigation. Studies have demonstrated that EtO treatment at 50% RH achieved ~5 log reduction of *Salmonella* and *E. faecium* at 46-60 °C temperature on cumin seeds and black peppercorns within 20 min (Wei et al., 2021a; Chen et al., 2020). On the other hand, gaseous chlorine dioxide required a treatment time of 300 min at 15 mg/L chlorine dioxide (ClO_2) concentration and 80% RH to attain >5 log *Salmonella* reduction on the same products (Wei et al., 2021b). For chia seeds, ClO_2 treatment at a concentration of 10 mg/L and 80% RH for 300 min resulted in a *Salmonella* reduction of less than 4 log CFU/g (Wason & Subbiah, 2023). A significant increase in peroxide value was also reported in chia seeds due to fat oxidation during the ClO_2 treatment. Unlike ClO_2 whose high oxidizing ability is responsible for biocidal activity, the main mechanism of EtO inactivation is by alkylation. EtO adds an alkyl group to sulfhydryl, hydroxyl, amino, and carboxyl groups of bacterial cells preventing cellular metabolism and leading to cell lysis (Wason et al., 2021). Additionally, the use of EtO does not impact the flavor or appearance of food products (ASTA, 2017). EtO is also used to sterilize other food products such as certain dried herbs, dried vegetables, some edible seeds, and walnuts (EPA, 2023a). However, EtO efficacy on pathogen inactivation in chia seeds remains unexplored. Furthermore, the EPA has emphasized the need to investigate alternative decontamination strategies for commodities that typically have high pathogen loads and currently lack an effective decontamination strategy (EPA, 2023b). Because

there is currently no standard processing method available for chia seeds, this study would provide useful information on the processing parameters for effective decontamination of chia seeds using EtO gas.

In addition to achieving desired microbial inactivation, it is important to assess the impact of EtO on the quality of treated chia seeds, considering their high nutritional value in terms of fatty acids and proteins. Furthermore, chia seeds are consumed as sprouts, and therefore germination ability or seed viability becomes a significant parameter for the chia seed industry. During fumigation, EtO can react with moisture to produce ethylene glycol while interaction with chlorides present in food could lead to ethylene chlorohydrin (ECH) production (EPA, 2020). Under the Food Quality Protection Act (FQPA) 1996, EPA has established tolerance limits for EtO and ECH residues on spices and seeds, which are set at 7 ppm and 940 ppm, respectively (EPA, 2023b).

The Food Safety Modernization Act mandates that industries develop validation programs as part of their preventive control. However, considering the risk of product contamination of *Salmonella* during validation studies, a non-pathogenic surrogate should be explored for adoption by the industries as an alternative to *Salmonella*. Surrogate microorganisms should possess certain characteristics such as being non-pathogenic (Biosafety level 1); exhibiting similar inactivation kinetics as the pathogen of concern; being easy to propagate and being easily distinguishable from other microorganisms. *E. faecium* B2354 has shown higher resistance to gaseous technologies than *Salmonella* and thus has been confirmed as an appropriate surrogate for *Salmonella* in low-moisture food products including ground black pepper (Wei et al., 2021a), cumin seeds (Wei et al., 2021b, Chen et al., 2020), dried basil leaves (Verma et al., 2022), and chia seeds (Wason & Subbiah, 2023). It is critical to evaluate the

surrogate for each food matrix and pathogen processing combination. There is little information on its use as a surrogate for Salmonella in chia seeds Therefore, it is essential to validate the suitability of *E. faecium* as a surrogate for gaseous EtO treatment of chia seeds. The objectives of the study were to a) investigate the efficacy of EtO gas to inactivate *Salmonella* on chia seeds, b) evaluate whether *E. faecium* is a reliable surrogate to *Salmonella*, and c) assess the influence of EtO on the byproduct formation and seed's germination and nutritional quality.

9.2 Materials & Methods

9.2.1 Bacterial strains and inoculation of chia seeds

Organic chia seed samples manufactured by Better Body Foods, Lindon, UT were bought from online sellers and kept at room temperature. Chia seeds from three different production lots (a mixture containing both black and white seeds) were utilized as replicates. Five serotypes of *Salmonella* enterica namely *S.* Agona 447967, *S.* Reading Moff 180418, *S.* Tennessee K4643, *S.* Montevideo 488275, and *S.* Mbandaka 698538 were selected for inoculation of chia seeds. These serotypes were chosen as they were linked to foodborne illness outbreaks in low moisture foods in the past (Wei et al., 2019; Verma et al., 2020). As a non-pathogenic surrogate for *Salmonella*, *E. faecium* NRRL B-2354 was used during EtO treatment in this study.

The inoculum preparation was similar to those earlier described in detail by Verma et al. (2021). New frozen cultures were used to repeat the procedure, inoculating samples from various lots for biological replication. Inoculation of chia seeds was performed by spraying 6 mL inoculum over a layer of chia seeds (300 g) using a manual spray head (ps20-410-natural, Midwest Bottle, Garrison, KY) fitted on a centrifuge tube (339650, Thermo Fisher Scientific, Waltham, MA). Thereafter, the samples were manually mixed for at least 10 minutes and placed

inside a RH chamber (Lau & Subbiah, 2019) for equilibrating the water activity to 0.53 (native a_w). The samples were stored inside the RH chamber for a minimum of 3 days to allow chia seeds to reach native water activity and also for the bacterial population to stabilize in the sample.

9.2.2 Ethylene oxide treatment

Ethylene oxide treatment was carried out in a Steri-Vac 5XL gas sterilizer from 3M Company, St. Paul, MN as described in Chen et al. (2020). Two grams of inoculated chia seeds were put in a heat-sealable tea bag for EtO fumigation. The influence of process parameters on *Salmonella* inactivation was evaluated by using a full factorial experimental design with three levels of temperature and RH, and five levels of exposure time were performed. To comply with ASTA recommendations (ASTA, 2009; Honeywell, 2008), the treatment temperature was set at or above 46 °C (115 °F). The treatment was conducted at different combinations of temperature (46, 53, 60 °C) and RH (30, 40, 50%). The maximum exposure time at each RH level was selected to reduce *Salmonella* by 3-5 log CFU/g. For instance, the samples were exposed for 0-120 min, 0-60 min, and 0-10 min for RH levels at 30, 40, and 50%, respectively due to rapid inactivation at higher RH.

The treatment process consisted of three steps - preconditioning, gas exposure, and aeration phase. During pre-conditioning, the chamber is set at target temperature and humidity levels and vacuumed to set pressure inside the chamber. The use of vacuum conditions has been demonstrated to enhance gas penetration (Fichet et al., 2007). Ethylene oxide cartridge (4-100, 3M 166 Company, Saint Paul, MN) was punctured after achieving the desired levels of temperature and RH to inject the gas inside the chamber. Based on the volume of the chamber (136 L) used, the gas concentration achieved inside the chamber was 735.3 mg/L. Following the

treatment, aeration was performed for 8 h as mandated by the equipment manufacturer for safety concerns. During aeration, EtO gas was flushed out from the chamber by passing air. The air along with EtO gas was evacuated to the outside of the building through the ceiling.

After the EtO treatment, chia seeds were diluted with 0.1% buffered peptone water in the ratio of 1:30 in a sterile Whirl-177 Pak bag (Nasco, Fort Atkinson, WI) and homogenized in a stomacher. Due to gelling Lau et al. (2021) recommended using a higher dilution rate (1:30) for chia seeds to recover bacteria efficiently. For enumeration, serial dilutions were prepared and plated on mTSAYE (TSAYE supplemented with ammonium iron citrate (Sigma-Aldrich, Co., 181 MO, USA), and sodium thiosulfate (Fisher Chemical, NJ, USA) for *Salmonella* inoculated samples (Garcia et al., 2022). For *E. faecium* inoculated samples, plating was performed on eTSAYE (TSAYE supplemented with ammonium iron citrate, and esculin hydrate (Acros Organics, NJ, USA). The plates were then incubated at 37°C for 24 h.

9.2.3 Model analysis

The survival data of *Salmonell*a or *E. faecium* NRRL B-2354 on chia seeds were fit using the Weibull model (Eqn. 1).

$$\log_{10}\left(\frac{N}{N_0}\right) = -\left(\frac{t}{\delta}\right)^p \quad (1)$$

N and N_0 (CFU/g) represent the microbial populations at time t (isothermal treatment time, min) and initial population, respectively; δ is the scale parameter, denoting the time required for first log reduction and p is the shape parameter indicating the concavity ($p < 1$) or convexity ($p > 1$) of the inactivation curve over time. Parameter estimation was performed using the GInafit - Version 1.6 add-in freeware (Geeraerd et al., 2005).

A fixed value approach was used to re-estimate the δ-value (delta*) by fixing the p-value as the average value from all the survival curve to remove any structural variations (Gautam et al., 2020; Zhang et al., 2020). The re-estimated δ*-values were further used for model analysis.

The influence of the process parameters (temperature and RH) on δ*-value of *Salmonella* inactivation in chia seeds was evaluated using modified version of a Bigelow-type relationship (Gaillard et al., 1998) model (Eqn. 2).

Modified Bigelow model

$$\delta^*(T, RH) = \delta^*{}_{ref} \cdot 10^{\frac{RH_{ref}-RH}{z_{RH}}} \cdot 10^{\frac{T_{ref}-T}{z_T}} \quad (2)$$

where T_{ref} and RH_{ref} are the optimized reference temperature and relative humidity, z_{RH} or $z_{T\,(°C)}$ is the RH or temperature increment needed to reduce the δ -values by 90%. All parameters for the Bigelow-type model were estimated using OLS minimization with nlinfit in MATLAB 2019 (The Mathworks, Inc., Natick, MA).

Response surface model (Eqn. 3) was developed for the primary models and only those parameters were included, which had a significant effect at $p < 0.05$. Response surface model was fitted in an open-source statistical software R (https://www.R-project.org/; Lenth, 2009).

$$\delta^*(T, RH) = \beta_o + \beta_1 * T + \beta_2 * RH + \beta_3 * T^2 + \beta_4 * RH^2 + \beta_5 * T * RH \quad (3)$$

To assess the goodness of fit of the model, the corrected Akaike information criterion (AIC_c), adjusted R^2, and RMSE (root mean square error, log CFU/g) were calculated.

9.2.4 Quality analysis

The impact of EtO treatment on the quality of chia seeds was assessed in terms of color, fatty acid composition, protein content, and germination ability. Uninoculated chia seeds were treated at conditions providing the highest log reduction at each RH and were used for the quality analysis. In addition, the residue and by-product formation were also determined to ensure the levels below the tolerance limit as per EPA. Since the byproduct formation is known to be dependent on moisture levels, the assessment of residues were conducted at different RH levels.

a. Color value

A colorimeter (Konica Minolta, model: BC-10, Osaka, Japan) was used to measure color (L*, a*, b*) at five random locations on a layer of chia seeds spread on a Petri dish. The protocol used for color analysis was earlier described in Wason et al. (2022b).

b. Protein content

The estimation of protein content was based on AOAC method 990.03, which uses the Dumas method of analysis. Chia seeds sample (0.2 g) was combusted within a LECO FP528 Nitrogen Determinator, and the percent nitrogen in the sample was determined by the system. This value was converted to percent protein by multiplying by the nitrogen conversion factor (6.25).

c. Fatty acid composition

The fatty acid in EtO-treated chia seeds followed the procedure outlined in Wason & Subbiah (2023). In summary, lipids were extracted from both treated and control chia seed samples using a methanol and chloroform solution (1:2) according to the method described by Folch et al. (1957). Ground chia seed samples (5 g) underwent a 2-hour extraction process with 150 mL of

the solvent mixture. The resulting extract was purified by adding moisture, filtering out solids, passing the solvent through anhydrous sodium sulfate, and drying the solvent using a rotovap to obtain the lipid extract.

The fatty acids in the extract were analyzed using the standard AOAC method 996.06 (AOAC, 2000). Approximately 100 to 200 mg of the lipid extract was mixed with ethanol, pyrogallol, and an internal standard solution in chloroform. Hydrochloric acid was added, and the sample was digested at 75 °C for 40 minutes. Lipids were extracted using a diethyl ether and hexane solution, dried, and then derivatized with boron trifluoride in methanol. The resulting samples were mixed with a solution of water, anhydrous sodium sulfate, and hexane. The organic phase was collected and injected into an Agilent 7820A GC system with a Flame Ionization Detector and a Supelco SP-2560 column. The GC parameters included specific temperature settings for the injection port, detector, and oven. The peaks obtained were identified and compared to an external standard.

d. Percent germination

Geneve et al. (2017) method was used to assess the germination ability of untreated and EtO-treated chia seeds. On a Petri dish, 50 chia seeds were dispersed over a wet blotting paper to facilitate germination at room temperature and RH. The seeds that germinated were counted at regular interval for a week until no increase in the number of sprouted seeds were noticed. Germination capacity was calculated as given below:

$$\text{Germination capacity} = \frac{\text{Number of germinated seeds}}{\text{Total number of seeds}} \text{ x } 100$$

e. EtO residue and byproducts

The EtO and ethylene chlorohydrin residues in both the treated and non-treated (control) chia samples (1.0 g) were directly analyzed using Thermo Scientific Gas Chromatography (Trace 1300) with an ISQ Mass Selective Detector (GC-MS) in the headspace injection mode. The samples were placed in 20 mL headspace vials with magnetic screw caps and incubated at an agitator temperature of 68 °C for 15 min. The gas released from the sample during incubation (2.0 mL) was injected into the GC-MS system using the splitless injection method. For the analysis, a CP-Wax 52 CB column (50m x 0.53mm ID, 2.0 µm) was utilized. The oven temperature was programmed to increase from 40 to 220 °C at a heating rate of 15 °C/min. The total run time for the analysis was 12 minutes. The inlet temperature was set to 230 °C. The ionization energy was 70 eV, and the mass range monitored was 10-550 amu. The solvent delay was set to 1.0 minutes. The mass detector's mass transfer line and ion source temperatures were maintained at 250 °C and 200 °C, respectively.

The peak shape of ethylene glycol in the chromatogram obtained from direct headspace injection was not good for accurate quantitation. Therefore, ethylene glycol in the samples and standards was derived by BSTFA/TMCS before analysis. For this purpose, a new set of chia seed samples were used. The 10 µl BSTFA/TMCS reagent and 500 µl pyridine were added into each sample and vortexed for 1 min before analysis. The headspace method and GC-MS conditions used for ethylene chlorohydrin residues were also used for ethylene glycol analyses. The detection limits for residues of ethylene oxide, ethylene chlorohydrin, and ethylene glycol are 15.30, 2.37, 10.12 ppm.

9.4 Results and Discussion

9.4.1 Antimicrobial efficacy of Ethylene oxide gas

The initial populations of *Salmonella* and *E. faecium* on chia seeds one day after inoculation were 8.0 ± 0.2 and 7.8 ± 0.3 respectively. According to Wason & Subbiah (2023), the bacterial population in chia seeds stabilized after three days of inoculation and remained stable for two weeks in the RH chamber set at 53% RH. Therefore, chia seeds were stored inside the RH chamber at room temperature and 53% RH for at least 3 days before EtO treatment. The moisture content and water activity were measured as 7.23 ± 0.11% (w.b) and 0.53 ± 0.01, respectively.

The survival trend of *Salmonella* and *E. faecium* on chia seeds after EtO treatment at different RH (30, 40, and 50%) and temperature (46, 53, and 60 °C) followed a non-linear pattern as depicted in Fig 1. The Weibull model was used to fit the survival data yielding high adjusted R^2 values above 0.95 and RMSE values below 0.48 log CFU/g, except for the case of *Salmonella* at 50% RH and 60°C (Table 1.). The shape factor (p value) of the Weibull model varied across all treatment conditions, therefore δ value cannot be compared. Notably, the shape factor (p) in the Weibull model was less than 1 for all treatment conditions, except for *Salmonella* at 50% RH and 60°C. This indicates a tailing effect, suggesting that the rate of bacterial inactivation decreased as the treatment time during EtO fumigation of chia seeds increased. The tailing effect was prominent for both bacteria after EtO exposure for 48 min at 30% RH and 24 min at 40% RH. Different bacterial cells may exhibit varying levels of sensitivity to EtO gas, resulting in a range of responses and inactivation rates This variation in bacterial cell response to EtO gas could contribute to this tailing effect, as reported in previous studies on EtO inactivation. Gilbert et al. (1964) investigated the effect of humidity on EtO inactivation of *Bacillus subtilis* spores and reported a non-homogeneous bacterial population

exhibiting varied resistance to EtO. A shape factor less than 1 and the tailing effect were also reported in EtO inactivation studies in black peppercorns (Wei et al. 2021) and cumin seeds (Chen et al., 2021). In general, δ values of *Salmonella* decreased with an increase in RH and temperature, indicating the significance of processing parameters on microbial inactivation. For example, at 46 °C, as RH increased from 30 to 40%, the δ value reduced from 17.25 to 3.75 min. Similarly, an increase in temperature from 46 to 60 °C at 40% RH reduced the δ value from 6.75 to 4.22 min. Previous studies have used the Weibull model to describe the EtO inactivation in black peppercorns (Wei et al., 2021) and cumin seeds (Chen et al., 2021). In contrast, a log-linear model was chosen to describe gaseous chlorine dioxide inactivation in chia seeds (Wason & Subbiah, 2023), black peppercorns (Wei et al., 2021) and dried basil leaves (Verma et al., 2022). The difference in microbial inactivation mechanisms between the two gaseous technologies could contribute to variation in the trend of bacterial inactivation. Chlorine dioxide oxidizes bacterial cell constituents while EtO acts on the sulfhydryl, hydroxyl, amino, and carboxyl groups of bacterial cells by adding alkyl groups to affect the cellular metabolism ultimately leading to cell lysis.

Comparing the thermal processing of chia seeds at 85 °C for 40 min, which provided a 5 log reduction of *Salmonella* (Lau et al., 2021), the EtO treatment required less than 10 min at 50% RH and 53-60 °C for a similar level of microbial inactivation. Additionally, thermal treatment was found to have a significant impact on the quality of chia seeds, affecting fatty acid content and lipid oxidation (Lau et al., 2021). Other non-thermal treatments such as the use of peracetic acid reported a 4-log reduction in *Salmonella* after soaking chia seeds in a 4 mL diluted peracetic acid solution (Hylton et al., 2019). However, a drying step at 70 °C is required after the treatment to bring down the moisture content to its native value. A dry inactivation process was

investigated by Reyes-Jurado et al. (2019) using high-intensity pulsed light for 15 s and showed *Salmonella* reduction by 4 log CFU/g. This technology has its drawback in upscaling the process because of the shadowing effect. In contrast, gaseous technologies including EtO provide a dry non-thermal treatment option that can effectively achieve the desired log reduction while preserving the chia seed viability and preventing gelling of seeds during the treatment.

9.4.2 Comparison of secondary models

To assess the impact of treatment temperature and relative humidity (RH) on the inactivation (δ-value) of *Salmonella* and *E. faecium* during EtO fumigation, a fixed value approach was used. The δ-values were re-estimated by fixing the p-value as the average value (shape parameter (p) = 0.47 for *Salmonella* and 0.45 for *E. faecium*) from all the survival curves to remove any structural variations Wason et al., 2021a). The re-estimated δ-values (δ*)) were used to develop secondary models such as modified Bigelow (Eqn. 2) and response surface model (Eqn. 2) to assess the impact of RH and temperature on the EtO inactivation.

Modified Bigelow model:

$$\textit{Salmonella} \quad \delta^*(T, RH) = 2.17 \cdot 10^{\frac{40-RH}{13.53}} \cdot 10^{\frac{53-T}{38.37}} \tag{4}$$

RMSE: 0.73 min; adj R^2: 0.99; AIC_c: -10.40

$$\textit{E. faecium} \quad \delta^*(T, RH) = 3.44 \cdot 10^{\frac{40-RH}{15.78}} \cdot 10^{\frac{53-T}{29.56}} \tag{5}$$

RMSE: 1.07 min; adj R^2: 0.98; AIC_c: 10.14

Response surface model:

$$\textit{Salmonella} \quad \delta^*(T, RH) = 208.62 - 2.75 * -5.69 * RH + 0.01 * T^2 + 0.04 * RH^2 + 0.04 * T * RH \tag{6}$$

RMSE: 1.09 min; adj R^2: 0.96; AIC_c: 95.46

E. faecium $\delta^*(T, RH) = 310.91 - 5.12 * T - 7.17 * RH + 0.02 * T^2 + 0.04 * RH^2 + 0.06 * T * RH$ (7)

RMSE: 1.07 min; adj R^2: 0.95; AIC_c: 116.84

The response surface model developed for δ*-values obtained for *Salmonella* and *E. faecium* demonstrated that both RH and temperature had significant ($p<0.05$) effects on the inactivation rate of the pathogens, with significant interaction between RH and temperature. For instance, the effect of temperature was more pronounced at higher RH (50%), where the time required to reduce *Salmonella* by 5 log CFU/g at 53 °C was more than double the time required at 60 °C. This indicated increased inactivation at high RH and treatment temperature. In addition, relative humidity showed significant quadratic effects ($p<0.05$) on microbial inactivation. Comparing the models, the modified Bigelow model had a lower RMSE and AICc value for both *Salmonella* and *E. faecium*, suggesting better goodness of fit. Fig 1. illustrates the experimental log reduction and reduction estimated by modified Bigelow Model developed based on Weibull model parameters under all tested conditions. It indicates the model's accuracy in predicting bacterial reduction after EtO treatment.

Fig 1. represents the experimental and Weibull-modified Bigelow model predicted log reduction at three levels of temperature (46, 53, and 60 °C) and RH (30, 40, and 50 %). High RH (50%) conditions provided the most rapid EtO inactivation of *Salmonella* on chia seeds, with >5-log reduction within 10 min. At a lower RH level (30%) and 53 °C, even 120 min of EtO exposure could not provide a 5-log reduction of Salmonella. In general, the model provided a fairly accurate prediction of *Salmonella* inactivation (RMSE =0.73 min) and *E. faecium* (RMSE =1.07 min) except at 50 % RH, where it underpredicted the log reduction. It is important to note

that the log reduction at 50 % RH exhibit greater variability compared to the model predictions. This difference may be attributed to the proximity of the observed values to the detection limit of 2 log CFU/g for 50 % RH conditions. Near the detection limit, small fluctuations in the microbial population can have a more pronounced effect on the measured values, leading to greater uncertainty in the data. Similar limitations were reported by Chen et al. (2020) for accurate prediction of bacterial inactivation at 50 %RH during EtO fumigation of cumin seeds. Overall, this underprediction can be seen as a conservative approach, providing a safety margin by ensuring a higher level of bacterial inactivation rather than overestimating the efficacy of the treatment. The modified Bigelow model can predict δ* values at any given water activity and temperature within the tested range. The predicted values of δ* from the modified Bigelow model (Eqn. 4 for *Salmonella*) were substituted into the Weibull model (Eqn. 1) to predict log reduction of *Salmonella* at all treatment conditions using a fixed p-value of 0.47 for *Salmonella*. These predictions were denoted as the Weibull-modified Bigelow model. The comparison of estimated δ-values with experimental values as represented in Fig 2., shows a good fit. Previous study on ClO_2 fumigation of dried basil leaves found that a modified Bigelow model provided a better prediction of *Salmonella* inactivation than the response surface model (Verma et al. ,2022). Furthermore, Wason & Subbiah (2023) utilized modified Bigelow model to assess the impact of gas concentrations and relative humidity during ClO_2 treatment on microbial reduction on chia seeds.

Fig 3. presents the contour surface plots based on Bigelow model for *Salmonella* and *E. faecium* δ- values at varied RH condition at different temperatures and treatment times. The gaps between contour lines tend to widen as relative humidity and temperature increase. At lower RH, a minimal decrease in temperature substantially increased the δ* compared to high RH. The

same trend was observed for *E. faecium* as well. Thus, high RH and the temperature had a considerable impact on the inactivation rate of *Salmonella* and *E. faecium* on chia seeds.

The developed models can serve as useful tools for predicting the efficacy of EtO treatment under different RH and temperature conditions. It is worth noting that the specific δ-values and mathematical models developed in this study may be specific to the conditions and pathogens investigated, and further research is needed to validate and generalize these models for other low moisture food ingredients and pathogens.

9.4.3 Effect of RH, temperature, and exposure time on bacterial inactivation

Higher treatment temperatures and RH during EtO treatment were found to enhance microbial inactivation. At 50% RH, increasing the temperature from 46 to 60°C improved the *Salmonella* log reduction from 3.45 to 4.49 log CFU/g within 4 min of exposure. Treatment at 30% RH for 120 min resulted in 2.31, 3.20, and 3.64 log reduction in chia seeds at 46, 53, and 60 °C respectively. These findings are in agreement with Wei et al. (2021) who reported an increase in log reduction from 3.23 to 4.05 as the temperature rose from 46 to 60 °C. Previous research on *Bacillus subtilis* for equipment sterilization also highlighted the significance of temperature on microbial inactivation (Mendes et al., 2017; Oxborrow et al., 1983).

At higher RH levels, microbial inactivation was notably higher and rapid, leading to shorter treatment times compared to lower RH processes. Similarly, Wei et al. (2021) and Chen et al. (2020) reported a dramatic increase in microbial inactivation at high RH and temperature conditions during EtO fumigation of spices. In other words, the time required for 5 log reduction was shorter at higher RH conditions. For instance, exposure to EtO for 24 min at 30 % RH resulted in *Salmonella* log reduction of only 1.11, 1.56, and 2.00 log CFU/g at 46, 53, and 60 °C,

respectively. In contrast, 2 minutes of exposure at 50% RH led to 3.15, 3.59, and 3.87 log reduction at the corresponding temperatures. The FDA (2020) recommends a 5-log reduction of pathogens for effective pasteurization. At 40% RH and 60°C, more than 5-log reduction of *Salmonella* required 60 min, while 10 min was sufficient at 50% RH. Similar findings were reported by Wei et al. (2021) in their study on EtO fumigation of black peppercorns, demonstrating an increase in *Salmonella* reduction from 3.81 to 4.74 log CFU/g as RH was increased from 30 to 40%. Prehumidification before gas injection provided high humidity environment beneficial for desiccated bacterial cells in chia seeds. Several studies have reported increased sensitivity of *Salmonella* at high humidity conditions for thermal (Wason et al., 2022a; Gautam et al., 2021) and non-thermal inactivation (Chen et al., 2020; Wei et al., 2021a, b; Verma et al., 2022; Wason & Subbiah, 2023) of low-moisture foods. Furthermore, Gilbert et al. (1964) reported that a higher humidity environment promotes the bactericidal action of EtO through the formation of cross-linkages within or between cell proteins, improving the accessibility of alkylating sites to EtO gas. In contrast, Oxborrow et al. (1983) observed no significant effect of RH on the D-value of *Bacillus subtilis* at or above 50 °C temperature while D-value increased at lower RH and temperature < 50°C. A previous study by Kelsey (1967) and Kaye & Philips (1949) emphasized the critical role of RH for microbial inactivation, where lower levels of moisture surrounding the bacteria hindered microbial inactivation while higher levels could lead to hydrolysis and production of ethylene glycol.

Additionally, treatment time significantly influenced bacterial inactivation. After 24 min of exposure at 46 °C, *Salmonella* log reduction of 1.11 and 2.41 was achieved at 30 and 40% RH, respectively. Increasing the exposure time from 24 min to 48 min at the same conditions led to a 1.55 and 3.84 log reduction of *Salmonella*. When chia seeds were exposed to EtO at 40% RH

and 46 °C, an increase in treatment time from 0 to 36 min reduced *Salmonella* by 3.20 log CFU/g, further increase in treatment time to 60 min only increased *Salmonella* reduction to 4.42 log CFU/g. In general, the majority of log reduction at each treatment condition occurred initially and then stabilized as the exposure time was increased.

9.4.4 *E. faecium* as a suitable surrogate

The comparison of *Salmonella* and *E. faecium* log reduction at various treatment conditions is presented in Fig. 4. The results indicate that *E. faecium* constantly exhibited lower inactivation as compared to *Salmonella* under the same combination of treatment RH and temperature. Additionally, *E. faecium* exhibited a similar inactivation trend with a tailing effect. These findings provide strong evidence for the suitability of *E. faecium* as a surrogate of *Salmonella* during in-plant validation. Several other studies have also identified *E. faecium* as a suitable surrogate in various treatments. The heat resistance of *E. faecium* is reported to be very high as compared to *Salmonella* on low moisture foods, thus it is a conservative surrogate during thermal inactivation (Wei et al., 2021; Ahmad et al., 2022; Verma et al., 2021; Wei et al., 2021; Chen et al., 2019). However, *E. faecium* is a suitable surrogate for *Salmonella* but not very conservative during gaseous treatments including EtO fumigation of cumin seeds (Chen et al., 2020) and black peppercorns (Wei et al., 2021); ClO_2 decontamination of black peppercorns (Wei et al., 2021); dried basil leaves (Verma et al., 2022). Similarly, Wei et al. (2021) and Newkirk (2019) found that *E. faecium* was not consistently exhibiting higher resistance to ClO_2 and EtO, respectively in cumin seeds. It could be due to higher susceptibility of *E. faecium* being a gram-positive bacteria to gaseous molecules as compared to Salmonella which is gram negative bacteria and has a thicker cell wall. Therefore, ist is imperative to evaluate the suitability of *E. faecium* in validating different products and processes.

9.4.5 Quality analysis

Relative humidity was found to be a significant factor influencing microbial inactivation. However, the presence of high levels of moisture in the treatment chamber could also lead to the production of EtO byproducts. Therefore, the impact of varying RH on the quality and byproduct formation in chia seeds was analyzed at the maximum tested treatment temperature. Uninoculated chia seeds were exposed to EtO gas (735.3 mg/L) at 60 °C and 30, 40, and 50% RH for 120, 60, and 10 mins durations, respectively. Fig 5. illustrates the total fat content, omega-3 fatty acids, and protein content in the chia seeds. The results indicate that chia seeds subjected to EtO at different treatment conditions did not significantly (P>0.05) differ from untreated control samples. The total fat content in the chia seeds remained consistent across all treatment conditions, ranging from 31.96 ± 2.13 to 32.51 ± 2.87 g/100 g. The omega-3 fatty acid was slightly higher (21.88 ± 0.79 g/100g) in chia seeds treated at 50 % RH at 60 °C for 10 min compared to other treatments, which were lower than 19.5 ± 0.33 g/100 g, but this difference was not statistically different (P >0.05). Similarly, the total protein content in chia seeds did not show a significant change after EtO treatment compared to the untreated control sample. Wason & Subbiah (2023) investigated the impact of ClO_2 gas treatment at different RH conditions (60, 70, and 80% RH) on the fatty acid content in chia seeds and found no significant effect. Conversely, thermal treatment was found to have a significant impact on the quality of chia seeds, affecting fatty acid content and lipid oxidation (Lau et al., 2021).

Additionally, calorimetric analysis of chia seeds revealed no significant difference in lightness (L*), red-green color (a*), and yellow-blue color (b*) values following EtO treatment (Table 3). The L* of the chia seeds ranged from 49.33 ± 0.58 to 50.7 ± 0.61 for both EtO treated and untreated controls chia seeds. Although the a* values indicating the red-green color of the

chia seeds were slightly higher (2.13 ± 0.15) compared to the control (1.23 ± 0.63), no significant difference was evident. Regarding the b* values, representing yellow-blue color, there was no significant difference between the EtO treated and untreated control samples at a significance level (α) of 0.05. However, the change in b* values was significantly different at α = 0.10 between the control (4.97 ± 0.3) and 50% R.H. at 60°C for 10 min with a value of 5.8 ± 0.21. Duncan et al. (2017) compared the effect of different processing technologies on the quality of spices. They reported that EtO processing of black peppercorns did not produce any off-flavors or color changes while steam processing increased monoterpene volatiles and loss of sesquiterpenes leading to odor differences. In addition, steam processing led to color differences which were also noticed in EtO-treated cumin seeds (higher b *; lower L *).

The germination test revealed that the EtO treatment did not significantly affect ($P > 0.05$) the germination of the chia seeds. On average, 70 ± 17.24 chia seeds germinated in the control group, while the treatment at 30% RH at 60°C for 120 min showed the highest germination (76 ± 13.86%), although the difference was not significant. Overall, the quality analysis of chia seeds following EtO treatment at different RH and exposure time did not influence the quality of chia seeds in terms of total fat content, omega-3 fatty acid, protein, color, and germination capacity.

Residue analysis revealed the absence of EtO residues in all treated samples, regardless of the specific treatment condition followed. No EtO residues were detected in the untreated control samples. The presence of EtO byproducts, ethylene chlorohydrin (ECH), and ethylene glycol (EG) compounds was also quantified in the treated samples (Table 4). Overall, the EtO residues and byproducts estimated in the treated chia seeds were much lower than the standard tolerance limits of 7 ppm for EtO and 940 ppm for ECH residues on spices and seeds (EPA, 2023b). No traces of EG residues were detected in any of the treatments, including control

samples. However, ECH residues were detected in all the treatment conditions except for the EtO treatment carried out at 53 and 60 °C with 50 % relative humidity (RH) exposure for 60 and 10 min, respectively. The presence of ethylene chlorohydrin residues in chia seeds following EtO treatment was found to be dependent on the treatment temperature. A significant reduction (P <0.05) in ECH residues, from 49.03 ppm to 15.86 ppm, was observed in chia seed samples when the treatment temperature was increased from 46 to 60 °C at 30% RH exposed for 120 min. Similar trends were observed at the 40 and 50% RH test conditions with exposures of 60 and 10 min, respectively. This aligns with the ASTA recommendation of using at least 46 °C to minimize the byproduct formation (ASTA, 2009). Furthermore, a significant decrease in ECH residue levels from 49.03 to 11.37 ppm, was observed when the relative humidity increased from 30 to 50 % at 46 °C exposed for 120 and 10 mins, respectively. Although high RH conditions are expected to produce higher EG byproduct due to the reaction of water with EtO gas, substantial differences in exposure time led to insignificant EG at increasing RH during EtO treatment. Similar decreasing trends were observed for treatments performed at 53 and 60 °C.

9.5 Conclusions

Ethylene oxide (EtO) fumigation demonstrated effective inactivation of *Salmonella* and *E. faecium* on chia seeds. Higher RH, temperature, and longer treatment time enhanced microbial reduction, whereas shorter treatment times were sufficient at higher RH levels. The survival data of *Salmonella* and *E. faecium* post EtO treatment showed a non-linear trend with a tailing effect indicating the variations in cell response to EtO gas. The modified Bigelow model combined with the Weibull model could be applied effectively to identify processing conditions for EtO fumigation. *E. faecium* was found to be suitable as a surrogate for *Salmonella* during EtO treatment, suggesting its potential for use in validation studies. Furthermore, EtO treatment

showed no significant impact on the quality of chia seeds in terms of fatty acid content and seed viability. These findings suggest that EtO gas can be a viable option for the decontamination of chia seeds without significantly affecting the nutritional quality and germination ability of seeds.

9.6 Acknwledgements

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Table 9.1. Weibull model parameters for *Salmonella* and *E. faecium* inactivation during EtO fumigation of chia seeds at different treatment conditions.

Microorganism	Temperature (°C)	RH (%)	δ (min)	p	RMSE (log CFU/g)	Adj R^2
Salmonella	46	30	17.25 ±3.84	0.46	0.14	0.97
		40	6.75 ±0.79	0.51	0.18	0.99
		50	0.04 ±0.02	0.29	0.08	0.99
	53	30	8.42 ±2.16	0.41	0.15	0.98
		40	5.90 ±2.22	0.59	0.36	0.95
		50	0.10 ±0.04	0.36	0.26	0.98
	60	30	5.28 ±1.58	0.41	0.18	0.98
		40	4.22 ±1.43	0.58	0.35	0.96
		50	0.03 ±0.02	0.36	0.43	0.98
E. faecium	46	30	37.34 ±7.07	0.59	0.16	0.95
		40	7.21 ±1.77	0.51	0.19	0.97
		50	0.14 ±0.09	0.31	0.18	0.97
	53	30	16.38 ±1.73	0.49	0.07	0.99
		40	3.56 ±1.09	0.40	0.18	0.97
		50	0.08 ±0.04	0.29	0.17	0.99
	60	30	14.88 ±2.63	0.52	0.14	0.98
		40	3.25 ±0.89	0.46	0.20	0.98
		50	0.12 ±0.05	0.31	0.20	0.98

Table 9.2. Color and percent germination in chia seeds treated with EtO at different treatment conditions.

Treatment	L*	a*	b*	ΔE	% Germination
Control	49.43 ± 0.58	1.23 ± 0.6	4.97 ± 0.30	0	80.7 ± 12.1
30% RH at 60 °C for 120 mins	50.53 ± 0.60	2.30 ± 0.12	5.10 ± 0.12	1.85 ± 0.75	82.7 ± 18.0
40% RH at 60 °C for 60 mins	50.30 ± 0.84	2.23 ± 0.15	5.73 ± 0.19	2.03 ± 0.74	74.0 ± 24.3
50% RH at 60 °C for 10 mins	50.70 ± 0.61	2.13 ± 0.15	5.80 ± 0.21#	2.00 ± 1.58	75.7 ± 20.0

*Not significant at $P < 0.05$, # significant at $P < 0.10$.

Table 9.3. Ethylene oxide residues and byproducts in chia seeds treated with EtO at different treatment conditions.

Treatment condition	Ethylene chlorohydrin (ECH) residues ± SD	Ethylene glycol (EG) residues	Ethylene oxide (EtO) residues
Control	n.d.	n.d.	n.d.
30% RH at 46 °C for 120 min	49.03 ± 4.89[a]	n.d.	n.d.
30% RH at 53 °C for 120 min	38.95 ± 3.63[ab]	n.d.	n.d.
30% RH at 60 °C for 120 min	15.85 ± 3.45[d]	n.d.	n.d.
40% RH at 46 °C for 60 min	32.06 ± 8.64[bc]	n.d.	n.d.
40% RH at 53 °C for 60 min	23.71 ± 5.31[cd]	n.d.	n.d.
40% RH at 60 °C for 60 min	13.25 ± 2.59[d]	n.d.	n.d.
50% RH at 46 °C for 10 min	11.37 ± 0.62[d]	n.d.	n.d.
50% RH at 53 °C for 10 min	Below the detection limit	n.d.	n.d.
50% RH at 60 °C for 10 min	Below the detection limit	n.d.	n.d.

Values within the same column followed by different letters are significantly different.
n.d. – not detected.
Detection limits for ECH, EtO, and EG are 2.37, 10.12, 15.30 ppm.
EPA tolerance limits for ECH and EtO are 940 and 7 ppm.

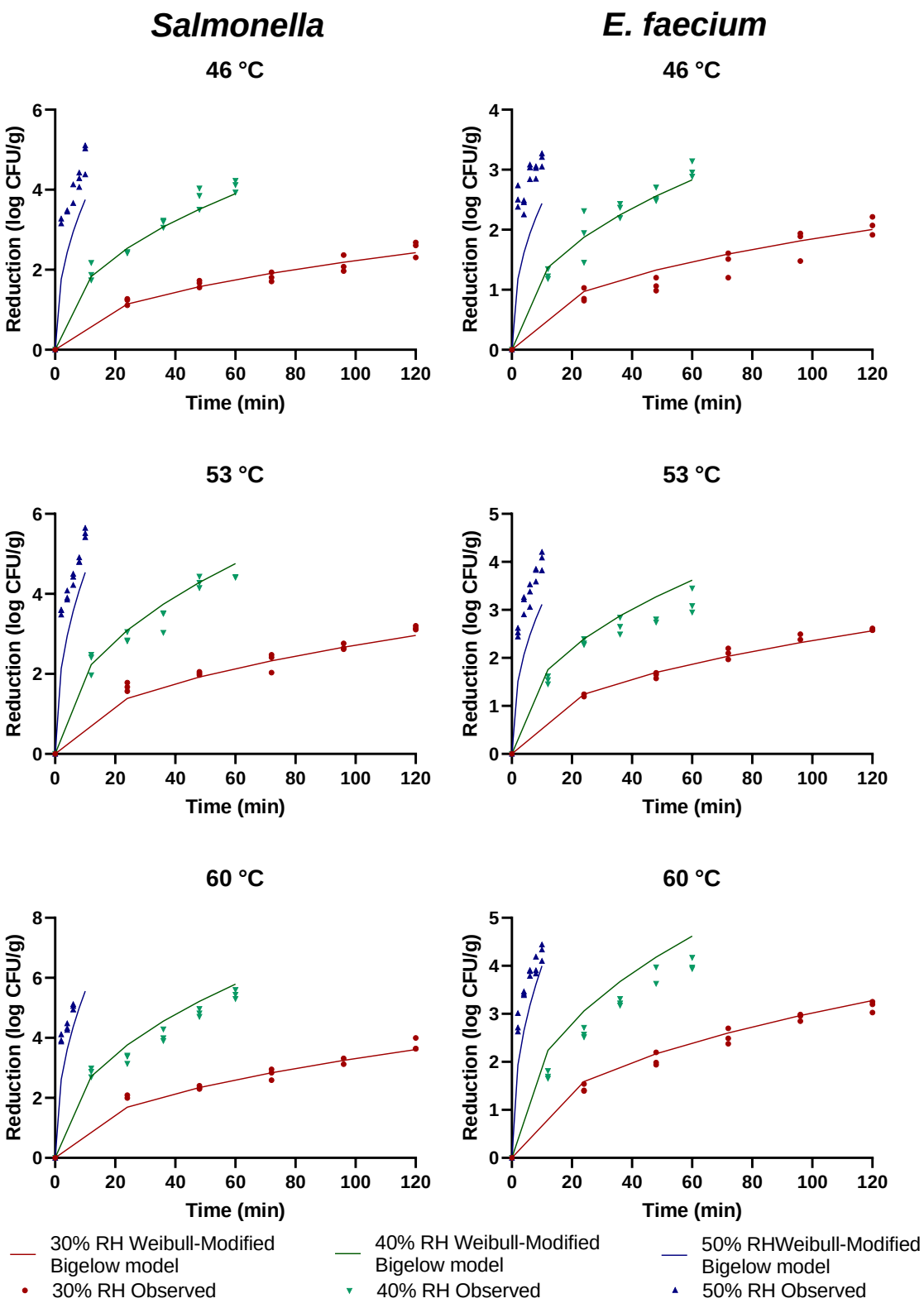


Fig 9.1. Experimental log reduction and Weibull-Modified Bigelow Model predicted δ*-value of *Salmonella* and *E. faecium* during EtO treatment of chia seeds.

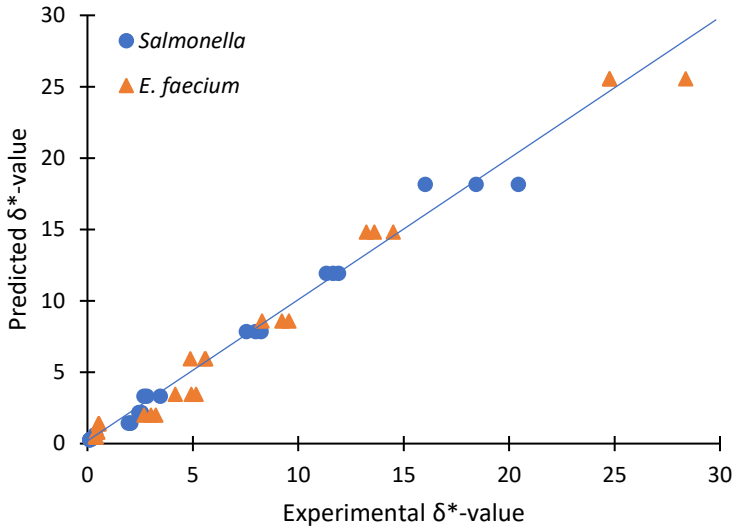
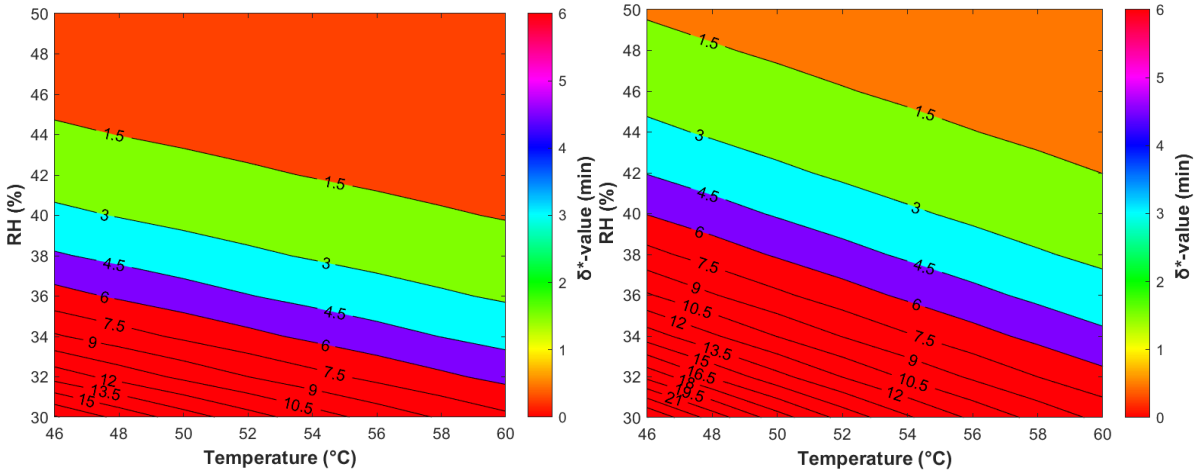


Fig 9.2. Comparison of experimental and predicted δ*-value using Weibull-Modified Bigelow Model at fixed shape parameter of 0.47 for *Salmonella* and 0.45 for *E. faecium*.



A. *Salmonella* B. *E. faecium*

Fig. 9.3. Contour plots showing the δ* for A. *Salmonella* (fixed shape parameter= 0.47) and B. *E. faecium* (fixed shape parameter = 0.45) using the modified Bigelow model.

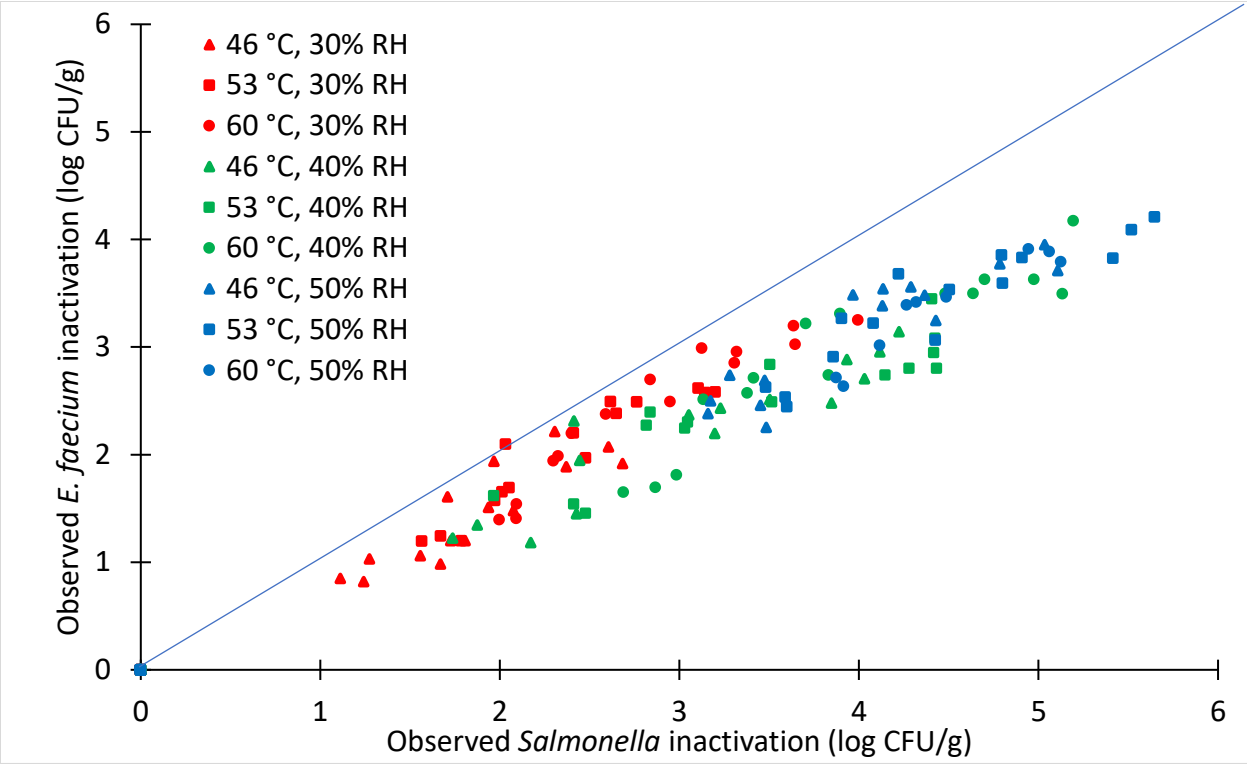


Fig 9.4. Comparison of *Salmonella* and *E. faecium* log reduction after EtO fumigation condition at different treatment conditions.

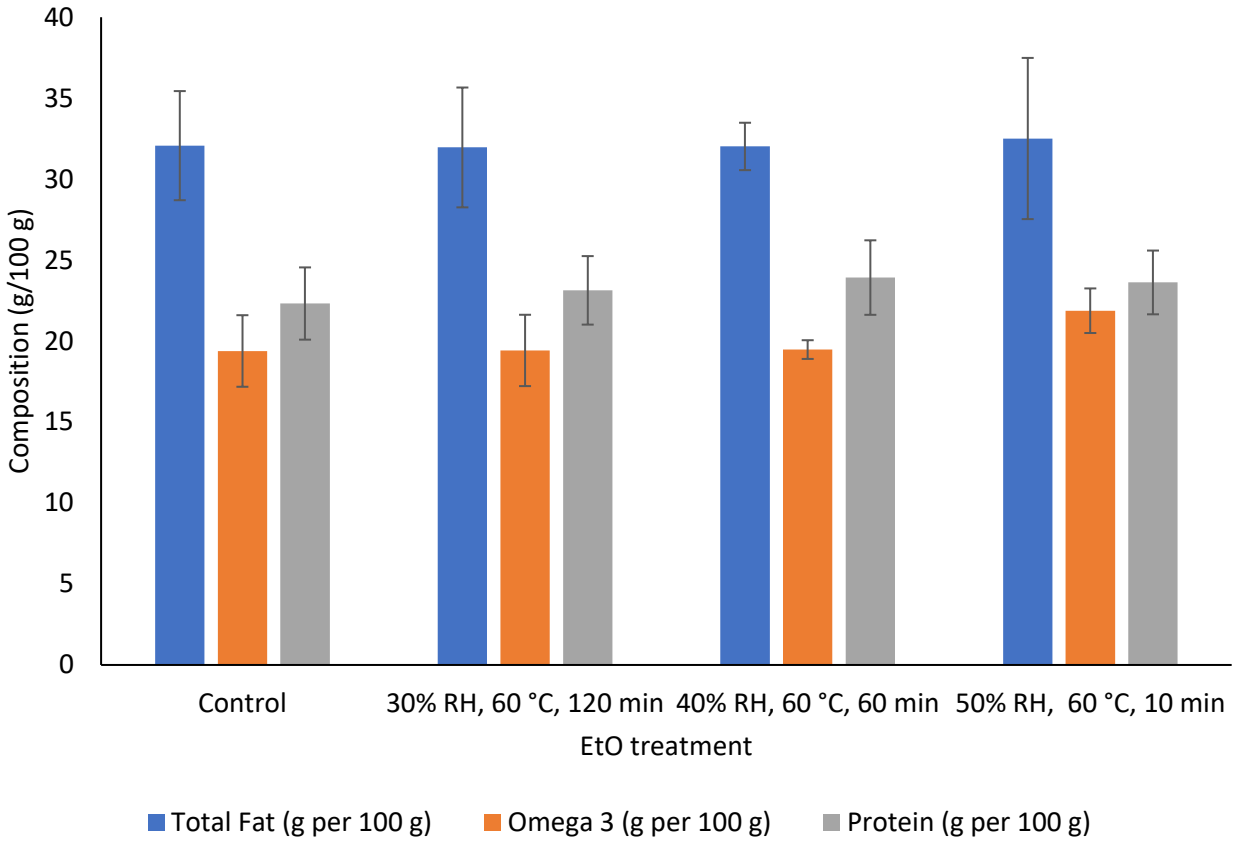


Fig 9.5. Total fat, omega 3 fatty acid, and protein content in chia seeds treated with EtO at different treatment conditions. Parameters are not significant among different treatment conditions at $P > 0.05$.

Chapter 10. Conclusion and suggestions for future work

10.1 Conclusions

The dissertation research focused on the development and optimization of pasteurization methods for spices, herbs, and seeds to ensure microbial safety. The studies investigated the thermal inactivation kinetics of *Salmonella enterica and Enterococcus faecium* NRRL B-2354 in fine ground black pepper, the effect of inoculation protocols on the decimal reduction time of *Salmonella* in black pepper, in-package radiofrequency pasteurization of dried basil leaves, in-package steaming for *Salmonella* inactivation on black peppercorns and dried basil leaves using radiofrequency, and the efficacy of gaseous chlorine dioxide and ethylene oxide for inactivating *Salmonella* in chia seeds.

Objective I: Investigate the parameters impacting the heat resistance of *Salmonella*.

Chapter 1 demonstrated that water activity and treatment temperature significantly enhanced the thermal inactivation of *Salmonella*. The Weibull model provided a better fit for the thermal inactivation kinetics, and the modified Bigelow model based on the Weibull model was effective in predicting the inactivation kinetics of *Salmonella*. The study also established that *Enterococcus faecium* NRRL B-2354 can be used as a suitable surrogate for *Salmonella* in black pepper. The findings of this study can be applied by the spice industry to identify treatment conditions and develop a pasteurization process for black pepper.

Chapter 2 highlighted the importance of considering the inoculation method when determining the thermal inactivation kinetics of pathogens in food products. Inoculation of black pepper pre-grinding provided a conservative estimation of the D-value compared to post-

grinding inoculation. Considering that contamination usually occurs during harvest and drying of whole black peppercorns, the conservative approach is recommended for validating the pasteurization process. Additionally, *Enterococcus faecium* NRRL B-2354 was confirmed as a suitable surrogate for *Salmonella* in both inoculation methods.

Objective II: Radiofrequency processing for enhancing the microbial safety of pre-packaged spices.

Chapter 3 evaluated in-package radiofrequency (RF) pasteurization using steam venting for dried basil leaves packed in a polypropylene bottle. Temperature distribution indicated that the steam generated in the package improved the overall heating uniformity during the RF pasteurization. The horizontal placement of samples achieved rapid and uniform heating, resulting in >5 log reduction of *Salmonella* within 35 s. The quality analysis indicated no significant effects on the color, phenolics, antioxidants, and volatile composition of the RF treated basil leaves. The use of in-package RF pasteurization with steam venting can enhance the safety of dried basil leaves while minimizing cross-contamination.

Chapter 4 investigated the effectiveness of in-package steaming using RF processing for pasteurizing black peppercorns and dried basil leaves in a steam vent package. Steam vent retained the steam within the package which provided a humid environment for better *Salmonella* inactivation and achieved uniform distribution of heat. RF heating of black peppercorns for 135 s and dried basil leaves for 40 s in a steam vent package reduced the *Salmonella* population to below the limit of detection. The spices and herbs in retail packages can be continuously processed on a conveyor belt, reducing cross-contamination risks.

Objective III: Antimicrobial gaseous technologies for improving the microbial safety of chia seeds.

Chapter 5 focused on the application of gaseous chlorine dioxide (ClO_2) for *Salmonella* inactivation in chia seeds. The antimicrobial efficacy of ClO_2 gas was influenced by gas concentration, relative humidity, and exposure time. A maximum log reduction of 3.7 CFU/g of *Salmonella* was achieved at the most severe treatment condition tested i.e., 10 mg/L concentration and 80% RH for 300 min *Enterococcus faecium* NRRL B-2354 was identified as a suitable surrogate for *Salmonella* during validation studies. The treatment did not significantly impact the color, fatty acid composition, and germination capacity of the chia seeds. However, the peroxide value increased post-treatment, indicating a need for further research on combination treatments to minimize oxidation.

Chapter 6 investigated the efficacy of mild heating and ambient storage in improving the antimicrobial efficacy of chlorine dioxide (ClO_2) gas against *Salmonella enterica* in chia seeds. The results demonstrated that ClO_2 gas treatment followed by mild heating effectively reduced *Salmonella* on chia seeds. Storage of ClO_2-treated chia seeds under ambient conditions further enhanced microbial reduction over time. The best treatment condition was found to be ClO_2 treatment at 90% RH, 3 mg/L for 2 hours, followed by mild heating at 60 °C for 2 hours and subsequent 7 days of ambient storage, which provided close to a 5-log reduction in *Salmonella*. The formation of ClO_2 byproducts in the treated seeds was within acceptable limits. In addition, the quality of chia seeds was minimally affected by ClO_2 treatment, both alone and in combination with mild heating, indicating the potential for maintaining the safety and quality of chia seed products.

Chapter 7 evaluated the antimicrobial efficacy of ethylene oxide (EtO) gas on chia seeds and assessed the impact of processing parameters on microbial reduction, byproduct formation, and seed quality. The results showed that EtO treatment effectively reduced bacterial populations on chia seeds, with the inactivation trend following a non-linear pattern. Higher relative humidity (RH), temperature, and longer exposure time resulted in greater log reductions of *Salmonella* and *E. faecium*. *E. faecium* was found to be a reliable surrogate for *Salmonella* during EtO treatment. EtO treatment showed no significant impact on the quality of chia seeds, suggesting that it can be a viable option for the decontamination of chia seeds without affecting their nutritional quality and germination ability. The findings provide valuable information for the chia seed industry to identify processing conditions for EtO fumigation.

Overall, thermal inactivation (radiofrequency) was rapid compared to non-thermal inactivation (antimicrobial gases). The antimicrobial efficacy of EtO was better than ClO_2 against *Salmonella* on chia seeds. Incorporating a steam vent during in-package RF pasteurization of dried basil leaves can eliminate potential post-process cross-contamination, improve heating uniformity by building steam pressure in a controlled manner, enhancing the pasteurization process without significantly impacting the quality. For radiofrequency pasteurization, dried basil leaves showed rapid and better *Salmonella* reduction than black peppercorns. Gaseous technologies (EtO & ClO_2) provided an effective waterless decontamination strategy for chia seeds that can maintain seed viability without gelling during treatment. Comprehensive data presented on inactivation efficacy of RF and gaseous technology, their effect on quality parameters and chemical residues in treated foods will be beneficial for industries and policymakers to utilize these technologies.

10.2 Suggestions for Future Research

The dissertation evaluated and optimized different pasteurizing technologies specific for a low moisture food ingredient. The findings from this research provide valuable insights into the efficacy of pasteurization techniques and their potential application in the food industry. However, there are still several areas that require further investigation to enhance our understanding and implementation of pasteurization methods. Some of them are suggested below:

1. *Investigation of different products and decontamination strategies:* The efficacy of intervention technology is dependent on various factors such as product matrix, food composition, pathogenic bacteria, etc. The developed decontamination strategies in this dissertation could be evaluated for different low moisture food ingredients. In addition, other pasteurization methods, such as cold plasma, vaporized hydrogen peroxide, ozone gas, pulsed UV light could be explored for decontamination of low moisture food ingredients. Recent research on decontamination of fresh produce using a combination of ozone, hydrogen peroxide, and UV (Advanced Oxidative Process) was reported to enhance radical production which significantly improves pathogen inactivation (Hasani et al., 2020). The interaction effect of UV, ozone and H_2O_2 on microbial reduction could be investigated for low moisture foods.
2. *Process Validation and Scale-up:* To facilitate the implementation of pasteurization methods in the food industry, process validation at industrial scale must be performed. For example, based on the treatment conditions optimized in objective II, in-package radiofrequency pasteurization of spices and herbs could be carried out on a conveyor belt for scaling up. Further investigation is required on the effect of continuous processing on heating uniformity

and *Salmonella* inactivation in spices and herbs packaged in a steam vent package. Another recommendation is to assess the efficacy of gaseous treatments on a larger sample size to assist in potential scale-up. Spice industry usually needs to process a large amount of products, which will require a better understanding of gas diffusion. The efficiency of gaseous inactivation could be strongly affected by product size, shape, and packaging; thus, it is important to study the gas diffusion in different food products. The development of gas diffusion model combined with gaseous microbial inactivation kinetics could provide the guidelines for scaling-up of the gaseous pasteurization process. Moreover, collaboration with regulatory agencies can help establish guidelines, standards, and maximum permitted levels of ClO_2 residues and byproducts in the treated sample, ensuring consumer safety.

3. *Kill ratio concept:* According to FDA (2000), a surrogate must be non-pathogenic and have characteristics similar to the pathogenic microorganism in terms of heat resistance in case of thermal processing. A surrogate is suitable when it exhibits higher thermal resistance than *Salmonella*. While this characteristic is important when defining the 'worst-case-scenario', it is important to consider the limitations of these assumptions, given that longer treatment times or increased treatment temperatures can negatively affect product quality. Therefore, the kill ratio must be selected to provide reasonable assurance and a margin of safety without compromising product quality. Monte Carlo simulation can be used to calculate the kill ratio which would assist in maintaining product quality while effectively pasteurizing the food product.
4. *Shelf-life Studies and Sensory Analysis:* Conducting shelf-life studies and sensory analysis is crucial to assess the quality and consumer acceptability of treated low moisture food ingredients. The assessment of shelf-life stability is crucial to ensure the long-term

microbial safety and nutritional quality of pasteurized spices and seeds. The efficacy of steam vent in preventing cross-contamination during storage under different environmental conditions should be assessed for commercialization. Comprehensive quality assessments could include the impact of pasteurization on the nutritional composition, flavor, aroma, and texture of treated low moisture food ingredients.

5. *Enhancement of Pasteurization Methods:* The ClO_2 treatment followed by mild heating enhanced *Salmonella* reduction in chia seeds. Further study could evaluate the impact of simultaneous ClO_2 treatment and mild heating on microbial inactivation. Additionally, agitation during the treatment could be employed to improve the treatment uniformity by exposing the all product surfaces to gases for potential improvement in microbial inactivation. Chia seeds are often sprouted to increase its nutritional quality, the assessment of *Salmonella* survival in chia seeds during germination seeds after gaseous treatment could be useful for chia seed industries to ensure its microbial safety.