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Microbial Challenge Studies of Radio Frequency Heating for Dairy Powders and Gaseous Technologies for Spices

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MICROBIAL CHALLENGE STUDIES OF RADIO FREQUENCY HEATING FOR DAIRY POWDERS AND GASEOUS TECHNOLOGIES FOR SPICES

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Persistence, thermal resistance, and survival of *Salmonella* in low moisture foods (LMF) have resulted in several foodborne illness outbreaks. Both existing and novel pasteurization technologies need to be validated for microbial safety of LMF. In this dissertation, a framework for using radio frequency (RF) heating to enhance microbial safety of milk powders and egg white powder was established. Thermal inactivation kinetics of *Salmonella* in milk powders were determined to guide the dairy industry for identifying thermal processing conditions for pasteurization. Storage time showed no effect on the thermal resistance of *Salmonella*, which can simplify the process validation study in the industry. Hot air-assisted RF heating followed by holding at high temperatures in a convective oven was developed and validated for pasteurization of milk powders. Continuous RF processing was evaluated for pasteurization of egg white powder (EWP), which can provide> 6.7 log reduction for *Salmonella* after RF-assisted thermal processing of EWP at 80°C for 2 h.

Ethylene oxide (EtO) and chlorine dioxide (ClO_2) gaseous technologies were evaluated as non-thermal pasteurization for improving microbial safety of spices. A response surface model was developed as a function of temperature, relative humidity,

and exposure time to predict the reduction of *Salmonella* or *Enterococcus faecium* NRRL B-2354 on whole black peppercorn during EtO fumigation, which can guide the spice industry in determining the process conditions for pasteurization. The effects of gas concentration, RH, and exposure time on *Salmonella* and *E. faecium* on whole black peppercorn and cumin seeds during ClO_2 gaseous treatment were evaluated which can provide technical information for implementation of this technology. *E. faecium* was found to be a suitable surrogate for *Salmonella* during both thermal (RF) and non-thermal (EtO and ClO_2) of dairy powders and spices, respectively. Therefore, the food industry can use *E. faecium* to replace *Salmonella* for conducting the industrial process validation.

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PREFACE

Chapter 2 in this dissertation has been published in the Journal of Dairy Science:

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Chapter 3 in this dissertation has been published in the Journal of Dairy Science:

Wei, X., Agarwal, S., & Subbiah, J. (2021). Evaluation of *Enterococcus faecium* NRRL B-2354 as a surrogate for *Salmonella enterica* in milk powders at different storage times and temperatures. *Journal of Dairy Science*, 104(1), 198-210.

Chapter 4 in this dissertation was submitted for publication in the Journal of Dairy Science:

Wei, X., Agarwal, S., & Subbiah, J. (2021). Heating of milk powders to 95℃ for 15 minutes using hot air-assisted radio frequency processing achieved pasteurization. *Journal of Dairy Science*.

Chapter 5 in this dissertation has been published in the Journal, Food Microbiology:

Wei, X., Lau, S. K., Reddy, B. S., & Subbiah, J. (2020). A microbial challenge study for validating continuous radio-frequency assisted thermal processing pasteurization of egg white powder. *Food Microbiology*, 85, 103306.

Chapter 6 in this dissertation has been published in the Journal, LWT - Food Science and Technology:

Wei, X., Chen, L., Chaves, B. D., Ponder, M. A., & Subbiah, J. (2021). Modeling the effect of temperature and relative humidity on the ethylene oxide fumigation of *Salmonella* and *Enterococcus faecium* in whole black peppercorn. *LWT*, 140, 110742.

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Chapter 1

INTRODUCTION

1.1 Overview of Low Moisture Foods (LMF) Safety

Salmonella persistence in LMF is an emerging and urgent challenge that has caused several nationwide foodborne outbreaks and/or recalls involving red and black pepper (CDC, 2010), almond (CDC, 2004), pistachio (CDC, 2016), hazelnuts (Izurieta & Komitopoulou, 2012), peanut butter (CDC, 2012), chia seeds (CDC, 2014), milk powder (Marler, 2018) and several pet foods (FDA, 2019). Historically, LMF were considered to be inherently microbiologically safe, because their low water activity (a_w) acts as a natural barrier to prevent microbial growth. Recent foodborne outbreaks linked to LMF have attracted public attention on the safety of LMF. Unfortunately, current decontamination methods for LMF are either insufficient to ensure food safety or compromising the food quality to achieve desired pasteurization. Because of the dry nature of LMF, dry heat is often used as a thermal treatment for the pasteurization of LMF. However, conventional dry heat methods, such as roasting, baking, drying, have limitations for pasteurizing products without affecting food quality due to the low thermal conductivities of LMF (Muramatsu et al., 2005). Thus, radio-frequency (RF) heating, a novel thermal treatment, which has been shown to rapidly heat up LMF (Boreddy et al., 2016; Jeong & Kang, 2014; Jiao et al., 2015; Lau & Subbiah, 2017; Tiwari et al., 2011; Villa-Rojas et al., 2017), could be used as an alternative thermal process technology for ensuring the safety of LMF.

Several non-thermal technologies, such as cold plasma (Hertwig et al., 2015; J. E. Kim et al., 2014), gamma irradiation (Song et al., 2014), ethylene oxide (EtO) fumigation (Gilbert et al., 1964; Gimeno et al., 2018), ozone (Khadre et al., 2005; Zhao & Cranston, 1995), chlorine dioxide (Annous & Burke, 2015; J.-M. Kim & Linton, 2008; Prodduk et al., 2014), hydrogen peroxide (Forney et al., 1991; Guan et al., 2013; Simmons et al., 1997) and UV-C radiation (Lacombe et al., 2016), have been investigated to improve the safety of LMF. However, some of them are not well-recognized and accepted in the market and by the customers, validated for getting FDA approval, or could not effectively ensure the safety of LMF. There is no single technology that could be universally suitable for ensuring the microbial safety of LMF.

Food Safety Modernization Act (FSMA) Preventive Controls rules require the food industry to scientifically prove that their preventive controls will limit effectively the hazards that are reasonably likely to occur (FDA, 2018). Due to the lack of understanding of critical factors involved in technology selection and implementation, most of the technologies have very limited market penetration in the food industry. Therefore, it is necessary to improve the implementation of these technologies and conduct product- or process-specific validation to provide enough inactivation data or models to guide the food industry on the selection of appropriate technology for improving their food product safety.

1.2 Current Research Gap

LMF typically have a_w lower than 0.70, which provides a natural barrier for the growth of most microorganisms (Blessington et al., 2013). Although *Salmonella* can only reproduce at a_w higher than 0.94, they are able to survive for a long period at low a_w storage conditions (Keller et al., 2013; Lian et al., 2015). As a_w decreases, *Salmonella* also exhibits increasing thermal resistance, which reduces the efficacy of most thermal processing technologies (Beuchat, 1981; Villa-Rojas et al., 2017; Syamaladevi et al., 2016). Unlike most thermal processing of high moisture foods, thermal processing of LMF usually experiences a dynamic moisture content change. As a_w decrease, long processing times would be required to achieve the desired inactivation of *Salmonella*. Therefore, a comprehensive study involving the effect of different a_w and temperatures on *Salmonella* inactivation is necessary for validating inactivation models in real-world processes.

Many LMF are ready-to-eat foods, such as nuts, spices, peanut butter, and dairy powders; thus the lack of appropriately applied lethal interventions could result in severe public concern about food safety. There are several pasteurization technologies such as RF heating, EtO fumigation, and gaseous chlorine dioxide technology that could be the potential solutions for improving the microbial safety of LMF.

Radiofrequency (RF) processing is a novel thermal processing technology based on dielectric heating using electromagnetic waves. The advantages of RF heating

compared to conventional thermal treatments are the rapid heating rate, better heating uniformity, and higher penetration depth than the microwave (Boreddy et al., 2016; Chen et al., 2013, 2017; Hou et al., 2016; Jiao et al., 2015; Lau & Subbiah, 2017; Villa-Rojas et al., 2017). RF heating has been widely applied by the food industry for food pasteurization (Al-Holy et al., 2005; Houben et al., 1991) and inactivation of *Salmonella* in many LMF, such as black pepper (Wei et al., 2018, 2019), cumin seeds (Chen et al., 2019), wheat flour (Liu et al., 2018) and almond (Li et al., 2017). It is important to scale-up the lab-scale experiment to an industrial application, which requires the understanding of microbial inactivation kinetics and RF heating profiles, and identification of a suitable surrogate that is specific to the technology and the food matrix.

Historically, EtO fumigation has been used to decontaminate pathogen in spices (Farkas & Andrassy, 1988; Gilbert et al., 1964; Heider et al., 2002; Vajdi & Pereira, 1973). However, most EtO fumigation studies of food commodities were conducted before the 1970s (Gilbert et al., 1964; Phillips & Kate, 1949; Wesley et al., 1965), since then, this technology has been commercialized for decontamination of spice products. There is no up-to-date standard protocol to guide the spice industry on how to properly conduct this pasteurization process and ensure the microbial safety of spice products. A recent study (Newkirk, 2016) found that there was a huge variance in microbial inactivation among two different EtO fumigation facilities and the size of the containers could also significantly affect the process. Thus, it is critical to evaluate the effect of EtO fumigation parameters

(relative humidity, temperature, exposure time) on microbial inactivation and identify the optimal condition as guidance on conducting process validation.

As gas molecules are much smaller than bacteria**,** antimicrobial gas has excellent permeability, which can reach all the niche places for bacteria. The antimicrobial gaseous technology has been well-investigated for high-moisture foods, such as fresh produce, seafood, and meat products (Bentley et al., 2012; Karaca & Velioglu, 2007; Khadre et al., n.d.; J.-M. Kim & Linton, 2008; Prodduk et al., 2014; Simmons et al., 1997), while only a few studies have evaluated thee gaseous technology for *Salmonella* inactivation in LMF. The waterless non-thermal gaseous technologies have a high potential for the pasteurization of LMF while minimizing quality deterioration. Thus, investigation of the gaseous technology and evaluatation of the effects of the process conditions (gas concentration, relative humidity, and exposure time) on microbial inactivation are important for developing the standard protocols and guidelines for LMF industries to apply the technology properly.

As required by the FSMA regulations, proper validations of these decontamination technologies should be performed. The lab-scale validation study should be conducted in such a way that it could be scaled-up easily and provide instructions for the industrial validation. One of the most effective forms of validation is to introduce a non-pathogenic surrogate into the processing facilities and conduct an in-plant validation. Therefore, it is critical to identify a proper surrogate for specific processes and products.

Enterococcus faecium NRRL B-2354 has been demonstrated as a non-pathogenic surrogate (Kopit et al., 2014) for *Salmonella* in various thermal processing of LMF, such as extrusion of oat flour (Verma et al., 2018) and carbohydrate-protein meal (Bianchini et al., 2014), RF heating of wheat flour (Liu et al., 2018; Villa-Rojas et al., 2017) and black pepper (Wei et al., 2018, 2019) and moist-air convection heating of almonds (Jeong & Kang, 2014). Although *E. faecium* has been evaluated as a good surrogate for *Salmonella* during some thermal processing of LMF, it needs to be validated for non-thermal technologies (Rachon et al., 2016). Therefore, it is necessary to evaluate the suitability of *E. faecium* as a non-pathogenic surrogate for specific processing technologies and products.

1.3 Goals of the Research

Salmonella-contaminated dairy powders have resulted in several foodborne outbreaks (Angulo et al., 2008; CDC, 1993; El-Gazzar & Marth, 1992; Park et al., 2004; Rowe et al., 1987), which calls for effective pasteurization technologies. Because *Salmonella* can survive after spray drying and cross-contamination can happen during the post-processing of dairy powders, a pasteurization process should be applied to dairy powders before being used as ingredients in ready-to-eat food products. As milk powder is used to feed young babies and infants and the milk powder is used as an ingredient in ready-to-eat foods like chocolates, a stringent pasteurization of this product is necessary without deteriorating the nutritional content. The RF heating process could be used as a

potential intervention technology for dairy powders. However, instead of conducting a simple case study for demonstrating the effectiveness of RF heating, comprehensive studies were performed for this technology, involving the evaluation of all critical processing parameters (temperature, time, $a_{w,}$ and moisture content). The thermal inactivation kinetics of *Salmonella* was determined at different a_w for estimating the proper time and temperature combination of RF heating treatment. Based on the results, a protocol with high repeatability and reliability was developed for validation of RF heating of dairy powders, which can guide dairy industries on the application of this novel technology.

Spices are the natural harbor for microorganisms and have been closely linked with *Salmonella.* Black pepper and cumin seeds contaminated with *Salmonella* have been reported to be the cause of several outbreaks (CDC, 1982, 2010) and were subjected to numerous recalls (Dey et al., 2013). Currently, several decontamination methods are available for spices; however, all come with some disadvantages which limit their applications. For instance, although gamma irradiation could effectively decontaminate *Salmonella* in black pepper (Song et al., 2014; Vajdi & Pereira, 1973), the poor consumer acceptance restricts the extensive application of this technology. The high-temperature steam treatment has been extensively used for decontamination of black peppercorn in the spice industry. However, due to its high temperature, significant color loss and quality deterioration are caused by steam treatment (Newkirk, 2016; Schneider, 1993).

Therefore, it is necessary to establish innovative technologies that could effectively pasteurize black pepper while minimizing quality deterioration. To provide spice industries with alternative technologies, the use of non-thermal gaseous technologies as intervention technologies for the pasteurization of black peppercorn and cumin seeds was explored. Besides, the measurements of residues and byproducts in black peppercorn and cumin seeds treated by these gaseous technologies were evaluated to determine the optimal condition which could deliver the desired inactivation of *Salmonella* while minimizing the formation of residues or byproducts and quality deterioration. Based on this study, mathematics models were developed for identifying proper process conditions for conducting validations of the gaseous technologies.

The graphical summary of the overall goal of this dissertation is shown in Figure 1.1. This dissertation aimed to fill the knowledge gaps, systematically evaluate different pasteurization technologies for LMF, and guide food manufacturers on how to conduct process validations properly.

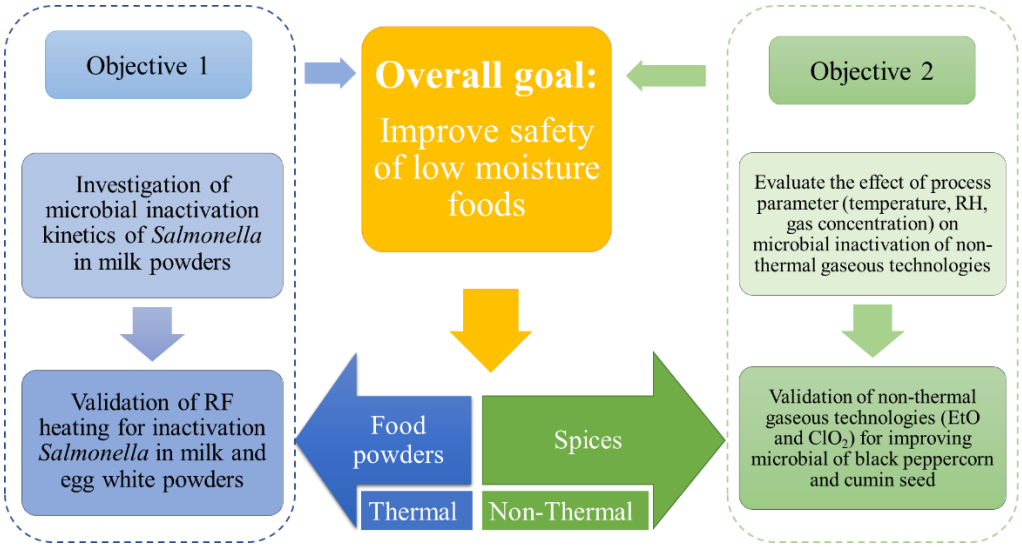

Figure 1.1. Objectives and the overall goal of this dissertation.

1.4 Objectives

This dissertation focused on improving the safety of LMF by using thermal and non-thermal process technologies. The overall goal was to enhance the safety of LMF by improving the implementation of pasteurization technologies and conducting validation studies, considering efficacy, regulatory requirements, and product quality. There were two specific objectives to achieve the overall goal:

A. Validation of thermal processing technology (RF heating) for enhancing the microbial safety of milk and egg white powders:

1. Evaluate the effect of a_w on microbial inactivation kinetics of *Salmonella* in milk powders,
2. Evaluate *E. faecium* as a surrogate for *Salmonella* in milk powders at different storage times and temperatures,

3. Validate RF heating assisted thermal processing as an effective pasteurization process for *Salmonella* in milk powders, and
4. Validate continuous RF heating assisted thermal processing for improving the microbial safety of egg white powders.

B. Validation of non-thermal gaseous technologies (EtO and ClO_2) for enhancing the microbial safety of spices (black peppercorn and cumin seeds):

5. Modeling the effect of temperature and relative humidity on the ethylene oxide fumigation of *Salmonella* and *E. faecium* in whole black peppercorn, and
6. Develop the response surface model for inactivation of *Salmonella* in spices under different gaseous ClO_2 treatment conditions (concentration, relative humidity, and exposure time).

1.5 Dissertation Organization

This dissertation consists of eight chapters. Chapter 1 provides an introduction of the entire dissertation and provides the background of LMF, related food safety issues and several pasteurization process technologies. The rationale and objectives of this dissertation are also presented in this chapter.

Objective 1 is covered in Chapters 2-5. This objective provides a comprehensive framework for adapting thermal technology to ensure food safety. In Chapter 2, the microbial inactivation kinetics of *Salmonella* were determined to identify proper process conditions for subsequent validation of RF heating-assisted thermal processing. Chapter 3

discusses the evaluation of the effect of storage time on the thermal resistance of *Salmonella* and demonstrates *E. faecium* is a suitable surrogate for *Salmonella* during thermal processing. The validation of RF heating-assisted thermal processing is presented in Chapter 4 that shows the technology can be used to properly pasteurize milk powders and ensure their microbial safety. In Chapter 5, continuous RF heating was shown to be an alternative technology for the pasteurization of egg white powder, which reduced the total processing time and improved the product quality.

Chapter 6-7 covers the validation of non-thermal technologies for improving the safety of spices (Objective 2). In Chapter 6, the efficacy of EtO fumigation for the inactivation of *Salmonella* in black peppercorn was evaluated under different treatment conditions. The response surface model was developed, which could provide technical information for guiding spice industries to apply this technology appropriately. Similarly, the antimicrobial gaseous (ClO_2) technology was evaluated to improve the food safety of black peppercorn and cumin seeds. For both gaseous technologies, *E. faecium* was evaluated as a suitable surrogate for *Salmonella*, which can be used for further industrial process validation.

The last chapter summarizes the whole dissertation and provides some suggestions for future research. -

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Chapter 2

EFFECT OF WATER ACTIVITY ON THE THERMAL INACTIVATION KINETICS OF *SALMONELLA* IN MILK POWDERS

2.1 Abstract

Salmonella persistence in milk powders has caused several foodborne outbreaks. The determination of proper pasteurization processing conditions requires identification of the thermal inactivation kinetics of *Salmonella* in milk powders. However, there is a lack of knowledge related to the effect of water activity (a_w) and fat content on *Salmonella* inactivation in milk powder during thermal processing.

Two types of milk powders, nonfat dry milk and whole milk powder, with different fat contents (0.62% and 29.46% w/w) were inoculated with a 5-strain *Salmonella* cocktail and equilibrated to three a_w levels (0.10, 0.20 and 0.30) for isothermal treatments at 75, 80 and 85°C to obtain the *D*- and *z*-values. Stability tests showed that the inoculation method used in this study could provide a high and stable population of *Salmonella* for thermal inactivation studies. The moisture sorption isotherm was measured to understand the relationship between a_w and moisture content of milk powders. The thermal resistance of *Salmonella* was found to significantly increase as a_w decreased, which suggested that a higher temperature or longer processing time would be required to achieve the desired inactivation of *Salmonella* at low a_w. Microbial inactivation kinetics was not significantly

different for two milk powders and therefore data were combined to develop a universal model. The response surface model was compared to the modified Bigelow model. The modified Bigelow model performed well to predict *D* values (RMSE = 1.47 min) and log reductions (RMSE = 0.48 log CFU/g). The developed modified Bigelow model can be used to estimate *D* value as a function of water activity and temperature for designing a thermal pasteurization system for milk powders.

Keywords: Heat resistance, dry milk powder, moisture sorption isotherm**,** low moisture food

2.2 Introduction

Annually, non-typhoidal *Salmonella* causes approximately 1.2 million illnesses and 450 deaths in the United States (Johnson et al., 2014). Historically, several foodborne illness outbreaks have been caused by *Salmonella*-contaminated milk powders, such as powdered infant formula (Angulo et al., 2008; Brouard et al., 2007; Park et al., 2004; Rodríguez-Urrego et al., 2010; Rowe et al., 1987), powdered milk products (CDC, 1993), infant milk powders (Jourdan-da et al., 2018), skim milk powder and dry milk powder (El-Gazzar and Marth, 1992). Milk powders are produced by spray drying of liquid milk. Although spray drying reduces the total bacterial population, it is not a pasteurization process and *Salmonella* may remain viable after the process (LiCari and Potter, 1970). Additionally, after spray drying, there are opportunities for milk powders to be cross-contaminated, including poor environmental sanitation practices and inadequate storage conditions (Podolak et al., 2010). Currently, there is no subsequent processing after spray

drying to pasteurize milk powders (LiCari and Potter, 1970). Considering that milk powders are commonly used as ingredients in many ready-to-eat foods, such as confectionery, drink mixes, seasoning, nutritional bars, and dry blend infant formula, that do not require further cooking steps before consumption, it is therefore necessary to identify an effective pasteurization process to improve the food safety of milk powders (Reij and Den Aantrekker, 2004).

Milk powders are considered as low water activity (a_w) foods with typical a_w values under 0.70 at which *Salmonella* is unable to multiply (Blessington et al., 2013). Although milk powders do not support the growth of *Salmonella*, higher survival of *Salmonella* was observed with declining a_w (Lian et al., 2015). It has also been shown that *Salmonella* could survive for a long period in low a_w food commodities (Keller et al., 2013; Tsai et al., 2019). Furthermore, *Salmonella* may possess higher thermal resistance with decreasing a_w (Smith et al., 2016; Syamaladevi et al., 2016; Villa-Rojas et al., 2013).

The enhanced heat resistance of *Salmonella* at lower a_w makes the elimination of *Salmonella* in milk powders a serious challenge for the food industry. Unlike pasteurization of high a_w foods, the thermal processing of low a_w food is a dynamic process. With the evaporation of moisture, a_w is expected to decrease during the thermal process, which could influence *Salmonella* inactivation due to its enhanced heat resistance at reduced a_w. To account for this dynamic thermal resistance during a thermal pasteurization process, the impact of a_w on *Salmonella* inactivation in milk powders

needs to be evaluated. In the dairy industry, the moisture contents of milk powders are monitored as part of the process controls (Hutson, 2017). Therefore, the development of moisture sorption isotherms of milk powder will be helpful to clarify the relationship between a_w and moisture content and subsequently link moisture content to the inactivation of *Salmonella*. Additionally, the different nutrition compositions like fat and carbohydrate contents of different types of milk powders could contribute to the variation of bacterial inactivation in milk powders. Relatively high fat content has been shown to have a protective effect on *Salmonella* during thermal processing (Ahmed et al., 1995; Verma et al., 2018). Thus, it is also important to evaluate the effect of fat content on the microbial inactivation kinetics of *Salmonella* in milk powders. A better understanding of the thermal inactivation kinetics of *Salmonella* in milk powders will be helpful to guide the dairy industry in designing thermal pasteurization processes for milk powders.

The objectives of this study were to 1) determine the moisture sorption isotherm of milk powders; 2) evaluate the effect of a_w on microbial inactivation kinetics of *Salmonella* in milk powders; and 3) assess the effect of fat content on microbial inactivation kinetics.

2.3 Materials and Methods

2.3.1 Milk Powder Sample

For this study, three production batches of two different milk powders, Grade A pasteurized non-fat dry milk (NFDM) and Grade A pasteurized whole milk powder (WMP)

were provided by Mars, Incorporated (McLean, VA). Upon receiving the samples, total plate counts performed to quantify the background microflora in milk powders following the standard procedure (Villa-Rojas et al., 2017; Liu et al., 2017). Three random 10-g of samples were removed randomly from each batch and diluted in 90 mL of 0.1% buffered peptone water (BPW; Becton, Dickinson and Company, Sparks, MD). The diluted samples were blended for 1 min in a paddle mixer (Neutec Group Inc, Farmingdale, NY, USA), serially diluted in 9 mL 0.1% BPW blanks, plated onto tryptic soy agar (TSA) supplemented with 0.6% (w/v) yeast extract (TSAYE) and incubated for 24 h at 37°C for enumeration.

2.3.2 Proximate Composition and Calories Estimation

AOAC official methods (AOAC, 2000) were used to determine ash (AOAC Official Method 985.01), fat (AOAC Official Method 989.05), and protein (AOAC Official Method 990.03) of NFDM and WMP. The moisture contents of both milk powders were measured by a halogen moisture analyzer (HR73, Mettler Toledo Laboratory and Weighing Technologies, Greifensee, Switzerland), and a_w values at 25°C were determined with a dew point water activity meter (Aqualab Series 4TE, METER Group, Pullman, WA). Carbohydrates were determined by subtracting other compositions from the total weight. Calories were calculated using the Atwater general factors, which are 4 kcal/g for protein, 9 kcal/g for fat, and 4 kcal/g for carbohydrate.

2.3.3 Moisture Sorption Isotherm

The moisture sorption isotherm of each milk powder product was generated with an AquaLab Vapor Sorption Analyzer (METER Group, Inc, Pullman, WA, USA) at 20°C. Approximately 1 g of sample was spread uniformly on a stainless steel sample cup for measurement of both absorption and desorption curve at a single temperature. The Dynamic Vapor Sorption method was used to measure the sorption isotherm (Garbalińska et al., 2017). For the sorption isotherm development, the moisture content at equilibrated points was determined in triplicate at a_w levels from 0.10 to 0.90 with 0.05 intervals. The trigger (%dm/dt; percent change in mass over a change in time for determination of the equilibrated point) was set at 0.020%/h with three events, which indicated that three readings in a row must be less than the set trigger value.

2.3.4 Bacterial Strain and Inoculation

The five strains of *Salmonella enterica* used in this study have been associated with different low a_w foodborne outbreaks. *S*. Agona 447967, *S*. Montevideo 488275 and *S*. Mbandaka 698538 were obtained from the FDA, Office of Regulatory Affairs Regional Lab in Jefferson, AR. *S*. Reading 180418 and *S*. Tennessee K4643 were obtained from the University of Georgia, Athens, GA. The frozen bacteria cultures were kept in trypticase soy broth (TSB) with 0.6% (w/v) yeast extract (TSBYE) supplemented with 20% glycerol and stored at -80°C until used.

Similar procedures were followed to prepare the *Salmonella* inoculum as described in

Hildebrandt et al. (2016) and Liu et al. (2017). To prepare the 5-strain *Salmonella* cocktail, one cryovial of frozen culture of each *Salmonella* strain was thawed at 22°C for 10 min. Then, one milliliter of each bacterial culture was transferred to 10 mL of TSBYE and incubated for 24 ± 2 h at 37°C. From the overnight culture, one loopful of culture was streaked onto the surface of TSAYE. After incubation at 37°C for 24 ± 2 h, the agar plate with isolated colonies was then wrapped with parafilm and stored at 4°C for up to a month. One isolated colony of each strain was transferred to 10 mL of TSBYE and incubated for 24 h at 37°C for second enrichment. To prepare the inoculum lawn, 0.1 mL of the overnight culture was spread plated onto TSAYE plates for incubation (24 ± 2 h at 37°C). The overnight lawns grown on the plate were harvested by adding 3 mL of 0.1% BPW and agitating the lawns with a sterile L-shaped spreader. Equal amounts of the dissolved lawns of each *Salmonella* strain were transferred to a sterile centrifuge tube and vortexed for 10 s to prepare the *Salmonella* cocktail. The initial bacterial population of the *Salmonella* cocktail was approximately 10.5 log CFU/mL.

The prepared *Salmonella* cocktail (10 mL) was sprayed onto 100 ± 0.1 g of WMP or NFDM samples in a sterile Whirl-Pak bag (23 x 12 cm). The inoculated sample was hand-massaged to exfoliate all powder from the inner surface of the bag and then homogenized in a paddle mixer for 6 min.

2.3.5 Sample Equilibration and Stability Tests

Inoculated samples were then uniformly placed on a sanitized aluminum tray (230 x

300 x 15 mm^3) and transferred to a custom designed humidity-controlled chamber. To evaluate the effect of a_w on thermal inactivation of *Salmonella*, the relative humidity in the chamber was set to 10.0%, 20.0% or 30.0% to equilibrate the inoculated sample to a target a_w of 0.10, 0.20 and 0.30, respectively. During the inoculation process, the addition of moisture from the inoculum caused the powders to cake and form clumps, and therefore additional steps were taken to reduce the clumps back into powder. Upon reaching the target a_w, inoculated samples were packed into sterile Whirl-Pak bags and then homogenized in a paddle mixer for 3 min to break the dried-up clumps. The homogenized samples were transferred back to the humidity-controlled chamber for re-equilibration.

Stability tests were conducted for both powder samples with a_w of 0.20 to evaluate the inoculation method used in this study. At Days 0, 1, 2, 3, 4, 5, 8, 12, 16, and 20, 2 ± 0.1 g of each inoculated powder sample from each batch were transferred to a sterile Whirl-Pak bag for enumeration. The samples were first tenfold diluted with 18 mL of 0.1% BPW and then stomached for 1 min. Blended samples were serially diluted in 9 mL 0.1% BPW and three dilutions of all samples were duplicate-plated onto TSAYE supplemented with 0.05% (w/v) ammonium iron citrate (Sigma-Aldrich, Co., MO, USA), and 0.03% (w/v) sodium thiosulfate (Fisher Chemical, Lenexa, KS, USA) (mTSA) and incubated for 24 h at 37°C (Liu et al., 2019; Smith et al., 2016). After incubation, colonies with a black center were enumerated as *Salmonella*. Homogeneity of inoculated samples was evaluated by enumerating six randomly selected subsamples (2 ± 0.1 g) from a 100 g batch of inoculated

powder on Day 1. Inoculated samples were considered to be homogenous when the standard deviation of the six enumerated samples was < 0.3 log CFU/g.

2.3.6 Isothermal Inactivation

In this study, the isothermal treatment of inoculated milk powder was conducted using a custom-designed thermal death time sandwich (Lau et al., 2019). Two grams of inoculated sample was packed into an aluminum-laminated plastic pouch (75 x 75 x 1 mm, Impak Corporation, Los Angeles, CA). To measure the temperature of the sample, a T-type thermocouple probe was inserted into the center of the aluminum pouch. The come-up time at each temperature was defined as the time required for the sample temperature from room temperature (22°C) to reach 0.5°C below the target temperature. For each target temperature, the average of three measurements of come-up time plus two standard deviations was used as time zero for the subsequent isothermal treatment. No thermocouples were inserted into the inoculated samples during the actual isothermal treatments.

The isothermal treatment temperatures used to obtain thermal death curves for *Salmonella* in both WMP and NFDM were 75, 80, and 85°C. The target isothermal temperatures were determined based on our preliminary experiments. Temperature higher than 85°C can result in discoloration of samples and temperatures lower than 75°C takes considerably long time for pasteurization. Six time points including time zero with equal time intervals were used for isothermal treatment to achieve at least 3 log reduction of

Salmonella. After each isothermal treatment, the treated aluminum pouch was immediately transferred to an ice-water bath for about 1 min to stop further thermal inactivation. The treated samples were enumerated using the same method as described previously in the sample equilibration and stability tests section. Three biological replicates (batches of milk powder samples from different production lots inoculated with independently grown bacterial cultures) were used in the experiments. Untreated inoculated samples from each batch were randomly selected and enumerated as the control.

2.3.7 Determination of *D*-value and z-value

The thermal inactivation kinetics of *Salmonella* in milk powders were modeled with the log-linear model to obtain the *D*-value:

$$D = \frac{t}{log_{10}\, N_0 - log_{10}\, N}$$

where N_0 (CFU/g) is the bacteria population at come-up time, N (CFU/g) is the bacteria population at time *t*, *t* (min) is the length of isothermal treatment time, and *D* (min) is the time required to achieve a 10-fold reduction of the bacteria at the isothermal treatment temperature. In addition to R^2 value, the goodness-of-fit of the models were quantified by the root mean square error (RMSE) (log CFU/g):

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

where $log_{10}(N)_{observed,i}$ is the observed log population, $log_{10}(N)_{predicted,i}$ is the predicted log population from the model, and *n* is the total number of observations.

The *z*-value is defined as the increase in temperature required to achieve a 90% reduction

of the decimal reduction time *D* and was determined by the equation:

$$z = \frac{T_2 - T_1}{log_{10}\, D_{T_1} - log_{10}\, D_{T_2}}$$

where $D_{T_1} and\ D_{T_2}$ are the *D*-values at temperatures T_1 and T_2, respectively.

2.3.8 Secondary Model Fitting

A split-plot analysis (a_w is the whole plot and temperature is the subplot) was used to determine the effect of temperature and a_w on the *D*-value and reduction of *Salmonella* in WMP and NFDM. The first secondary model evaluated in this study response surface model and only factors with significant effects ($P < 0.05$) were considered in this model. The parameters for the response surface model were estimated using the analysis of variance and response surface method functions in the open-source statistical software, R (Lenth, 2009).

The second secondary model evaluated in this study was a modified version of a Bigelow-type relationship (Gaillard et al., 1998):

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

where D_{ref} is the time (min) needed to achieve a ten-fold reduction in the population at T_{ref} and $a_{w,ref}$, T is the temperature (°C), T_{ref} and $a_{w,ref}$ are the optimized reference temperature and water activity (Dolan et al., 2013), z_{aw} or z_T is the a_w or temperature increment needed to decrease the D-values by 10-fold, respectively. All parameters for Bigelow-type model were estimated using OLS minimization with nlinfit in MATLAB 2019.

The corrected Akaike information criterion (AIC_c) was also calculated for evaluating each model (Motulsky and Christopoulos, 2004):

$$\mathrm{AICc} = \mathrm{nln}(\frac{SS}{n}) + 2\mathrm{K} + \frac{2K(K+1)}{n-K-1}$$

where n is the total number of the data points, SS is the sum of squares of the residuals, and K is the number of parameters being estimated plus 1. Lower AIC_c values indicate that the determined model is more likely to be correct for the data.

2.4 Results and Discussion

2.4.1 Moisture Sorption Isotherm

The moisture sorption isotherm results are shown in Figure 2.1. For both milk powders, the moisture contents slowly increased with increasing a_w. After a_w reached 0.40, the moisture content was found to increase sharply with rising a_w until it reached 0.55, which may be attributed to capillary condensation (Tham et al., 2016). When the powder started to absorb moisture, part of the powder became crystalline while part of it remained amorphous. Compared to the corresponding pure amorphous substance, powders containing a mixture of amorphous and crystalline components have been shown to have a greater water sorption capacity (Hartmann and Palzer, 2011). Consequently, a higher absorption rate of moisture was observed after the initial uptake of moisture.

After a_w reached 0.60, the moisture contents of both milk powders declined slightly, which may be due to lactose crystallization (Tham et al., 2016). According to McCarthy et al. (2013), a sudden drop in water content was observed because the amorphous lactose

had lower water sorption prior to lactose crystallization. Subsequent increases in a_w were followed by an increase in the moisture content. At high a_w, lactose crystallization is an irreversible change (Berggren and Alderborn, 2004), therefore the trends observed from 0.4 to 0.6 a_w in the absorption curve were not observed in the desorption curve. At the same a_w level, NFDM had higher moisture content than WMP (Figure 2.1) which was due to the higher protein content of NFDM (Table 2.1). Proteins have been shown to increase the moisture absorption in milk powder, by binding more water molecules at the same a_w level (Ibach and Kind, 2007; Shrestha et al., 2007). Therefore, NFDM was found to have higher moisture content than WMP at the same a_w level due to interaction between moisture and protein.

2.4.2 Stability Tests of Powder Inoculation

The aerobic bacteria counts of both types of uninoculated milk powders were estimated to be < 100 CFU/g. As the inoculated population of *Salmonella* was considerably higher and the differential medium (mTSA) was used for enumeration, the overall microbial results should not be affected by the background microflora. The detection limit of the enumeration method used in this study is <10 CFU/g.

The initial *Salmonella* populations, after inoculation, in WMP and NFDM ranged from 7.7 to 7.9 and 8.1 to 8.5 log CFU/g, respectively (Figure 2.2). One day after the samples were transferred to the equilibration chamber, the a_w levels of WMP and NFDM were adjusted to the target a_w. About 1.1 and 0.5 log CFU/g reductions of *Salmonella* in

WMP and NFDM, respectively, were observed at the end of the a_w adjustment (Figure 2.2). The *Salmonella* populations in WMP and NFDM were then monitored during the following 20 days, and less than 0.2 log CFU/g reduction of *Salmonella* population was found in both samples. The inoculation method used in this study was shown to provide stable *Salmonella* populations in both types of milk powders prepared for thermal treatment. Different inoculation methodologies have been shown to significantly influence the thermal inactivation of *Salmonella* in low a_w foods (Hildebrandt et al., 2017, 2016; Liu et al., 2019). A proper inoculation method which simulates the real route of sample contamination could help yield more reliable inactivation kinetics. Hildebrandt et al. (2016) suggested that lawn-based pelletized inoculum, which was used in this study, could provide a stable *Salmonella* population prior to isothermal treatment and yield consistent *D*-values. Several studies have shown that this lawn-based inoculation method provides consistent and stable *Salmonella* inoculated samples of different low a_w foods, such as spices (Chen et al., 2019; Wei et al., 2019, 2018), oat and wheat flour (Liu et al., 2017; Verma et al., 2018) and egg white powder (Wei et al., 2020). Also, this wet inoculation method could simulate a common contamination scenario of milk powders, in which *Salmonella*-contaminated liquid drips from the roof of a processing plant into the powders being processed.

The 20-day stability tests indicated that *Salmonella* could survive in milk powders for a long time and no considerable decrease in *Salmonella* population was found after the

inoculated samples were stabilized. Several studies (Abd et al., 2012; Keller et al., 2013; Lian et al., 2015) also showed that *Salmonella* could survive in low a_w foods for a long period, and higher survival of *Salmonella* was found at a lower a_w level.

During the a_w adjustment of milk powders and the corresponding desiccation stress, *Salmonella* bacteria would start to enhance their thermal resistance. (Gruzdev et al., 2011). Therefore, the inoculated samples were equilibrated for at least five days before the isothermal treatment to allow the inoculated *Salmonella* to fully adapt to the low a_w environment and presumably attain a high thermal resistance.

2.4.3 Thermal Resistance of *Salmonella* in WMP and NFDM

This study aimed to determine the effect of a_w on the thermal inactivation kinetics of *Salmonella* in WMP and NFDM. At 75, 80 and 85°C, WMP yielded come-up times of 63, 74 and 75 s, respectively. Similarly, the come-up times of NFDM were found to be 87, 87 and 88 s at 75, 80 and 85°C, respectively. The thermal death time sandwich has a PID control system to achieve the target temperature. When two standard deviations were added to the mean time to reach the target temperature, the come-up time values were similar for all isothermal temperatures. The isothermal inactivation curves of *Salmonella* in WMP and NFDM at a_w of 0.10, 0.20 and 0.30 are shown in Figure 2.3.

Thermal resistance parameters of WMP and NFDM are reported in Table 2.2. According to Liu et al., (2019), the *D*-value of *Salmonella* in NFDM with a_w of 0.25 at 85°C was found to be 16.05 min. Michael et al. (2014) reported that the *D*-values of

Salmonella in NFDM were 23.02, 10.45, 8.63 and 5.82 min at 75, 80, 85 and 90°C, respectively (a_w data were not reported). In this study, at a_w of 0.20, the *D*-values of *Salmonella* at 75, 80 and 85°C were 22.70, 12.11 and 6.17 min for WMP and 26.76, 10.46 and 5.60 for NFDM, respectively (Table 2.2). The minor differences in *D*-values for the similar products could be attributed to the use of different bacterial strains (Ma et al., 2009; Quintavalla et al., 2001), different inoculation method (Bowman et al., 2015; Liu et al., 2019) or different a_w (Syamaladevi et al., 2016; Tsai et al., 2019; Villa-Rojas et al., 2013). For both types of milk powders, at all temperatures and a_w levels, the isothermal inactivation curves were found to follow a log-linear trend with high R^2 values ($R^2 > 0.94$).

The effect of temperatures and a_w on $\log_{10}$ *D*-values are shown in Figure 2.4. The z_T-values of *Salmonella* in both milk powder at different a_w levels ranged from 14.75 to 17.68°C and z_{aw}-values ranged from 0.44 to 0.66 (Table 2.2). Liu et al. (2019) reported that the *z*-values of *Salmonella* in NFDM at a_w of 0.25 varied between 16.0 to 16.5°C, which were similar to the *z*-values of *Salmonella* in NFDM found in this study. No significant difference ($P < 0.05$) was found for the *z*-values of *Salmonella* in NFDM at different a_w levels. The estimated *z*-values could be used to predict the *D*-values at different temperatures, which could assist the determination of the desired thermal processing temperature. If a food manufacturer wants to estimate a *D* value simultaneously in water activity and temperature, these independent z values will not be sufficient. Therefore, the response surface model and Bigelow were developed.

Response surface models were fit for *D*-values of *Salmonella* in WMP and NFDM under isothermal treatment at different a_w and temperatures. The resulting *D*-value models were as follows:

WMP: $D = 1162.581479 - 24.321946T - 896.135507a_w + 8.788976Ta_w + 0.128314T^2 + 303.089547{a_w}^2$ RMSE = 1.70 min, AIC_c = 119.20

NFDM: $D = 1347.962075 - 28.889744T - 867.512273a_w + 8.825606Ta_w + 0.5587T^2 + 239.108313{a_w}^2$ RMSE = 1.36 min, AIC_c = 107.32

where *D* the *D*-values of WMP and NFDM, T is the isothermal treatment temperature (°C), and a_w is the water activity of the sample.

The fitted modified Bigelow model were as follows:

WMP: $D = 33.13 * 10^{\frac{0.12-a_w}{0.54}} * 10^{\frac{75.8-T}{15.4}}$ RMSE = 1.57 min, AIC_c = 31.33

NFDM: $D = 32.58 * 10^{\frac{0.12-a_w}{0.47}} * 10^{\frac{75.7-T}{16.65}}$ RMSE = 1.25 min, AIC_c = 18.81

For both RSM models, temperature and a_w showed significant linear and quadratic effects, and a significant interaction between temperature and a_w was also observed. In these two models, the estimated value of each parameter was not significant from each other ($P > 0.05$). Therefore, data were combined for both milk powders and the following universal response surface model and modified Bigelow model were generated:

The universal models also provided a reasonable prediction of *D*-values for both types of

Both WMP and NFDM: $D = 1255.271777 - 26.605845T - 881.82389a_w + 8.807291Ta_w + 0.1421T^2 + 271.09893{a_w}^2$ RMSE = 1.61 min, AIC_c = 218.43

$D = 32.58 * 10^{\frac{0.12-a_w}{0.47}} * 10^{\frac{75.7-T}{16.65}}$ RMSE = 1.47 min, AIC_c = 47.40

milk powders and may be more convenient to be used as a reference for the food industry. The predictions and residual analyses of both models are shown in Figure 2.5. While the RMSE is acceptable, the residuals at lower D values (at a higher temperature and higher a_w), the maximum error could be up to +/- 41.8% error for RSM model and +/- 24.0% for modified Bigelow model. The fat content did not show a significant effect on the *D*-values of the response surface model and therefore was not included in the final model. Although the protective effect of fat on *Salmonella* inactivation has been observed in many high moisture foods (Ahmed et al., 1995; Verma et al., 2018), a similar protective effect was not found in milk powders. The protection effect of fat was not observed in milk powder during this short storage time (5 days).

For the convenience of application of the response surface model, the response surface model for directly predicting log reductions was also generated by including time (t) as the independent variable as follow:

Both WMP and NFDM:	$Log\ (No/N) = 106.5685 - 2.585471T - 57.91383a_w - 0.6847655t + 0.714746Ta_w + 0.008980569Tt + 0.2262141a_wt + 0.01567976T^2 + 6.369736{a_w}^2 + 0.00004763879t^2$	$RMSE$ = 0.48 log CFU/g AIC_c = 617.19

where $Log\ (No/N)$ is the log reduction of *Salmonella* in WMP and NFDM and t is the holding time of isothermal treatment. This model could predict *Salmonella* reduction directly for both types of milk powders, which is more convenient for the industrial application. To compare its performance with the combined Bigelow model, the log

reduction could also be predicted by using the *D*-values generated from the universal Bigelow model, which gave a RMSE of 0.47 log CFU/g and AIC_c of -456.93. The predictions and residual analyses of both models are shown in Figure 2.6.

Based on the AIC_c and RMSE values, the modified Bigelow model was a better fit for the range of conditions evaluated when compared to the RSM model, which agreed with Smith et al. (2016). While RSM model is an empirical (data-driven) model, the Bigelow model is a mechanistic model with phenomenological meaning and is more preferable.

2.4.4 Influence of Temperature and a_w on the Thermal Resistance of *Salmonella*

The contour plots based on the developed universal model for predicting *D* values are shown in Figure 2.7. According to the results, temperature showed a significant positive effect ($P < 0.05$) on the inactivation of *Salmonella* in both milk powders. A significant negative effect ($P < 0.05$) of a_w was observed on the inactivation of *Salmonella* in both milk powders based on the models. Therefore, a higher temperature would be required to obtain similar inactivation kinetics at lower a_w levels, thus supporting the observation that *Salmonella* thermal resistance increases at lower water activity values.

During the thermal processing of low a_w foods, a_w is a critical parameter to be controlled as it considerably affects the heat resistance of bacteria (Aljarallah and Adams, 2007; Podolak et al., 2010). Low a_w has been reported to show a protective effect on different foodborne pathogens, such as *Staphylococcus epidermidis* (Verrips and Van

Rhee, 1981), *Listeria monocytogenes* (Valdramidis et al., 2006), and *Salmonella enterica* (Smith et al., 2016; Villa-Rojas et al., 2013). In this study, *D*-values of *Salmonella* in both WMP and NFDM increased with decreasing $a_{w,}$ which indicated that a longer thermal treatment time would be required to achieve the desired reduction of *Salmonella* for the milk powder with lower a_w.

Because of the moisture evaporation during the thermal processing, the a_w of milk powder is expected to decrease throughout the thermal pasteurization process. The reduced a_w could enhance the thermal resistance of *Salmonella*, which will require a more severe treatment. It is essential to understand the effect of a_w on thermal inactivation of *Salmonella* in milk powders when developing the proper pasteurization process. The models developed in this study will help the food industry to understand the influence of temperature and a_w on the thermal inactivation of *Salmonella* in milk powders and identify the proper temperature and time combinations for the development and implementation of pasteurization process to ensure food safety.

2.5 Conclusions

The inoculation method used in this study provided high and stable populations of *Salmonella* in both WMP and NFDM. The thermal resistance of *Salmonella* has been shown to increase significantly with decreasing a_w. However, different fat contents in WMP and NFDM did not significantly influence the thermal resistance of *Salmonella*. The universal models developed in this study could be a valuable tool to predict a

reference *D*-value of *Salmonella* at selected a_w and temperature for the food industry. The thermal inactivation kinetics of *Salmonella* in milk powders could provide technical information for developing and validating thermal processing for controlling *Salmonella* in milk powders.

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2.7 References

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Table 2.1. Proximate composition (%) of whole milk powder (WMP) and nonfat dry milk (NFDM), n = 3 samples.

Milk powder	Component	Concentration (%)	Water activity	Calories (kcal/kg)
WMP	Ash	4.91 ± 0.53	0.175 ± 0.001	5176.2 ± 17.10
	Protein	25.50 ± 0.12		
	Lipid	29.46 ± 0.52		
	Carbohydrates	37.62 ± 1.27		
	Moisture	2.51 ± 0.43		
NFDM	Ash	7.97 ± 0.24	0.158 ± 0.002	3583.0 ± 14.10
	Protein	36.41 ± 0.24		
	Lipid	0.62 ± 0.03		
	Carbohydrates	51.77 ± 0.52		
	Moisture	3.23 ± 0.29		

Table 2.2. Thermal resistance of *Salmonella* in whole milk powder (WMP) and nonfat dry milk (NFDM) at different water activities (a_w).[1]

Milk powder	a_w	Temp (°C)	*D*-value (min)	RMSE (log CFU/g)	*z*-value (°C)
WMP	0.10	75	41.1 ± 2.7 [a]	0.2	16.4 ± 0.6 [ab]
		80	22.8 ± 2.5 [d]	0.3	
		85	10.1 ± 0.9 [g]	0.2	
	0.20	75	22.8 ± 1.7 [c]	0.2	17.7 ± 1.0 [a]
		80	12.1 ± 0.4 [f]	0.3	
		85	6.2 ± 0.1 [ij]	0.4	
	0.30	75	17.0 ± 0.1 [e]	0.2	14.8 ± 0.3 [c]
		80	8.1 ± 0.62[hj]	0.2	
		85	3.6 ± 0.1 [k]	0.1	
NFDM	0.10	75	40.6 ± 0.5 [a]	0.3	16.0 ± 0.9 [bc]
		80	19.7 ± 0.6 [d]	0.2	
		85	9.6 ± 0.8 [gh]	0.3	
	0.20	75	26.9 ± 2.1 [b]	0.3	14.9 ± 0.1 [bc]
		80	10.5 ± 0.2 [fg]	0.2	
		85	5.7 ± 0.7 [j]	0.3	
	0.30	75	16.9 ± 0.5 [e]	0.2	15.0 ± 1.5 [bc]
		80	9.8 ± 0.7 [gh]	0.2	
		85	3.6 ± 0.5 [k]	0.2	

[1]Values are mean ± standard deviation. [a-k]*D*- and *z*-values with differing letters are significantly different ($P < 0.05$).

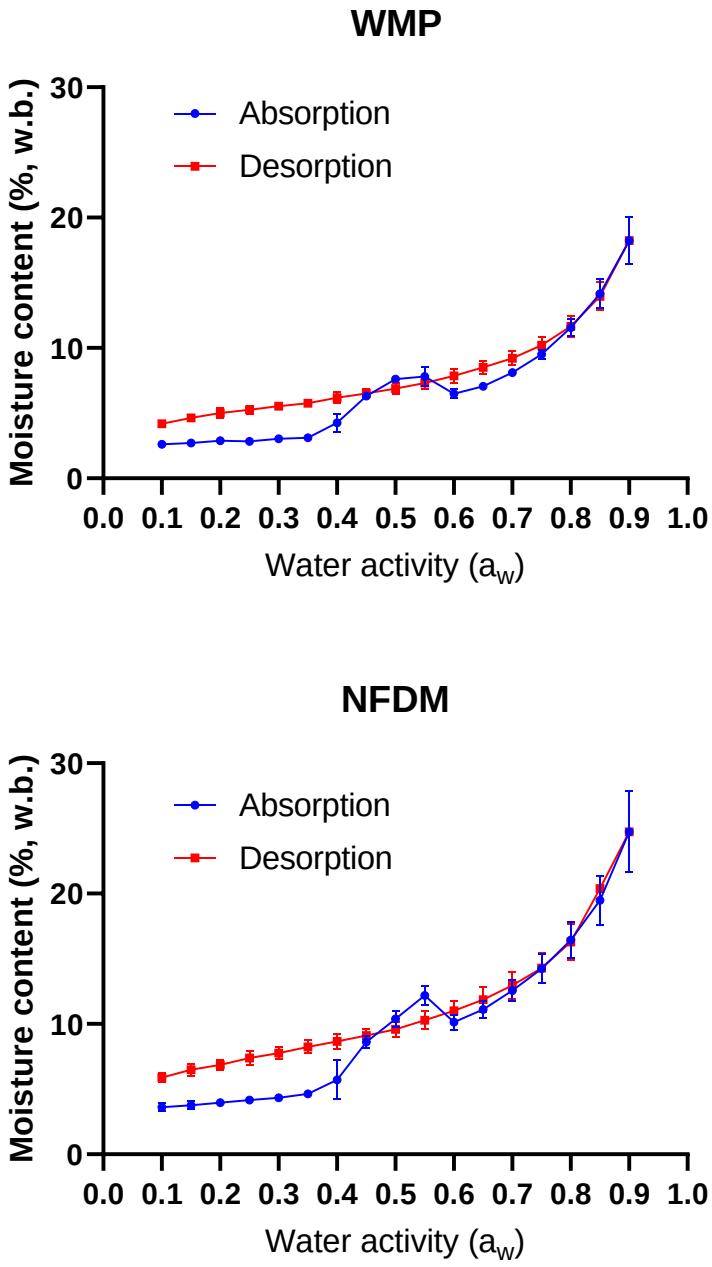

Figure 2.1. Moisture sorption isotherm of whole milk powder (WMP) and nonfat dry milk (NFDM) at 20°C. The moisture content at equilibrated point was determined in triplicates at the a_w level from 0.10 to 0.90 with a 0.05 interval. Error bars represent standard deviations of three replications.

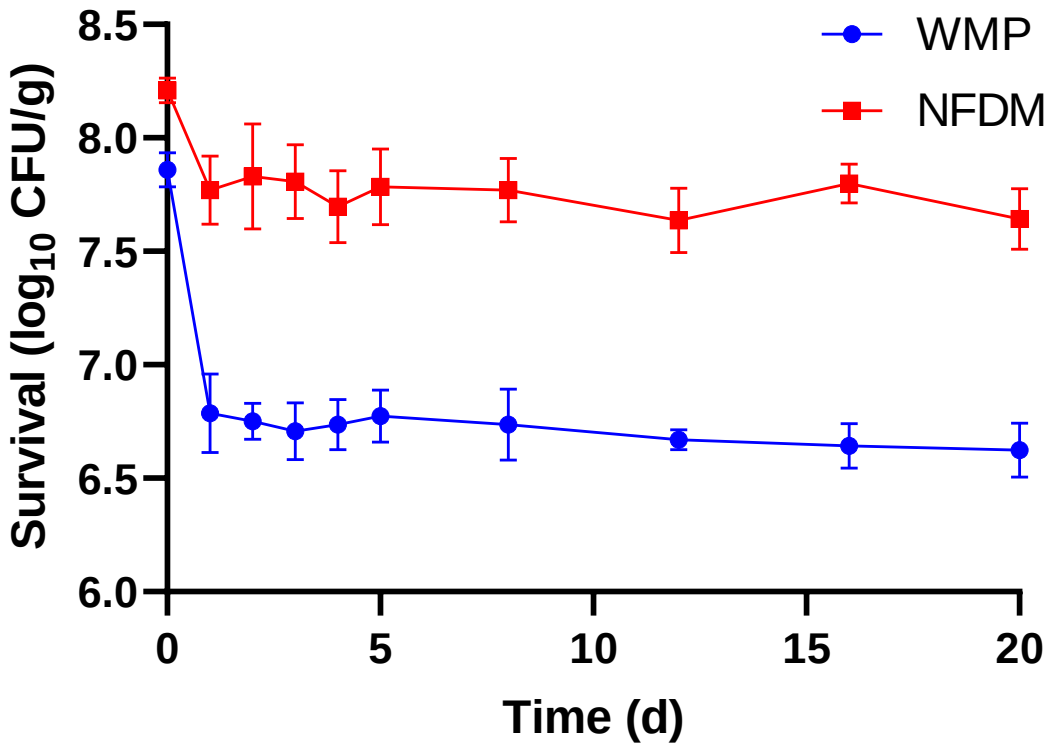

Figure 2.2. Stability tests of *Salmonella* in whole milk powder (WMP) and nonfat dry milk (NFDM). WMP and NFDM samples from each independent batch (n = 3) were collected and enumerated at day 0, 1, 2, 3, 4, 5, 8, 12, 16, and 20. Sample inoculation was conducted in day 0. Error bars represent standard deviations of three replications.

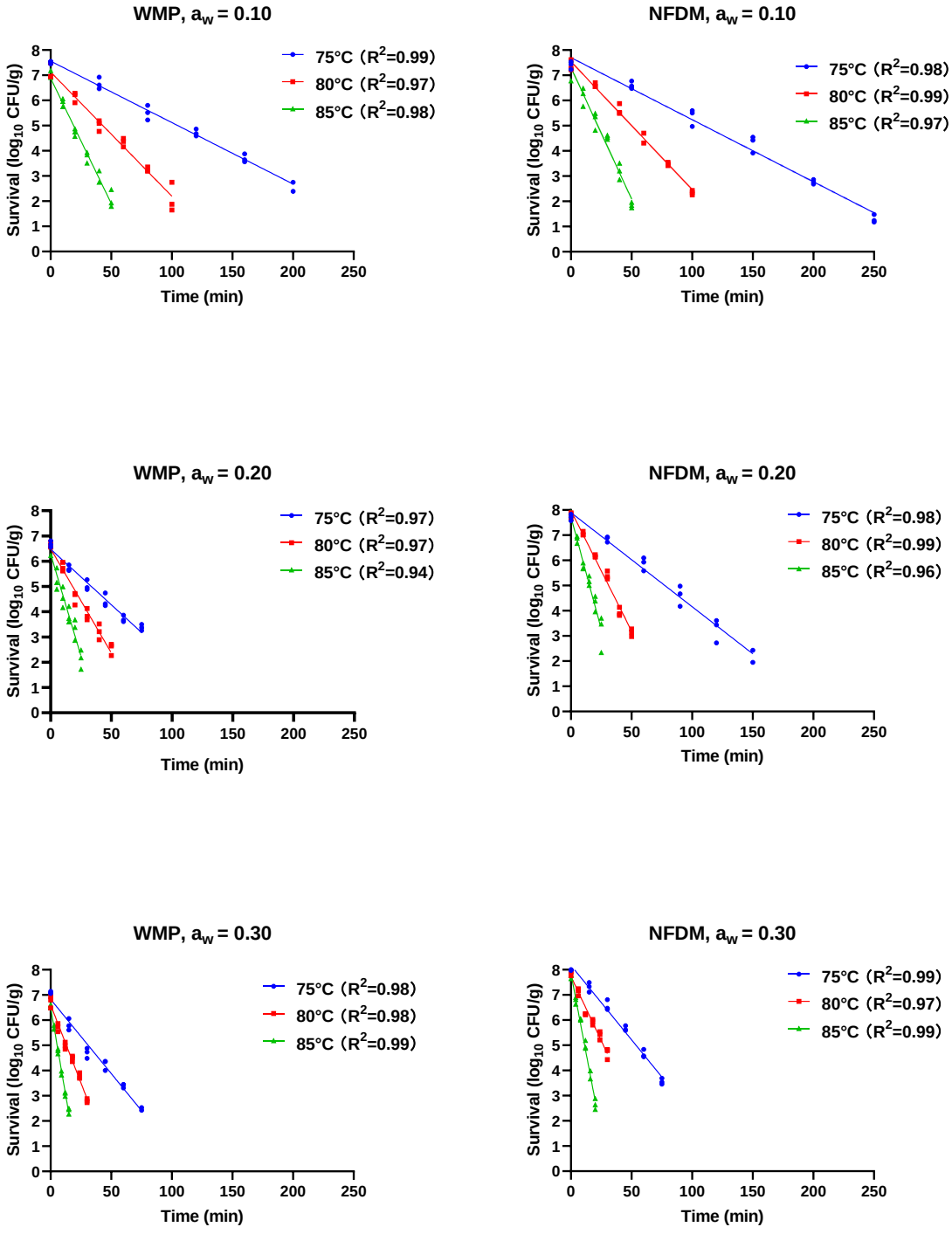

Figure 2.3. Thermal inactivation kinetics of *Salmonella* in whole milk powder (WMP) and nonfat dry milk (NFDM) equilibrated to the different a_w (0.10, 0.20 and 0.30) at various temperatures. Each isothermal treatment condition was performed in triplicates.

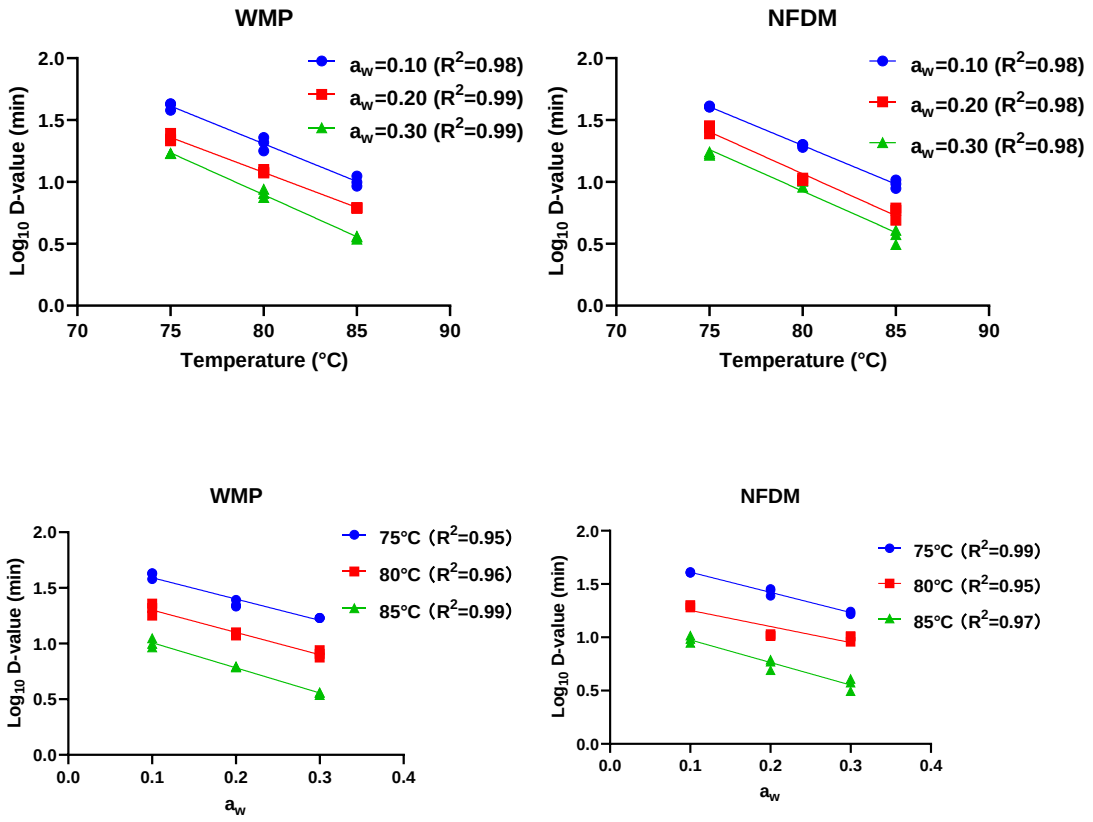

Figure 2.4. z_T and z_{aw} values of *Salmonella* in whole milk powder (WMP) and nonfat dry milk (NFDM) at different temperatures (top row) and a_w levels (bottom row).

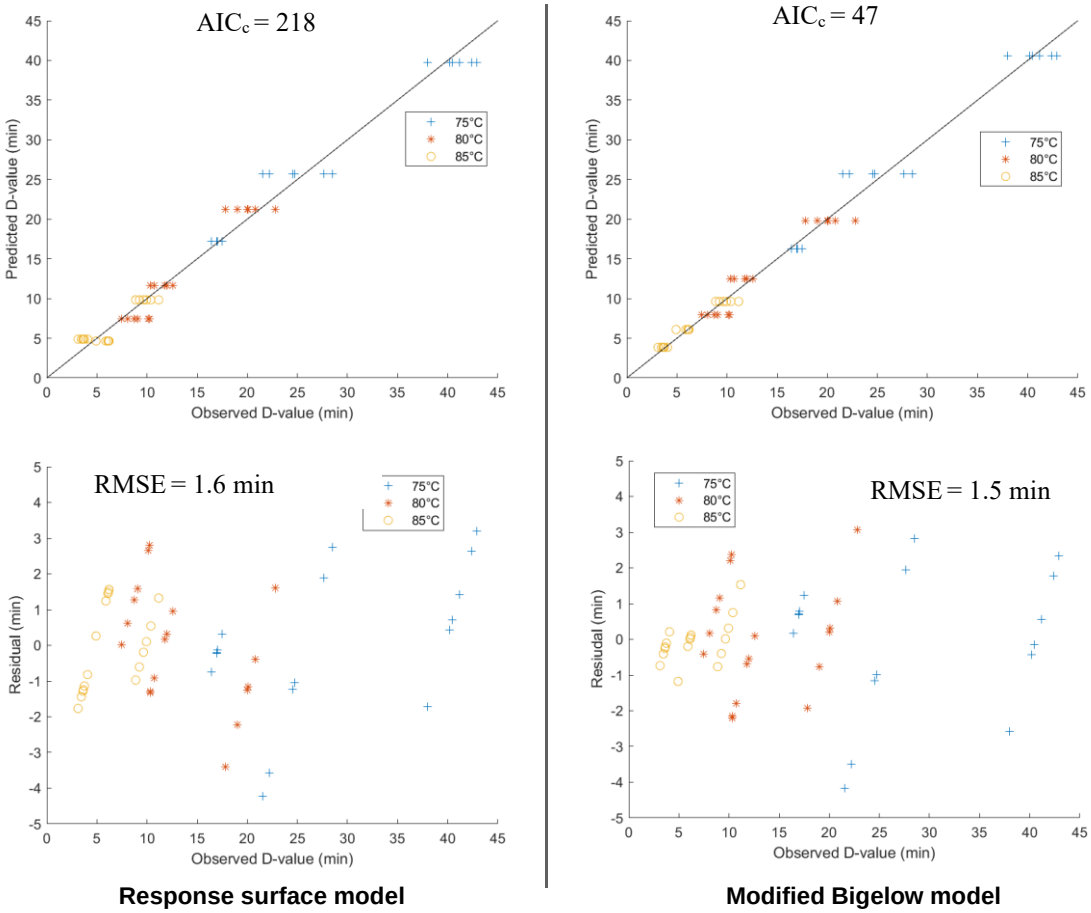

Figure 2.5. Comparison of the response surface model and modified Bigelow model for predicting *D*-value of *Salmonella* in milk powders. Root mean square error (RMSE) and corrected Akaike information criterion (AICc) were used for evaluation of model fitting.

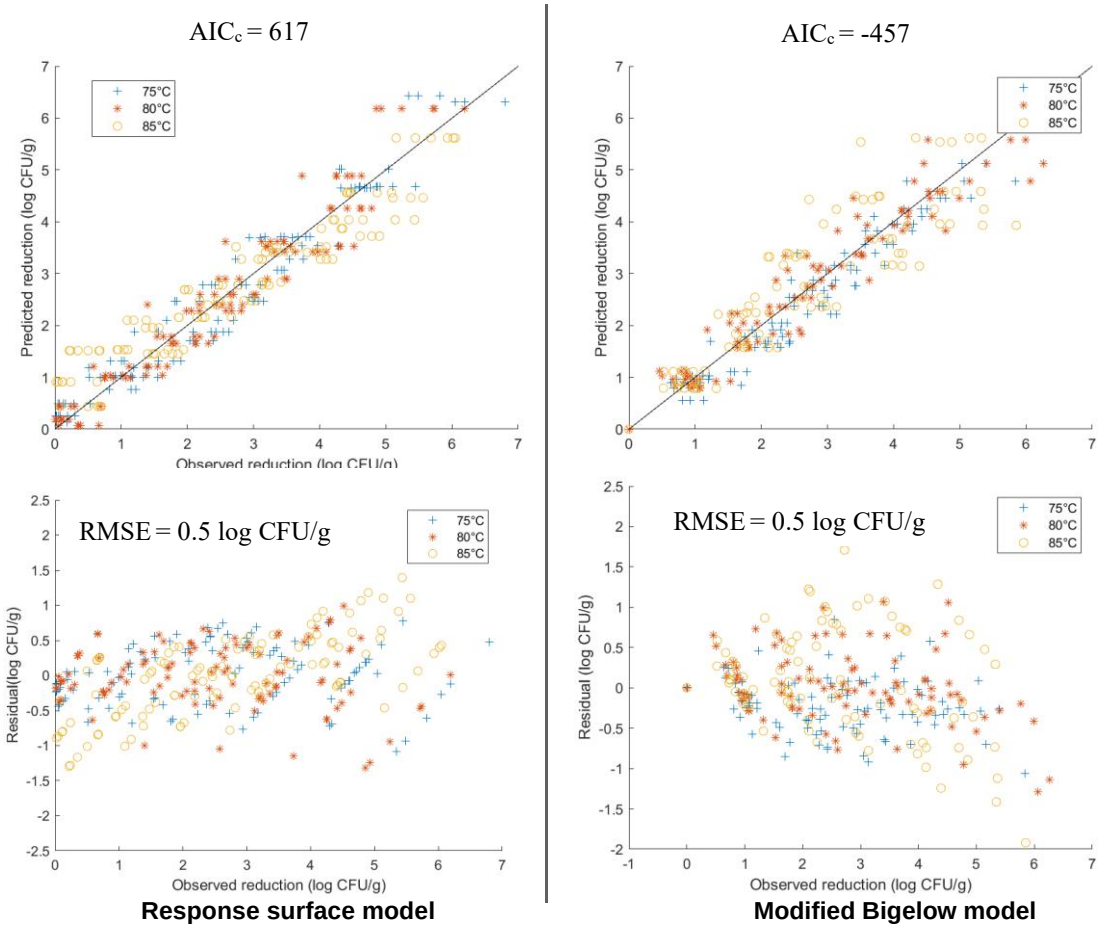

Figure 2.6. Comparison of response surface model for predicting log reduction and modified Bigelow model for predicting *D*-values by evaluating log reductions of *Salmonella* in milk powders. Root mean square error (RMSE) and corrected Akaike information criterion (AICc) were used for evaluation of model fitting.

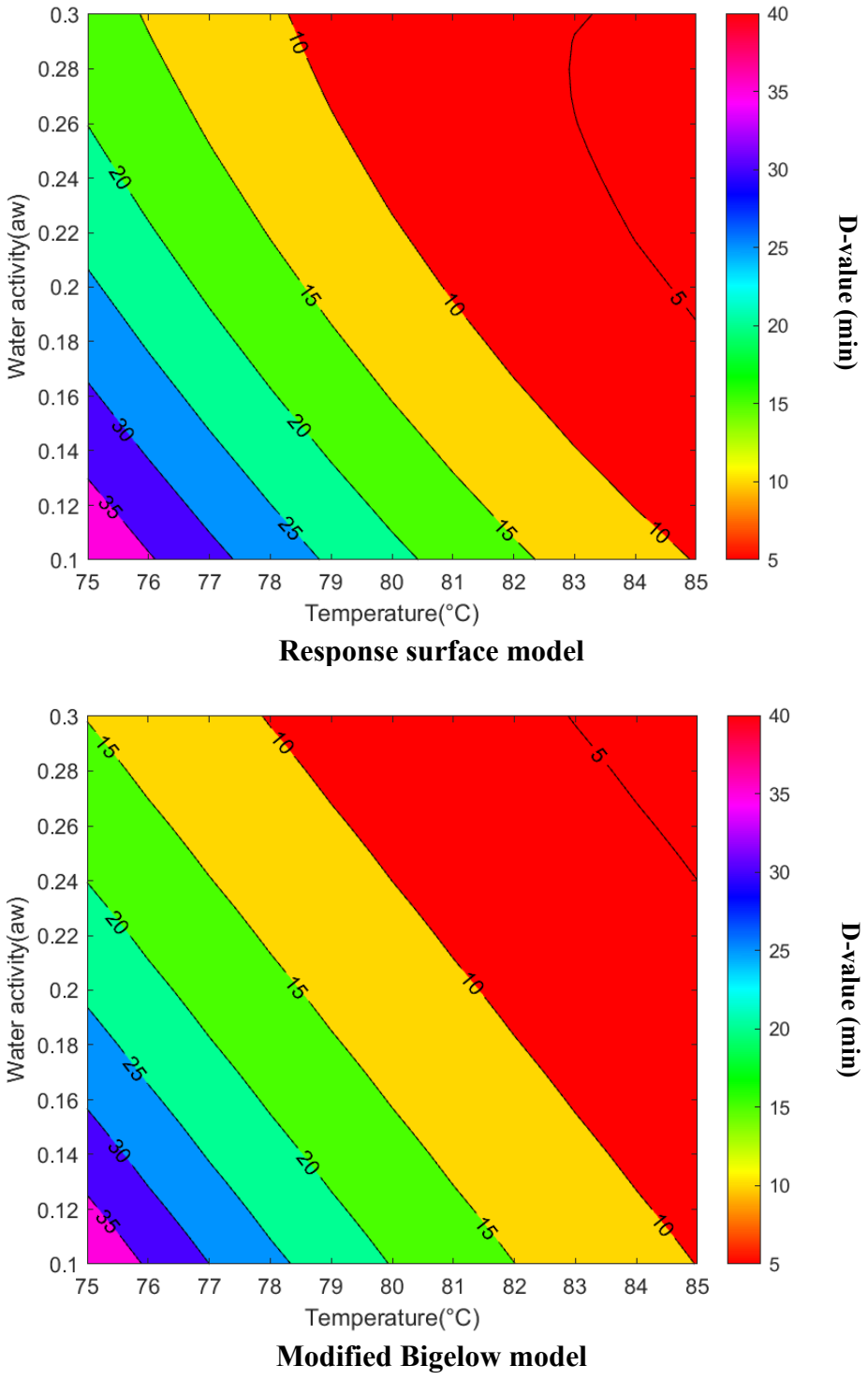

Figure 2.7. Contour surface plots of predicted *D*-values (min) of *Salmonella* in both types of milk powders as a function of temperature and water activity (a_w).

Chapter 3

EVALUATION OF *ENTEROCOCCUS FAECIUM* NRRL B-2354 AS A SURROGATE FOR *SALMONELLA ENTERICA* IN MILK POWDERS AT DIFFERENT STORAGE TIMES AND TEMPERATURES

3.1 Abstract

While the increase in thermal resistance of microorganisms at reduced water activity is demonstrated for low-moisture food products, the effect of storage time on the thermal resistance of microorganisms in low moisture foods is not well established. As low moisture foods are stored for long periods and are used as ingredients, cross-contamination can occur at any time period before the lethality step. Therefore, this study was designed to investigate the effect of storage time (30, 60, 90 d) on the thermal resistance of *Salmonella* and *Enterococcus faecium* NRRL B-2354 in milk powders at a low water activity of 0.10 (conservative level). In this study, two milk powders, whole milk powder (WMP) and nonfat dry milk (NFDM) were inoculated with a five serotypes *Salmonella* cocktail or *E. faecium* and equilibrated to a water activity of 0.10. The thermal resistance of *Salmonella* and *E. faecium* in WMP and NFDM were determined at different storage times (30, 60, 90 d) at 85°C. The storage time had no impact on the thermal inactivation kinetics of *Salmonella* within 90 days of storage at 85°C. In the second part of this study, isothermal treatments were also conducted at higher temperatures (90 and 95°C) to evaluate the suitability of *E. faecium* as a surrogate for *Salmonella* in milk powders. The *D*-values of *Salmonella* in WMP with 30 days of storage at 85, 90, and 95°C were 7.98, 3.35, and 1.68 min. The corresponding values for *E. faecium* were 16.96, 7.90, and 4.16

min. Higher *D*-values of *E. faecium* indicates that it is a conservative surrogate. Similar results were found for NFDM. In general, *D*-values of both microorganisms are slightly higher in NFDM than WMP. Two primary models (log-linear and Weibull) were compared for their goodness-of-fit. The Weibull model was found to be more appropriate than the log-linear model. This study provides valuable information for establishing process validation for the pasteurization of milk powders.

Keywords: Surrogate, Weibull, model analysis, process validation, low moisture food

3.2 Introduction

Food products with water activities < 0.70 are categorized as low water activity foods (Blessington et al., 2013). Most of the foodborne pathogens require water activity > 0.9 for proper growth, thus they were historically not a concern in low water activity foods. However, the survival of *Salmonella* in low water activity foods, such as almond (CDC, 2004), milk powder (Angulo et al., 2008; CDC, 1993), peanut butter (CDC, 2009) and black pepper (CDC, 2010), have resulted in several foodborne outbreaks in the United States, indicating that low water activity food can no longer be considered as inherently microbiologically safe.

Milk is a nutrient-rich liquid that is often spray-dried into powder forms for a longer shelf-life. Milk powders are commonly used as ingredients in many food products including ready-to-eat foods, such as confectionery, drink mixes, seasoning, nutritional bars, and dry blend infant formula. However, no pathogen lethality step is applied to eliminate foodborne pathogens in milk powders after spray-drying (LiCari and Potter, 1970), which could potentially result in serious food safety issues. For instance, 37 babies in France were known to have fallen sick due to the presence of *Salmonella* Agona in

powdered milk in 2018 (Marler, 2018). There are also several recalls and outbreaks that have been linked to *Salmonella* contaminated dry milk powder products (Brouard et al., 2007; Jourdan-da et al., 2018; Park et al., 2004; Rodríguez-Urrego et al., 2010).

After spray drying of milk powders, there is a risk of cross-contamination through poor post-processing practices, such as poor environmental sanitation, inappropriate storage conditions, or mishandling during transportation (Podolak et al., 2010). Before further processing of milk powders especially for ready-to-eat foods, an effective pasteurization process should be integrated to minimize the risk of contamination of final products.

As required by the U.S. Food and Drug Administration (2018), validation studies are required to scientifically demonstrate that processing technologies effectively limit the hazards they are designed to address. Also, due to the difficulty of directly introducing the target pathogen into the processing plant, the use of a surrogate to replace the pathogen is recommended by FDA (2013) to conduct the validation study in the industrial processing plant. Several studies (Bianchini et al., 2014; Liu et al., 2018; Verma et al., 2018; Wei et al., 2018) have previously demonstrated that *Enterococcus faecium* NRRL B-2354 is a suitable surrogate for *Salmonella* during thermal processing of low water activity foods.

In commercial practice, ingredients such as milk powders are usually stored at 23°C for a long period before usage. It has been reported that high storage temperature (45°C) could greatly affect lipid oxidation in whole milk powder, and the maximum level of free radicals was detected after 47 days of storage at 45 °C (Stapelfeldt, et al. 1997). According to US Dairy Export Council (2017), the typical storage time for whole milk powder (WMP) is 6 to 9 months at room conditions (< 27°C, < 65% RH). Flavor deterioration of whole

milk powder has been reported to occur after 3 months of storage (Drake et al., 2003). However, the effect of storage time on the thermal inactivation kinetics of *Salmonella* in milk powders is currently unknown. Therefore, it is important to understand the effect of storage time on thermal inactivation kinetics for process validation studies. It is also important to evaluate the suitability of surrogate at different storage times and temperatures.

The overall goal of this study is to evaluate the effect of storage time on thermal inactivation kinetics of *Salmonella* and *E. faecium* NRRL B-2354 in milk powders. The specific objectives were to:

1. Determine the effect of storage time (30, 60, 90 d) on the thermal inactivation kinetics of *Salmonella* and *E. faecium* NRRL B-2354 in milk powders at 85°C and water activity of 0.10, and
2. Evaluate *E. faecium* NRRL B-2354 as a surrogate for *Salmonella* at different temperatures (85, 90, 95°C) after 30 days of storage of milk powders at a water activity of 0.10.

3.3 Materials and Methods

3.3.1 Milk Powder Products

Three batches of Grade A pasteurized WMP and non-fat dry milk (NFDM) from different production lots were received from Mars, Incorporated (McLean, VA). The milk powders were stored inside polyethylene bags and held at ambient temperature (23 ± 2 °C) prior to inoculation. On receiving the samples, the proximate analysis, and background microorganism tests were conducted as described in Wei et al. (2020). A water activity meter (4TE, METER Group, Pullman, WA) was used to measure the a_w of milk powders

at 25°C.

3.3.2 Inoculum Preparation

The details of the bacterial serotypes used in this study were described in Table 3.1. The *Salmonella* cocktail, consisting of five serotypes of *Salmonella enterica* subsp. *enterica* which were previously isolated from different foodborne outbreaks and was used to inoculate both powder samples. *E. faecium* NRRL B-2354 was used to inoculate both powder samples for surrogate tests.

The lawn-based pelletized inoculum method was used to prepare the inoculum based on a standard procedure as described in Hildebrandt et al. (2016), which yields a stable bacterial population prior to isothermal treatment and consistent *D*-value. The frozen stock of each bacteria was thawed at 37°C for 5 min, then was subjected to two sequential transfers (24 h at 37°C) in 10 mL of trypticase soy broth (TSB, Becton, Dickinson and Company, Sparks, MD) with 0.6% (w/v) yeast extract (YE, Becton, Dickinson and Company). A loop (~10 µL) of the incubated culture was streaked onto tryptic soy agar (TSA, Becton, Dickinson and Company, Franklin Lakes, NJ) supplemented with 0.6 % (w/w) YE and incubated at 37°C for 24 h to prepare the working stock plate. A 10 µL loop was used to harvest one isolated colony from a working stock plate and was then transferred to 10 mL of TSBYE and incubated for 24 h at 37°C. The incubated culture (0.1 mL) was then spread plated onto a TSAYE plate and incubated for 24 h at 37°C. The grown bacterial lawn was then harvested with 3 mL of 0.1% buffered peptone water (BPW; Becton, Dickinson and Company) with an L-shaped spreader. By mixing the equal amount of lawn from each *Salmonella* serotypes, the *Salmonella* cocktail inoculum was prepared with the bacterial population of ca. 10^{10} CFU/mL. Similarly, *E. faecium* NRRL B-2354 inoculum

was prepared with the bacterial population of ca. 10^9 CFU/mL.

3.3.3 Milk Powder Inoculation and Equilibration

The WMP or NFDM from each batch was weighed (100 g) and transferred to a resealable 1-gallon polyethylene bag. A sterile 15 mL centrifuge tube (Thermo Fisher Scientific, Rochester, NY) with a finger-operated spray head contained 10 mL aliquot of the prepared *Salmonella* cocktail or *E. faecium* NRRL B-2354 inoculum was used to spray-inoculate both milk powder samples. The inoculated sample was then mixed by hand for 5 min to manually detach powders that were stuck to the inner lining of the bag. Subsequently, the sample was transferred into a sterile Whirl-Pak bag (2.04 L, Nasco, Fort Atkinson, WI) and then placed in a paddle mixer (9000471, Neutec Group Inc, Farmingdale, NY) and mixed for 20 min to homogenize the inoculum distribution and minimize the clumps. The mixed inoculated sample (5 mm thickness) was then placed on a sterile aluminum tray (230 x 300 x 15 mm) and transferred into a custom made relative humidity chamber (Lau et al., 2020) set to a target relative humidity of 10% to equilibrate the inoculated sample to a water activity of 0.10 at 25°C. Based on the previous study (Wei et al., 2020), a low water activity of 0.10 was selected which could represent the worst-case scenario where *Salmonella* exhibits high thermal resistance. This low water activity (0.10) represents a conservative study, as *Salmonella* resistance is higher at lower water activity. After five days of equilibration in the chamber, the inoculated sample was packed into heat-sealable aluminized pouches (7.62 cm x 7.62 cm x 0.10 cm, IMPAK Corporation, Los Angeles, CA) in quantities of approximately 2 g and then sealed for storage at 23°C for 30, 60 and 90 days. The whole packing process was conducted inside the relative humidity chamber to ensure a minimal change in the water activity.

3.3.4 Determination of Thermal Resistance at Different Storage Times

Stored inoculated milk samples were sampled on 30, 60, and 90 days to evaluate the effect of storage time on the heat resistance of *Salmonella* and *E. faecium*. Isothermal treatment was conducted at 85°C by using the custom-designed thermal death time (TDT) sandwich (Lau et al., 2020) for both WMP and NFDM to obtain the isothermal death curve of *Salmonella* and *E. faecium* at 85°C. The TDT sandwich has two resistive heating elements that could apply dry heat to samples. Each packed inoculated sample was placed in between the two heating elements in one TDT sandwich unit for isothermal treatment of one time and temperature combination.

Before conducting the isothermal treatment, the come-up time was measured in triplicates by a type T thermocouple (5TC-TT-T-40-36, Omega Engineering Inc., Norwalk, CT) with an accuracy of ± 0.5°C inserted to the inside center of an aluminized pouch. The come-up time was the average time required for the sample to be heated to 84.5°C by the TDT sandwiches plus twice the standard deviation, which would ensure that at least 95% of TDT sandwiches would reach at least 84.5°C at time zero in the inactivation curve.

Six-equally spaced time points including time zero were used for the isothermal treatment of inoculated samples to obtain thermal death curves for *Salmonella* or *E. faecium* in both WMP and NFDM. Immediately after each treatment, the treated pouch was transferred into an ice-water bath (0°C) for at least one minute before it was enumerated for bacterial survivors.

3.3.5 Surrogate Evaluation at Different Temperatures

To evaluate the suitability of *E. faecium* as a surrogate for *Salmonella* at different temperatures, isothermal treatments at the higher temperatures (90 and 95°C) were

conducted for *Salmonella* or *E. faecium* in inoculated milk powders. Based on the results of the storage study as described in the previous section, a single storage time was used to determine the thermal inactivation kinetics for both bacteria at higher temperatures. Inoculated samples were isothermally treated by the TDT sandwiches as described in the previous section to obtain thermal inactivation curves for *Salmonella* or *E. faecium* in both WMP and NFDM at 90 and 95°C. All isothermal treatments for inoculated WMP and NFDM were performed in three independent replicates by using inoculated samples from each batch.

3.3.6 Bacterial Enumeration

Enumeration was performed as previously described (Wei et al., 2019). Briefly, the *Salmonella* or *E. faecium* survivors in the thermally treated sample were enumerated by serially 10-fold diluting samples in 0.1% BPW and were then spread-plated onto TSAYE supplemented with 0.05% (w/v) ammonium iron citrate (Sigma-Aldrich, Co., MO, USA), and 0.03% (w/v) sodium thiosulfate (Fisher Chemical, NJ, USA) (mTSA) for *Salmonella* or TSAYE supplemented with 0.05% (w/v) ammonium iron citrate, and 0.025% (w/v) esculin hydrate (Acros Organics, NJ, USA) (eTSA) for *E. faecium* and incubated for 24 h at 37°C (Liu et al., 2017; Smith et al., 2016; Wei et al., 2020). Colonies with a black center were counted as *Salmonella* on incubated mTSA plates and black colonies were counted as *E. faecium* on incubated eTSA plates, respectively. The untreated WMP or NFDM samples were enumerated as a control to monitor the bacterial population change during the 90 days of storage.

3.3.7 Curve Fitting of the Survivor Models

The *Salmonella* or *E. faecium* survivors in both milk powders were fitted with two

primary inactivation models, the log-linear model (1) and the Weibull-type model (2) as described in Peleg and Cole (1998):

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

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

where N and N_0 (CFU/g) are the *Salmonella* or *E. faecium* survivors at time t and 0, t is the instantaneous time of the thermal treatment (min), D is the decimal reduction time which is the time required to reduce the microbial population by 10-fold, δ (min) is the scale parameter and β is the shape parameter of the Weibull inactivation curve, where $\beta >$ 1 indicates a concave downward trend, $\beta <$ 1 indicates a concave upward trend of the inactivation curve and β = 1 indicates a linear trend.

The *z*-value is defined as the increase in temperature required to achieve a 90% reduction of the decimal reduction time *D* and was determined by the equation:

$$z = \frac{T_2 - T_1}{log_{10}\, D_{T_1} - log_{10}\, D_{T_2}} \quad (3)$$

where $log_{10} D_{T_1} - log_{10}\, D_{T_2}$ are the *D*-values at temperature T_1 and T_2, respectively.

The bacterial survival data for each isothermal treatment were fitted to equations 1 and 2 using the Levenberg–Marquardt algorithm for non-linear least squares regression as implemented in the curve_fit function of the Python SciPy library (Newville et al., 2015). The statistical significance of differences between different storage times and different milk powder products were analyzed using Fisher's Least Significant Difference test as implemented in the LSD_test function of the R agricolae library (de Mendiburu and de Mendiburu, 2019).

3.3.8 Global Models

The log-linear and Weibull models were also globally fitted across all three storage times. To evaluate the appropriateness of model fitting, model selection analyses were performed using root mean squared error (RMSE) and corrected Akaike Information Criteria (AIC_c) (Motulsky and Christopoulos, 2003):

$$\text{RMSE} = \sqrt{\frac{\sum_{i=1}^{n}[log_{10}(N)_{observed,i} - log_{10}(N)_{predicted,i}]^2}{n}} \quad (4)$$

$$\text{AICc} = \text{nln}\left(\frac{SS}{n}\right) + 2\text{K} + \frac{2K(K+1)}{n-K-1} \quad (5)$$

where $log_{10}(N)_{observed,i}$ is the observed log population, $log_{10}(N)_{predicted,i}$ is the predicted log population from the model and *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. Lower RMSE and AIC_c values indicate that the model prediction is more likely to be correct for the experimental data. According to Motulsky and Christopoulos (2003), the percent likelihood of a given model is more correct which can be estimated using the equation (5) below:

$$Probability\ (Model\ A\ over\ B) = \frac{e^{\left(\frac{AIC_{C,B} - AIC_{C,A}}{2}\right)}}{1 + e^{\left(\frac{AIC_{C,B} - AIC_{C,A}}{2}\right)}} \quad (6)$$

3.4 Results and Discussion

3.4.1 Survival of *Salmonella* and *E. faecium* in WMP and NFDM

The bacterial populations of *Salmonella* and *E. faecium* in the prepared inoculum were 10.84 and 9.92 log CFU/mL, respectively. Immediately after inoculation, *Salmonella* and *E. faecium* populations were determined to be 8.13 and 8.86 log CFU/g in WMP and 8.25 and 8.92 log CFU/g in NFDM, respectively. After 24 h equilibration in the relative

humidity chamber, the inoculated samples were equilibrated to the water activity level of 0.10 ± 0.01, and the *Salmonella* and *E. faecium* population were dropped to 7.75 and 8.61 log CFU/g in WMP and 7.86 and 8.78 log CFU/g in NFDM, respectively. The standard deviations of three subsamples from inoculated WMP and NFDM were determined to be 0.18 and 0.15 log CFU/g, respectively, which indicated a good homogeneity of the inoculated samples.

The survival results of *Salmonella* and *E. faecium* in WMP and NFDM are shown in Figure 3.1. During 90 days of storage, the reductions of *Salmonella* in WMP and NFDM were observed to be 0.79 and 0.86 log CFU/g, while the reductions of *E. faecium* in WMP and NFDM were observed to be 0.10 and 0.16 log CFU/g (Figure 3.1). Most of the decrease in the initial population of *Salmonella* was observed in the first 30 days of storage. The results shown in this study that *Salmonella* in milk powder experienced a rapid death rate in the first few weeks of storage followed by a slower death rate are in agreement with several published studies (Lian et al., 2015; LiCari and Potter, 1970) A similar reduction in *Salmonella* population has been observed during the post-inoculation process of low water activity foods, such as black pepper (Wei et al., 2018, 2019), cumin seeds (Chen et al., 2019), egg white powder (Wei et al., 2020), almond (Abd et al., 2012), wheat flour (Forghani et al., 2019) and walnut kernel (Blessington et al., 2012). *Salmonella* cells need time to adapt to a low water activity environment. Thus, a higher death rate was observed during the adaption period and then the death rate leveled off in the remaining storage time.

According to Lian et al., (2015), the population of *Salmonella* in skim milk powder dropped by 4.23, 4.69, and 5.58 log CFU/g after two months of storage at the water activity of 0.33, 0.53 and 0.81, respectively. As the water activity of milk powder is this study was

much lower at 0.10, a much lower reduction of 0.86 log CFU/g of the *Salmonella* population in NFDM after 90 days of storage was observed. Enhanced survivability of *Salmonella* was observed at lower water activity level during the storage of low water activity foods, as desiccated conditions have been reported to increase survivability (Jung and Beuchat, 1999; Kataoka et al., 2014; Lian et al., 2015). Differences in reductions of *Salmonella* found in this study when compared to Lian et al., (2015) may also be due to differences in inoculum protocols (Hildebrandt et al., 2016).

Similar to *Salmonella*, several other foodborne pathogens, such as *Enterobacter sakazakii* (Gurtler and Beuchat, 2007) and *Listeria monocytogenes* (Ballom et al., 2020) have been shown to survive well in milk powders at a reduced water activity level. Therefore, the long-term survival of foodborne pathogens in milk powders should be of concern to the dairy industry and it is important to have a kill step to enhance the safety of milk powders.

3.4.2 Effect of Storage Time on the Thermal Resistance of *Salmonella* and *E. faecium*

The effect of storage time on the thermal resistance of *Salmonella* and *E. faecium* was investigated at 85°C and at the water activity of 0.1. The low water activity of 0.1 was selected in this study to represent the worst-case scenario, as increased thermal resistance of *Salmonella* has been reported at reduced water activity levels in both WMP and NFDM (Wei et al., 2020). The isothermal treament temperature of 85°C was chosen because there was a minimum quality impact on sensory of milk powders at this temperature.

The model parameters of the log-linear model and the Weibull model for both WMP and NFDM were compared across the three storage times in Table 3.2. For both WMP and

NFDM, the *D*-values of *Salmonella* were not significantly ($P < 0.05$) different among the storage times. Similarly, there were no significant ($P < 0.05$) differences of *D*-values among the storage times for the Weibull model parameters of both WMP and NFDM. The fitted models were plotted against the inactivation data in Figure 3.2 for WMP and Figure 3.3 for NFDM. The proximity (closeness) and similar curvatures (slope and shape) of the curves for different storage times also reveal the similarity in model parameters.

These results suggest that the thermal resistance of *Salmonella* is not affected by storage time. Abd et al. (2012) and Limcharoenchat et al. (2019) also reported that the storage time had no impact on the heat resistance of *Salmonella* in almond which is in agreement with this study. Similarly, the thermal resistance of *L. monocytogenes* adapted to NFDM (Ballom et al., 2020), chicken meat powder, and pet food (Rachon et al., 2016) was shown to remain constant during prolonged storage.

Therefore, a conclusion can be drawn that when viable cells of *Salmonella* reach stable status in the low water activity environment, their heat resistance would not significantly change with increasing storage time up to 90 days. Although *Salmonella* can survive in low water activity foods for extended periods, this study suggests that their resistance to thermal treatment does not change significantly during the storage of 90 days at 85°C. While the effect of storage period on thermal resistance at other pasteurization temperatures needs to be evaluated, this result implies that the pasteurization protocol does not need to consider the prior storage period up to 90 days at 85°C. This may increase the flexibility of the application of process control if the results from the effect of storage period hold at other pasteurization temperatures.

In Table 3.2, it has been shown that *D* and δ values of *E. faecium* were significantly

higher ($p < 0.05$) than *Salmonella* at the same storage condition in both WMP and NFDM. It demonstrated that *E. faecium* consistently exhibited greater thermal resistance ($p < 0.05$) than *Salmonella* during the storage of WMP and NFDM.

3.4.3 Thermal Inactivation Kinetics of *Salmonella* and *E. faecium* in WMP and NFDM

Because there was no impact of storage time (90 d) on the heat resistance of *Salmonella*, the isothermal treatments at 90 and 95°C were conducted by using the inoculated samples at day 30 of storage time to acquire thermal inactivation kinetics of *Salmonella* and *E. faecium*. The bacterial inactivation data were fitted to both log-linear and Weibull models in Figure 3.4. The model parameters for both WMP and NFDM are summarized in Table 3.3. For both *Salmonella* and *E. faecium*, D and δ values were found to significantly ($p< 0.05$) decrease with increased temperature, which indicated that a higher microbial reduction could be achieved at a higher temperature for the same treatment time. Significantly higher ($p< 0.05$) D and δ values of *E. faecium* were also observed at 85,90 and 95°C than *Salmonella*. Similarly, Liu et al. (2019) conducted isothermal treatments for NFDM with storage time of 48 h after inoculation and found that the decline of *Salmonella* exceeded that of *E. faecium* during isothermal treatment of NFDM at all three temperatures (85, 90, and 95°C). These results suggest that *E. faecium* is a suitable non-pathogenic surrogate for *Salmonella* in the thermal processing of milk powder at different process temperatures with 30 days of storage.

Figure 3.5 shows the effect of temperature on the D-values of *Salmonella* and *E. faecium* in dairy powders. The z-values of *Salmonella* and *E. faecium* were determined to be 14.77 and 16.39°C for WMP and 12.82 and 16.58°C for NFDM, respectively. The z-

values of *Salmonella* and *E. faecium* found in this study were very close to the *z*-values of *Salmonella* and *E. faecium* (16.4 and 16.5°C) reported by Liu et al. (2019) and *z*-values of *L. monocytogenes* (14.6 to 16.0°C) in NFDM at different water activities as reported by Ballom et al. (2020). The determined microbial inactivation kinetics of *Salmonella* and *E. faecium* could be a practical tool for the food industry to identify appropriate thermal processing conditions for pasteurization of milk powders.

Tables 2 and 3 show that *D*-values of both microorganisms in NFDM were slightly higher than WMP. Although the initial water activity of both milk powders was equilibrated 0.10 at 25°C the water activity at 85°C could be different because of the differences in food matrix. As water activity at high temperature plays an important role in thermal inactivation of low moisture foods, the water activity at 85°C for both milk powders may need to be determined to explain the slight differences in *D*-values. Fat has been shown to have a protective effect on high moisture foods; however, protective effect of fat in low-moisture foods is not known and need to be investigated in future.

3.4.4 Bacterial Inactivation Model Comparison

Storage time (90 days) did not have an impact on the thermal resistance of bacteria in WMP and NFDM. Therefore, the thermal inactivation data at different storage times were combined and globally fitted to both log-linear and Weibull models for model comparison. AIC_c and RMSE analysis (Table 3.4) indicated that the Weibull model was more likely correct ($p > 0.72$ for *Salmonella* and > 0.99 for *E. faecium*) for all cases. The visualization of the models fitting on the combined dataset was shown in Figure 3.6.

Several studies have reported that the Weibull model is a better fit than the log-linear model in low water activity food, such as *Salmonella* in whey protein powder

(Farakos et al., 2013), *Salmonella* in almond kernels (Villa-Rojas et al., 2013), and *Listeria monocytogenes* and *E. faecium* in low water activity food powders (Rachon et al., 2016). Although Smith et al. (2016) reported that the log-linear model provided a better prediction than the Weibull model for *Salmonella i*nactivation in wheat flour with minimal difference in AIC_c values for both models at most water activity levels. In terms of model fitting, the Weibull model was shown to be more suitable than the log-linear model for describing the thermal inactivation of *Salmonella* and *E. faecium* in WMP and NFDM.

3.5 Conclusions

In this study, both *Salmonella* and *E. faecium* showed good survivability in WMP and NFDM at low water activity with < 0.9 log CFU/g reduction of *Salmonella* and < 0.2 log CFU/g reduction of *E. faecium* after 90 days of storage. The thermal resistance of *Salmonella* and *E. faecium* did not change significantly during the 90 days storage of WMP and NFDM. This finding simplifies the process validation study in the industry. *Enterococcus faecium* was a suitable surrogate for *Salmonella* at different temperatures (85, 90, and 95°C). The thermal death time comparison between *Salmonella* and *E. faecium* in milk powders provides an important foundation for the dairy industry for evaluating different thermal treatment technologies that can achieve a suitable reduction of target pathogens. Use of *E. faecium* which is a conservative surrogate for process validation may result in over-processing beyond what is needed for *Salmonella* reduction, which could affect product quality. Therefore, identifying the kill ratio between *Salmonella* and *E. faecium* could contribute to efficient process validations. Based on microbial inactivation kinetics of *Salmonella* and *E. faecium*, the food industry could establish an appropriate thermal process validation for pasteurization of milk powders.

3.6 Acknowledgement

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3.7 References

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Table 3.1. Bacterial serotypes used in this study.

Bacterial name	Source	Related outbreak	Reference
Salmonella Agona 447967	FDA, ORA Regional Lab (Jefferson, AR)	Oats cereal	(CDC, 1998)
Salmonella Montevideo 488275	FDA, ORA Regional Lab (Jefferson, AR)	Black and red pepper	(CDC, 2010)
Salmonella Mbandaka 698538	FDA, ORA Regional Lab (Jefferson, AR)	Sprouts	(Jackson et al., 2013)
Salmonella Reading Moff 180418	FDA Culture Collection (Bedford Park, IL)	Alfalfa sprout	(CDC, 2016)
Salmonella Tennessee K4643	University of Georgia (Athens, GA)	Peanut butter	(CDC, 2007)
Enterococcus faecium NRRL B-2354	USDA, ARS (Peoria, IL)	--	(Almond Board of California, 2014)

Table 3.2. Effect of storage time on model parameter estimates for the log-linear and Weibull models at 85°C.

Sample	Bacteria	Storage time (d)	D (min)	δ (min)	β
WMP	*Salmonella*	30	7.9 ± 0.8 ^{f}	7.2 ± 0.3 ^{e}	0.9 ± 0.1 ^{f}
		60	8.0 ± 0.1 ^{f}	7.5 ± 1.2 ^{e}	1.0 ±0.2 ^{def}
		90	7.8 ± 0.3 ^{f}	7.7 ± 0.8 ^{e}	0.9 ± 0.1 ^{ef}
	E. faecium	30	16.9 ± 0.7 ^{cd}	23.3 ± 1.8 ^{b}	1.3 ± 0.1 ^{abc}
		60	18.4 ± 1.5 ^{bc}	26.1 ± 0.8 ^{a}	1.4 ± 0.1 ^{a}
		90	16.4 ± 0.6 ^{d}	20.0 ±0.8 ^{c}	1.2 ± 0.1 ^{bcd}
NFDM	*Salmonella*	30	10.9 ± 0.5 ^{e}	11.5 ± 0.9 ^{d}	0.9 ± 0.1 ^{f}
		60	11.4 ± 0.9 ^{e}	12.9 ± 2.9 ^{d}	1.1 ± 0.2 ^{de}
		90	10.7 ± 0.2 ^{e}	13.4 ± 0.7 ^{d}	1.2 ± 0.1 ^{bcd}
	E. faecium	30	19.8 ± 1.8 ^{ab}	26.1 ± 2.9 ^{a}	1.3 ± 0.1 ^{abc}
		60	19.5 ± 0.3 ^{ab}	23.1 ± 0.6 ^{b}	1.1 ± 0.1 ^{cd}
		90	20.7 ± 0.8 ^{a}	27.5 ± 1.8 ^{a}	1.4 ± 0.1 ^{ab}

Values are mean ± standard deviation of triplicates. $^{a\text{-}f}D$ (decimal reduction time), δ (time scale parameter for Weibull model) and β (shape parameter for Weibull model) values with differing superscript letters are significantly different within a column ($P < 0.05$).

Table 3.3. Effect of temperature on model parameter estimates for the log-linear and Weibull models after one month of storage.

Sample	Bacteria	Temperature (°C)	D (min)	δ (min)	β
WMP	*Salmonella*	85	7.9 ± 0.9 ^{e}	7.2 ± 0.3 ^{e}	0.9 ± 0.1 ^{e}
		90	3.4 ± 0.2 ^{g}	3.5 ± 0.2 ^{gh}	1.0 ± 0.0 ^{cd}
		95	1.7 ± 0.1 ^{h}	1.4 ± 0.2 ^{i}	0.8 ±0.1 ^{e}
	E. faecium	85	17.0 ± 0.7 ^{b}	23.3 ± 1.8 ^{b}	1.3 ± 0.1 ^{b}
		90	7.9 ± 0.2 ^{e}	12.2 ± 0.8 ^{d}	1.5 ± 0.1 ^{ab}
		95	4.2 ± 0.1 ^{fg}	3.7 ± 0.4 ^{g}	0.5 ±0.1 ^{de}
NFDM	*Salmonella*	85	11.0 ± 0.5 ^{c}	11.5 ± 1.0 ^{d}	0.9 ± 0.1 ^{de}
		90	4.8 ± 0.5 ^{f}	5.3 ± 0.8 ^{fg}	1.1 ± 0.2 ^{c}
		95	1.8 ± 0.2 ^{h}	1.7 ± 0.4 ^{hi}	0.9 ± 0.1 ^{de}
	E. faecium	85	19.8 ± 1.8 ^{a}	26.1 ± 2.9 ^{a}	1.3 ± 0.1 ^{b}
		90	9.7 ± 0.8 ^{d}	14.7 ± 0.6 ^{c}	1.5 ± 0.1 ^{a}
		95	4.9 ± 0.0 ^{f}	6.9 ± 0.2 ^{ef}	1.4 ± 0.0 ^{ab}

Values are mean ± standard deviation of triplicates. $^{a\text{-}f}D$ (decimal reduction time), δ (scale parameter for Weibull model) and β (shape parameter for Weibull model) values with differing superscript letters are significantly different within a column ($P < 0.05$).

Table 3.4. Model parameter estimates for the log-linear and Weibull models globally fitted across all storage times at 85°C.

Sample	Bacteria	Model	D^1 or δ^1 (min)	β^1	RMSE ($\log_{10}$CFU/g)	AIC_c	Probability
WMP	*Salmonella*	Log-Linear	7.9 (0.3)	-	0.2	-165	0.04
		Weibull	7.1 (0.3)	0.9 (0.1)	0.2	-172	0.96
	E. faecium	Log-Linear	17.2 (0.8)	-	0.3	-116	~ 0
		Weibull	23.1 (0.6)	1.3 (0.1)	0.2	-158	~ 1
NFDM	*Salmonella*	Log-Linear	11.00(0.3)	-	0.3	-119	0.28
		Weibull	12.0 (0.3)	1.1 (0.1)	0.3	-121	0.72
	E. faecium	Log-Linear	20.0 (1.4)	-	0.3	-133	~ 0
		Weibull	25.5 (0.8)	1.3 (0.1)	0.2	-166	~ 1

[1]Microbial population from three storage periods and triplicates were simultaneously used to fit model to estimate model parameters along with the standard error of estimate reported in parenthesis. When the probability is < 0.001 or larger than 0.999, the corresponding values were reported as ~ 0 or ~ 1.

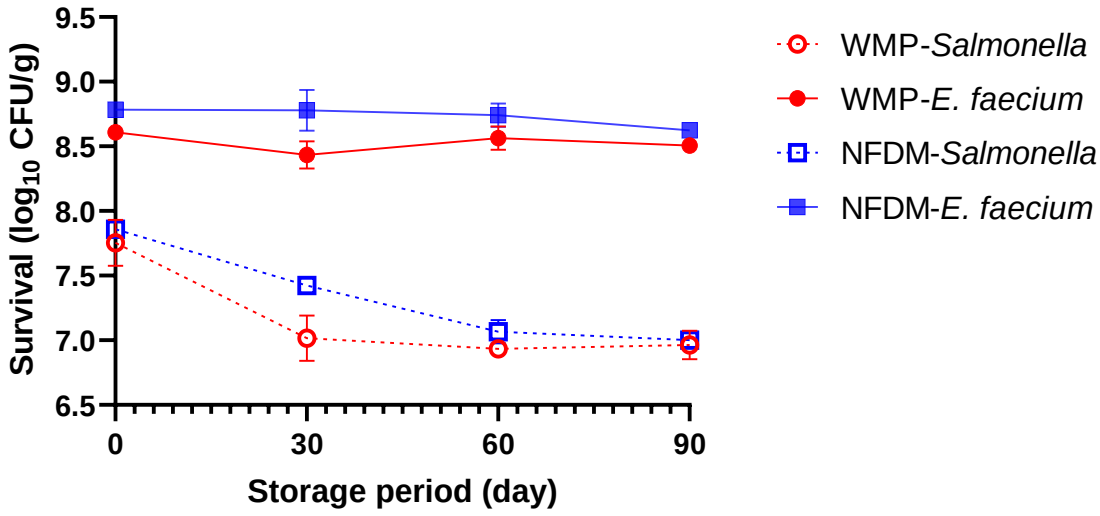

Figure 3.1. Viability of *Salmonella* and *E. faecium* population in whole milk powder (WMP) and non-fat dry milk (NFDM) during storage period (23°C). Each test was conducted in triplicates. Error bars represent standard deviations of three replications.

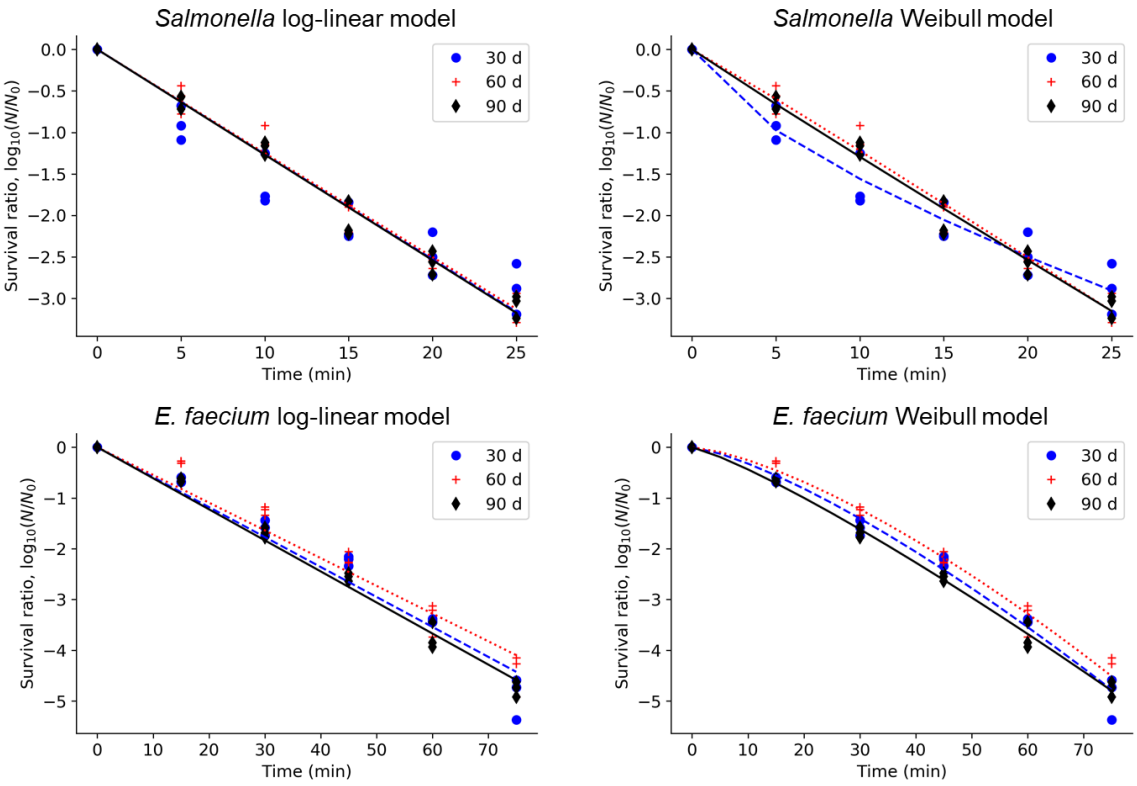

Figure 3.2. Experimental and predicted survival of *Salmonella* and *E. faecium* in whole milk powder stored for 30 days (●, dashed lines), 60 days (+, dotted lines), and 90 days (♦, solid lines) at 85°C.

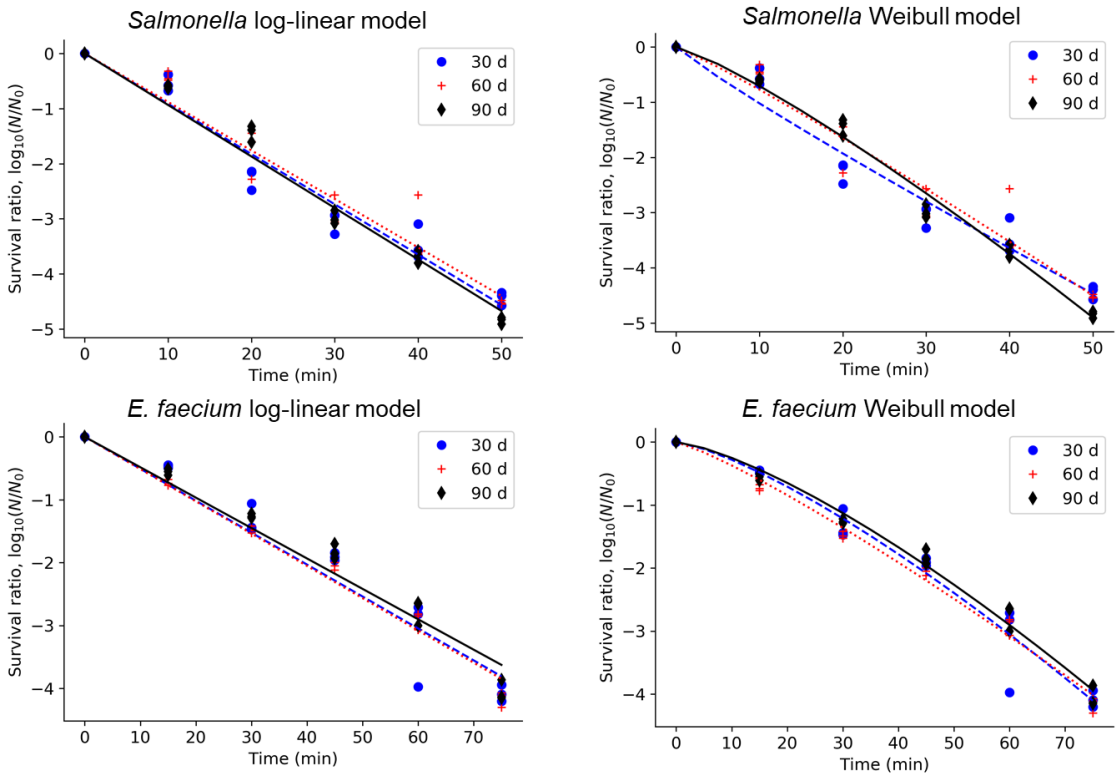

Figure 3.3. Experimental and predicted survival of *Salmonella* and *E. faecium* in non-fat dry milk stored for 30 days (●, dashed lines), 60 days (+, dotted lines), and 90 days (♦, solid lines) at 85°C.

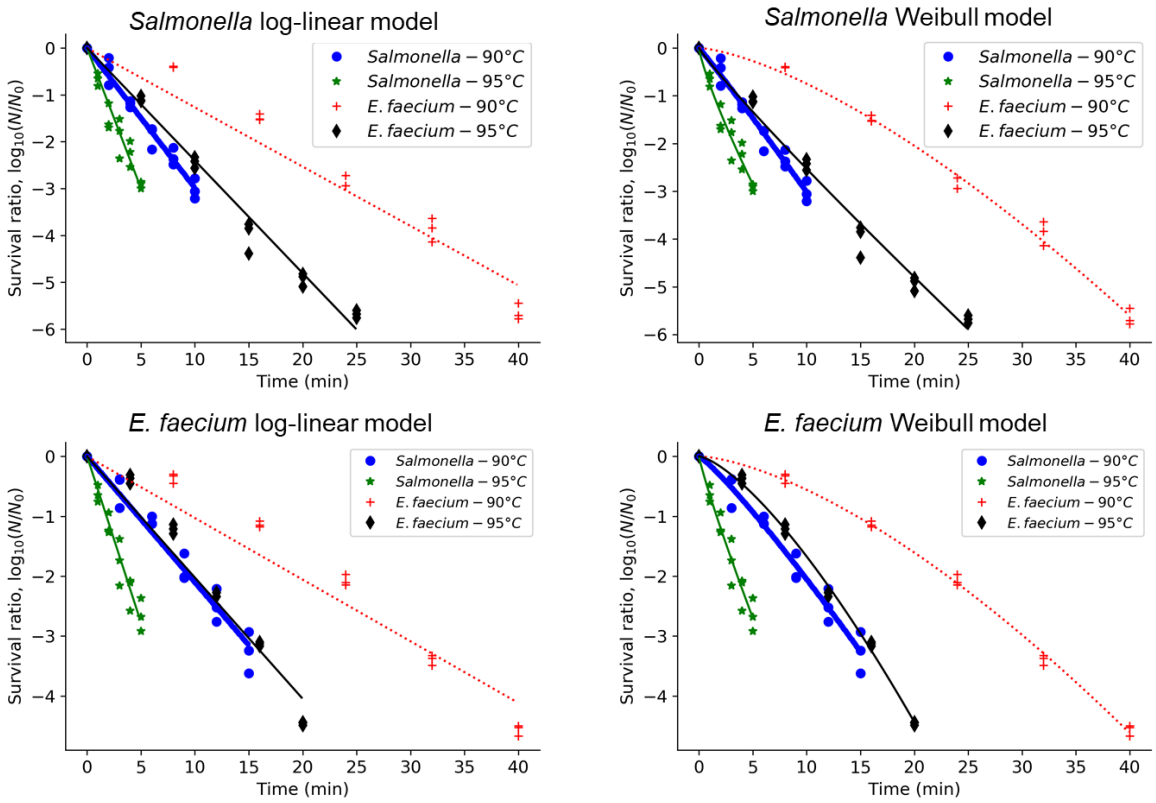

Figure 3.4. Log-linear and Weibull models fitted to *Salmonella* and *E. faecium* inactivation data of 90 and 95°C. *Salmonella*-90°C (●, dashed lines), *Salmonella*-95°C (★, dash-dot line), *E. faecium*-90°C (+, dotted lines), and *E. faecium*-95°C (♦, solid line).

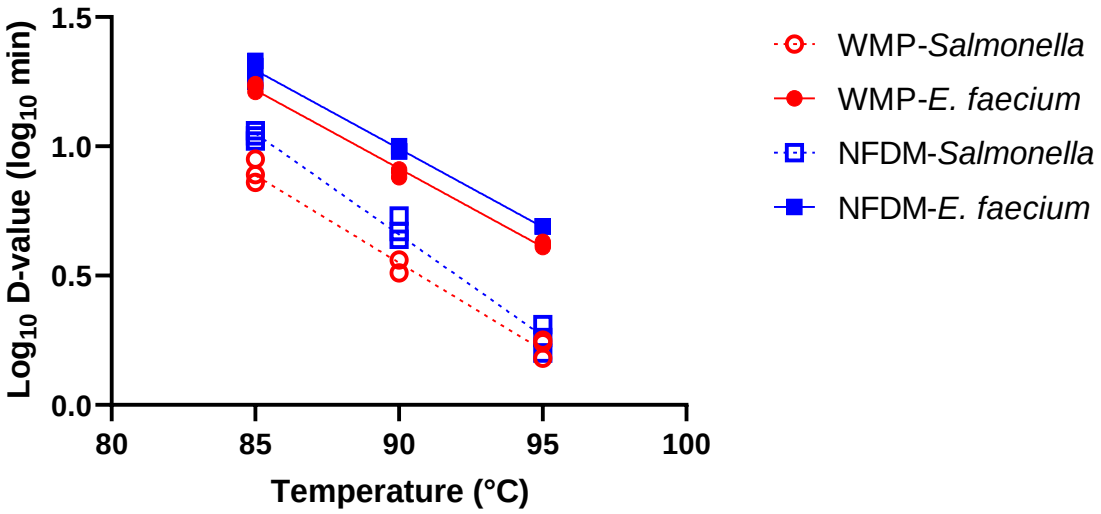

Figure 3.5. Thermal resistance constants (z-values) of *Salmonella* and *E. faecium* in whole milk powder (WMP) and nonfat dry milk (NFDM). Each test was conducted in triplicates.

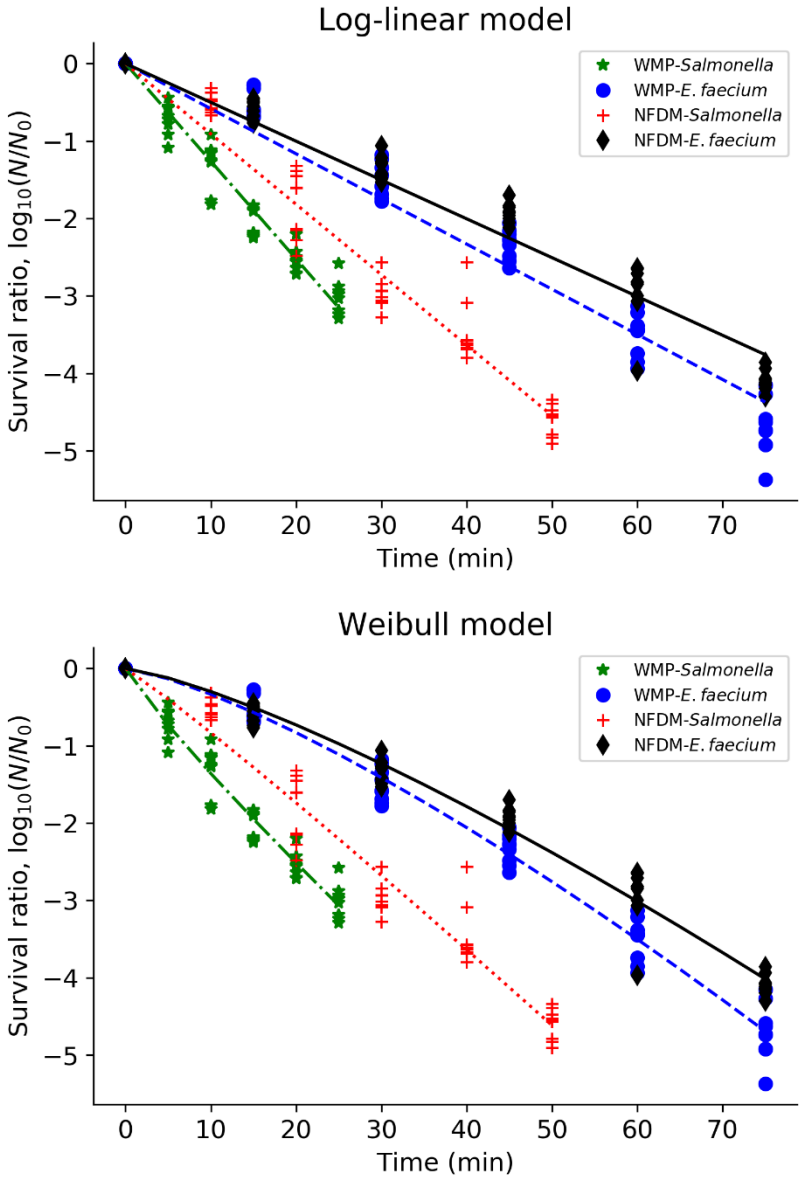

Figure 3.6. Global log-linear and Weibull models fitted to *Salmonella* and *E. faecium* inactivation data combined across all storage times at 85°C at WMP-*Salmonella* (★, dash-dot line), WMP-*E. faecium* (●, dashed lines), NFDM-*Salmonella* (+, dotted lines), and NFDM-*E. faecium* (♦, solid line).

Chapter 4

HEATING OF MILK POWDERS TO 95ºC FOR 15 MINUTES USING HOT AIR-ASSISTED RADIO FREQUENCY PROCESSING ACHIEVED PASTEURIZATION

4.1 Abstract

Salmonella persistence in milk powders has caused several multistate foodborne outbreaks. Therefore, effective antimicrobial treatments need to be identified and validated to ensure the microbial safety of milk powders. In this study, hot air-assisted radio frequency (HARF) followed by holding at high temperatures in a convective oven was developed and validated for pasteurization of milk powders. Whole milk powder (WMP) and nonfat dry milk (NFDM) were inoculated with a 5-serotype *Salmonella* cocktail and equilibrated to a water activity of 0.10 for the microbial challenge study. After heating the sample to 95°C using HARF followed by 10 and 15 min of holding in the oven, more than 5 log reduction of *Salmonella* was achieved in WMP and NFDM water activity of 0.10. *E. faecium* was demonstrated to be a suitable surrogate due to its higher thermal resistance than *Salmonella* in both milk powders. This HARF process can be implemented in the dairy industry to enhance microbial safety of milk powders.

Keywords: Dielectric heating, low-moisture foods, *Salmonella*, *Enterococcus faecium*, Whole milk powder, nonfat dry milk

4.2 Introduction

Milk plays an important role in the human diet because of its high nutritive value as a source of proteins, fats, carbohydrates, vitamins and minerals (Neumann et al., 2002). The high-quality protein supplied by milk is readily absorbed, which can promote the proper growth of adults and infants (Poonia et al., 2017). Compared to liquid milk which usually requires refrigerated storage conditions and cold chain transportation, it is easy to transport and store milk powder (Barbano, 2017). In addition, milk powder is an important ingredient in various ready-to-eat (RTE) food products such as infant milk formula, chocolates, granola and drink mixes, seasonings, protein bars, and confectionery. In the industrial process, milk powder is produced by spray-drying of pasteurized liquid milk. Although pasteurization of milk inactivates pathogens, and milk powders are manufactured in a relatively clean environment, pathogens like *Cronobacter sakazakii* and *Salmonella* are found to cross-contaminate the product at low levels. Often, these bacteria can survive through the spray-drying process (LiCari and Potter, 1970; Osaili et al., 2009). Because pathogens like *Salmonella* survives in low moisture foods for a long period of time (Podolak et al., 2010; Keller et al., 2013; Chuang et al., 2020) and these products are often consumed as RTE or as ingredients in RTE foods by young infants and kids, there is a need to pasteurize milk powders.

Recently, various outbreaks and recalls have been associated with powdered infant formula (CDC, 1993) and milk powder (Brouard et al., 2007; Jourdan-da et al., 2018; Marler, 2018). Currently, the microbial safety problems of milk powders are mainly due to lack of the key lethal treatment before consumption or used as ingredients (Carrasco et al., 2012; Silva and Gibbs, 2012). For example, after spray-drying of milk powder, no

pasteurization step is currently applied to eradicate pathogens before using the milk powder as an ingredient in RTE products (LiCari and Potter, 1970). Therefore, an effective pasteurization process should be included in the production line, which uses milk powders as ingredients in RTE products.

Thermal processing is one of the most common and effective methods for the inactivation of pathogens in food products. Currently, one challenge in the thermal processing of the milk powder is the increased thermal resistance of food pathogens at reduced water activity. Several studies (Liu et al., 2019; Wei et al., 2020a) reported that the higher *D*-value of *Salmonella* in nonfat dry milk (NFDM) was observed at a lower water activity. Similar, increased *D*-values of *Escherichia coli*, *Salmonella* Typhimurium, *C. sakazakii* were observed when the water activity decreased from 0.58 to 0.11 at different temperatures (Lang et al., 2017). When heating the milk powder, the water activity of the milk powder decreases due to moisture evaporation. This increases the difficulty to reduce the microbial population in the milk powder and therefore, requires a longer holding time at the specific treatment temperature. The other challenge for thermal processing of the milk powder is the difficulty of heating the milk powder using the traditional thermal processing technologies, such as dry heat or hot room method, because of the low thermal conductivity of milk powder (Muramatsu et al., 2005). The slow heating rate of milk powder using traditional thermal processing would require an extended treatment time and over-processing on edges, resulting in considerably quality deterioration (Karagül-Yüceer et al., 2001). The best way to preserve food quality during pasteurization is to heat the product to the maximum possible temperature uniformly (shortest come-up time) and rapidly with the shortest holding time.

Radio-frequency (RF) heating is a kind of dielectric heating methods, in which the heat is generated within the food product by friction from the vibration of polar dielectric molecules or moving ions under the alternating electric field, which is similar to microwave heating (Chen et al., 2013, 2017; Pitchai et al., 2014). RF heating is independent of the product's thermal conductivity, so it can provide a rapid heating rate to milk powder regardless of its low thermal conductivity. Compared to conventional heating, RF heating has the advantages of rapid and uniform heating and high energy efficiency (Zhao et al., 2000). RF preferentially vibrates bound water while the microwave vibrates free water in food products (Awuah et al., 2014). Because of that, RF heating is found to be suitable for pasteurizing various low moisture foods such as wheat flour (Liu et al., 2017), black pepper (Wei et al., 2019), cumin seeds (Chen et al., 2020a), basil leaves (Verma et al., 2021a) almonds (Li et al., 2017) and walnuts (Wang et al., 2007). Recently, RF heating-assisted traditional thermal processing has been shown to effectively reduce the target food pathogen in infant formula milk with a shorter processing time and lower lipid oxidation compared to the conventional thermal method (Lin et al., 2020). Similarly, Michael et al. (2014) reported that RF heating can be a post-process lethality treatment for inactivation of *C. sakazakii* and *Salmonella* in NFDM. However, the RF heating validation was only conducted for NFDM, and a surrogate was not identified for subsequent industrial-scale validation. RF does not heat the air and therefore the product can lose temperature to the air. After RF heating of low moisture food products, the cold spot was usually found on top center and edges were overheated. By heating surrounding air to the desired temperature, the uniformity of the heating can be further increased. Several studies have shown that the hot-air assisted RF heating (HARF) can improve the heating rate and uniformity for food

products like nuts (Wang et al., 2014; Chen et al., 2020b), rice bran (Ling et al., 2018), and spices (Liu et al., 2021).

The Food Safety Modernization Act (FSMA) requires food producers to provide scientific proof that their established preventive controls could ensure the food safety of their products (FDA, 2015). Thus, the process validation is usually conducted to demonstrate that the implemented pasteurization process is capable of reducing the target foodborne pathogen in the final products as intended. Using pathogens in the industrial facility for process validation is risky, which could cause potential health threats for the operator or contaminate the food products if the sanitation was not appropriately applied. The recommended strategy for thermal process validation is to use a non-pathogenic surrogate, which has been evaluated to present similar or higher thermal resistance compared to the target food pathogen (FDA, 2013).

The objectives of this study were to evaluate the HARF heating-assisted thermal processing for WMP and NFDM pasteurization at different holding temperatures and to investigate a suitable surrogate, which can be used for in-plant thermal process validation.

4.3 Materials and Methods

4.3.1 Milk Powder Samples

The WMP and NFDM were acquired from Mars Inc. (McLean, VA) and were held at ambient temperature (23 ± 2°C). Upon receiving the milk powder samples, the moisture content and water activity (25°C) were determined by a halogen moisture analyzer (HR73, Mettler Toledo Laboratory and Weighing Technologies, Greifensee, Switzerland) and a dew point water activity meter (Aqualab Series 4TE, Meter Group, Pullman, WA), respectively. The background microorganisms were quantified by the aerobic plate method

as described in Wei et al. (2020). Briefly, milk powder samples (10 ± 0.1 g) were ten-fold diluted in 0.1% buffered peptone water (BPW; Becton, Dickinson and Company, Sparks, MD) and homogenized in a paddle mixer (9000471, Neutec Group Inc., Farmingdale, NY) for 60 s. The homogenized sample was then serially ten-fold diluted in 9 mL of 0.1% BPW, plated onto tryptic soy agar with 0.6% (w/v) yeast extract (TSAYE, Becton, Dickinso and Company), and incubated at 37 °C for 24 h for enumeration.

4.3.2 HARF of Milk Powders

HARF of the milk powder was conducted in a pilot-scale parallel-plate RF heating system (6 kW, 27.12 MHz; Model: SO-6B, Monga Strayfield, India). Due to the difference in density, different amounts of WMP (400 ± 0.1 g) and NFDM (500 ± 0.1 g) were weighed and filled in a laminated paper tray (ConAgra Brands, Omaha, NE) and placed at the center of the bottom electrode (Figure 4.1a). Based on our preliminary experiments, the electrode gap between the top and bottom electrodes was set to 130 mm, which would provide a rapid heating rate as well as a good heating uniformity during HARF heating of milk powders. Two fan-forced heaters (1.5 kW, HHF370B, Honeywell, Charlotte, NC, USA) were placed separately at the two sides of the HARF heating system and were used to preheat the RF chamber to around 75°C to minimize the heat loss from milk powders during HARF heating. During HARF heating, six fiber optic sensors (accuracy of 0.6°C; Neoptix, Quebec City, Quebec) were inserted into the sample tray through pre-drilled holes and used to trace the temperature at different locations (Figure 4.1b) to determine the cold spot.

In this study, the inoculated pouch method was used as previously described in Wei et al. (2019) and Liu et al. (2018). In brief, inoculated sample (2.0 ± 0.1 g) would be packed

into a paper bag (7 x 3 mm) and placed at the cold spot during HARF heating. The inoculated pouch separated the inoculated samples from the uninoculated sample in the paper tray and allowed for easier determination of microbial inactivation at the cold spot with higher sensitivity. The use of inoculated pouch method would avoid the overestimation of microbial inactivation and evaluate the worst-case scenario of microbial inactivation within the whole sample tray during HARF heating.

4.3.3 Bacterial Cultures and Inoculum Preparation

The five serotypes of *Salmonella enterica* used in this study were selected based on their implication in outbreaks and recalls related to low-moisture foods or high thermal resistance. The details of each *Salmonella* serotype are shown in Table 4.1. *Enterococcus faecium* (NRRL B-2354) obtained from the United States Department of Agriculture, Agriculture Research Services (USDA, ARS) in Peoria, IL was used for surrogate evaluation. Each bacterial culture was stored in trypticase soy broth (TSB; Becton, Dickinson and Company) supplemented with 0.6% (w/v) YE and 40% (v/v) glycerol (G31-1, Fisher Chemicals) in a cryogenic vial at -80°C.

The same inoculation method as described in Wei et al. (2020) was followed in this study, which has been shown to provide a high and stable bacterial population in milk powders stored at low water activity. Briefly, each bacterial culture was thawed in an incubator at 37°C for 5 min and then transferred into a 10 mL TSBYE tube, incubated at 37°C for 24 h for enrichment. The enriched bacterial culture was then streaked onto a TSAYE plate and incubated at 37°C for 24 h to obtain isolated colonies, which would be used as a working plate and stored at 4°C. Then, a sterilized loopful (10 µL) was used to transfer one bacterial colony from the working plate to a 10 mL TSBYE tube and then

incubated at 37°C for 24 h. The overnight culture (100 µL) was spread plated onto a TSAYE plate and incubated at 37°C for 24 h to create bacterial lawns. Finally, the bacterial lawns were harvested by agitating the lawns with 3 mL of 0.1% BPW. An equal amount of each harvested *Salmonella* lawn was mixed in a 15 mL sterile conical tube (339650, Thermo Scientific) to prepare the *Salmonella* inoculum. The harvested *E. faecium* lawns were used as the *E. faecium* inoculum. The inoculum of both bacteria was used within 30 min for milk powder inoculation. New working cultures were used to inoculate different batches of the milk powder, which represented the biological replication.

4.3.4 Milk Powder Inoculation and Water Activity Equilibration

The sample inoculation was conducted in a biological safety cabinet. Each milk powder sample (100 ± 0.1 g) was aseptically weighed and placed into a sterile Whirl-Pak bag (2.04 L, Nasco, Fort Atkinson, WI). A finger-operated spray head (ps20-410-natural, Midwest Bottles LLC, Garrison, KY) was used to spray the inoculum (10 mL) onto the milk powder sample. The inoculated sample was hand-massaged for 5 min to manually detach powders from the inner lining of the bag. Then, inoculated milk powder sample was homogenized in the paddle mixer for 6 min to achieve a uniform distribution of bacterial cells inside the sample. Subsequently, the inoculated sample (~5 mm thickness) was transferred onto a sanitized aluminum tray (230 × 300 × 15 mm) and placed into a custom-built environmental relative humidity chamber (Lau and Subbiah, 2020).

Based on the results found in Wei et al. (2020), a higher thermal resistance of *Salmonella* was observed at a lower water activity; natural water activities of both milk powders ranged from 0.18 to 0.20. During heating and holding milk powders at high temperatures, the water activity of the product decreases due to moisture evaporation. In

this study, an extremely low water activity of 0.10 was selected to evaluate the worst-case scenario for HARF heating of milk powders. So, the environmental relative humidity was set to 10% to equilibrate the inoculated milk powder samples to the water activity of 0.10 for 5 days. This equilibration period also allowed the bacteria to acclimatize and adapt to the low moisture environment prior to HARF treatment.

4.3.5 HARF Microbial Inactivation Validation

The inoculated milk powder (2 ± 0.1 g) was packed into a heat-sealable paper bag (7 x 3 mm) to prepare the inoculated pouch. Two inoculated pouches (one *Salmonella* and one *E. faecium*) were placed next to each other at the predetermined cold spot along with the remaining (396 and 496 g for WMP and NFDM) uninoculated milk powders in the paper tray. The tray was then placed at the center of the bottom electrode for HARF heating (Figure 4.1a). One fiber optic sensor was placed onto the pouch to measure the temperature of the cold spot during the HARF of milk powder. The samples were HARF heated to 85, 90 and 95°C before transferring to the hot-air oven (preheated to target holding temperature) for subsequent holding. According to the previous study (Wei et al., 2021), the *D*-values of *Salmonella* in WMP (water activity = 0.10) at 85, 90 and 95°C were determined to be 7.98, 3.35 and 1.68 min, respectively. Similarly, the *D*-values of *Salmonella* in NFDM (water activity = 0.10) at 85, 90 and 95°C were found to be 10.98, 4.82 and 1.82 min, respectively. Initially, the maximum holding time at 85, 90 and 95°C were selected to be 60, 20 and 10 min (> 5*D*-value) to achieve more than 5 log reduction in this study. However, due to the moisture loss during the HARF heating and oven holding, the thermal resistance of *Salmonella* increased dramatically. The maximum holding times were then extended to 120, 30 and 15 min at 85, 90 and 95°C, respectively. After holding in the oven, the inoculated

pouch was immediately transferred into a sterile Whirl-Pak bag and immersed into an ice-water bath (0°C) for stopping the thermal inactivation.

The treated inoculated pack (2 ± 0.1 g) was diluted by adding 18 mL of 0.1% BPW and was homogenized for 1 min in a paddle mixer. Then, the homogenized sample was 10-fold diluted using 0.1% BPW and spread-plated onto TSAYE supplemented with 0.05% (w/v) ammonium iron citrate (Sigma-Aldrich, Co., MO, USA), and 0.03% (w/v) sodium thiosulfate (Fisher Chemical, NJ, USA) for *Salmonella* enumeration or 0.025% (w/v) esculin hydrate (Acros Organics, NJ, USA) for *E. faecium* and incubated at 37°C for 24 h. The grown yellow colonies with a black center were counted as *Salmonella* survivors, while the *E. faecium* survivors were enumerated by counting the black colonies. All the HARF heating inactivation treatments were conducted in triplicates.

4.4 Results and Discussion

4.4.1 HARF of Milk Powder

The WMP (water activity = 0.205 ± 0.004; moisture content = 3.07 ± 0.22 %) and NFDM (water activity = 0.220 ± 0.002; moisture content = 3.15 ± 0.05 %) were HARF heated for around 15 min to reach 95°C at the coldest location (Figure 4.2). As shown in Figure 4.2, the top center (1) was determined to be the cold spot among the six locations, which were measured during the HARF heating, for both WMP and NFDM. Within each layer, it can be observed that the heating rate of the edge was faster than the center. Similar observations were also found during RF heating of other food products, such as infant formula milk (Lin et al., 2020), peanut kernels (Zhang et al., 2021a), wheat and corn flour (Ozturk et al., 2017; Villa-Rojas et al., 2017) and almonds (Li et al., 2017). The edges received more electromagnetic energy from multiple directions in addition to the fringe

effect of the electromagnetic field. In general, the dielectric properties increase with temperatures which leads to the hot spot heating at a faster rate than the cold spot. This is known as thermal runaway heating (Zhang et al., 2021b). The top layer of both milk powders had the slowest heating rate compared to the other two layers. Because RF heating does not heat the air, the temperature gradient between the top layer of milk powder and air would result in considerable heat loss from the top layer. Thus, the fan-forced heater was used to preheat the RF chamber and reduce the thermal gradient in this study. The high environmental temperature minimized the heat loss from the milk powder during RF heating and improved the heating uniformity. The HARF has been shown to provide a faster heating rate and better heating uniformity for several low moisture food products (Ling et al., 2018; Chen et al., 2020b; Liu et al., 2021). The heating uniformity of milk powders can be further improved by establishing a continuous RF process or adding a mixing step between two RF units (Zhou and Wang, 2019). Besides, recent studies (Chen et al., 2016, 2017; Huang et al., 2018; Zhang et al., 2021a) have shown that computer simulations could be used to design, scale-up and optimize the RF heating process to provide a better heating uniformity.

The average heating rates for the cold spot temperature to reach 95°C were determined to be 6.4 and 6.2°C/min for WMP and NFDM, respectively. The WMP and NFDM were packed in the same tray and heated the temperature of the cold spot (top center) to 95°C using a convection oven preheated to 105°C. The temperature histories of both milk powders during oven heating were shown in Figure 4.3. The average heating rates for oven heating of WMP and NFDM were 0.42 and 0.32°C/min, respectively. The HARF heating considerably shortened the amount of time required for heating the milk powder to

95°C. This shorter come-up time of HARF allowed for high-temperature short-time pasteurization which has been shown to minimize quality deterioration.

In this study, only small sample sizes (400 g for WMP and 500 g for NFDM) of milk powders were heated, while a much larger size sample would be needed for the industrial-scale processing. With a larger sample size, the oven heating may require few days to heat the milk powder to the target pasteurization temperature due to its poor thermal conductivity. For example, the traditional hot-room pasteurization of egg white powder in commercial packages (9.07 kg) requires 15 days of holding time, which takes 4-7 days to heat the sample to a target temperature of 67°C (Baron et al., 2003; Boreddy et al., 2016; Wei et al., 2020b). Because RF heating can heat the milk powder volumetrically, the increase in come-up time would be relatively small with an increase in sample size. A longer heating time at high temperatures would result in larger loss of moisture and caking of milk powder. This may necessitate an additional unit operation to break the clumps. Also, a continuous RF heating process can be developed for milk powder pasteurization, which could maintain a similar heating profile as shown in this study and may provide a better heating uniformity.

4.4.2 Inactivation of *Salmonella* in Milk Powders during HARF

The background microorganisms were determined to be < 10 CFU/g in both WMP and NFDM. After inoculation and equilibration, the inoculation control samples had a *Salmonella* population of 6.47 ± 0.03 log and 6.54 ± 0.14 log CFU/g in WMP and NFDM, and *E. faecium* population of 8.49 ± 0.04 log and 8.13 ± 0.08 log CFU/g in WMP and NFDM. Higher *E. faecium* population compared to *Salmonella* was observed in both milk powders, which indicated *E. faecium* was sturdier than *Salmonella* in the low water activity

environment and this result is corroborated by other studies (Liu et al., 2019; Chen et al., 2020a; Wei et al., 2020a; Verma et al., 2021). The inoculation method has been shown to significantly affect the *Salmonella* inactivation kinetics and subsequent thermal inactivation study (Hildebrandt et al., 2016). The lawn-based liquid inoculum method used in this study has been exhibited to a suitable method for inoculation of low moisture foods, which could provide a stable *Salmonella* population in different low moisture foods (Hildebrandt et al., 2016; Verma et al., 2018a; Chen et al., 2019).

Before the microbial challenge study, the inoculated samples were equilibrated to the water activity at 0.10 and acclimated for 5 d for the adaption of bacteria to the low moisture condition. The acclimation of inoculated samples before the microbial challenge study is essential for improving the external validity of the process validation (Allison and Fouladkhah, 2018). The fresh inoculated sample could be sensitive to the subsequent inactivation treatments, while the acclimation would enhance the resistance of *Salmonella* which simulates the real scenario (Piao et al., 2007; Lambertini et al., 2016; Lang et al., 2017). In the industrial process, the milk powder could get contaminated during shipping or storage; thus the bacteria usually have sufficient time to adapt to the low water activity environment before further process. After the inoculation, the thermal resistance of *Salmonella* and *E. faecium* has been shown to remain consistent during the extended storage time up to 180 d (Sekhon et al., 2021; Wei et al., 2021). Thus, HARF heating of the inoculated milk powder was conducted between 5-15 d after the inoculation.

The survival curves of *Salmonella* and *E. faecium* in WMP and NFDM after HARF heated to 85, 90, and 95°C were shown in Figure 4.4. More than 5 log reductions of *Salmonella* was achieved in WMP after holding at 90 and 95°C for 30 and 10 min,

respectively, while more than 5 log reduction of *Salmonella* was only observed after holding at 95°C for 15 min in NFDM. Thus, HARF heating-assisted thermal processing was demonstrated to be an effective pasteurization process for the milk powder. Due to the presence of antimicrobial compounds (Tang et al., 2017), the thermal resistance of *Salmonella* in black pepper is low ($D_{75°C}$-value = 7.8 min at a natural water activity of 0.45) at a high temperature (Vasquez, 2018; Gautam et al., 2020). There is no need for additional holding time in RF heating of spice products, such as black pepper (Wei et al., 2018), cumin seeds (Chen et al., 2019) and basil leaves (Verma et al., 2021b). However, the thermal resistance of *Salmonella* is relatively high ($D_{75°C}$-value = 26.9 min at a natural water activity of 0.20) in NFDM (Liu et al., 2019; Wei et al., 2020a), which usually requires a holding process after the RF heating to achieve the desired inactivation of *Salmonella* in powdered milk products (Michael et al., 2014; Lin et al., 2020). During the holding period, a large amount of moisture is lost due to the evaporation. The thermal resistance of *Salmonella* was enhanced at this reduced water activity environment resulting in an extended holding time requirement for achieving desired pasteurization. For instance, after holding at 85°C for 120 min, only 3.21 and 4.03 log reductions were observed in WMP and NFDM. According to Wei et al. (2020a), the determined $D_{85°C}$-values of *Salmonella* in WMP and NFDM were 10.1 and 9.6 min, respectively, at a water activity of 0.10. This indicated that a 5 log reduction of *Salmonella* was expected after holding the WMP and NFDM at 85°C for 50.5 and 45.0 min. The initial water activity and moisture content of inoculated WMP were found to be 0.10 and 2.07%, while those corresponding values decreased to 0.064 and 1.09%, respectively, after holding in the oven for 120 min at 85°C. The required longer holding time to achieve the desired inactivation because of the

considerably enhanced thermal resistance of *Salmonella*. Therefore, the *Salmonella* inactivation efficiency of HARF was low at 85°C, and a higher temperature (90 or 95°C) was recommended for pasteurization of milk powders.

Microbial inactivation kinetics of food pathogens in low moisture foods have been collected extensively and modeled at different water activities, such as *Listeria monocytogenes* in cocoa powder (Tsai et al., 2019b), *Salmonella* in black pepper powder (Gautam et al., 2020) and dried basil leaves (Verma et al., 2021), and *Escherichia coli* in confectionery and chicken meat powder (Daryaei et al., 2018). Those data will be very helpful for determining the adequate thermal process conditions for pasteurization of low moisture foods considering the dynamic moisture change during the process. In this study, because the microbial inactivation kinetics data of *Salmonella* in milk powders at the extremely low water activity (< 0.10) were not available, it is difficult to predict the *Salmonella* inactivation based on the thermal process data. To develop a reliable model, the thermal inactivation kinetics data as a function of water activity, especially in the low range, is required.

4.4.3 Surrogate for Process Validation

The identification of a suitable surrogate for replacing the target food pathogen is essential for industrial process validation. In this study, higher survival of *E. faecium* compared to *Salmonella* in both milk powders was observed at all temperatures and holding times. Thus, *E. faecium* was determined to be a suitable surrogate for *Salmonella* during HARF assisted thermal process of milk powders.

E. faecium has been demonstrated to be a suitable surrogate for several thermal processing of low moisture foods, such as roasting of almond (Almond Board of California,

2007), RF heating of wheat flour (Villa-Rojas et al., 2017), egg white powder (Wei et al., 2020b), spices (Chen et al., 2019; Wei et al., 2019) and extrusion of oat flour (Verma et al., 2018b). However, no proper guidance was provided for applying *E. faecium* as a surrogate for the process validation. In low moisture foods, *E. faecium* is usually a conservative surrogate and exhibits significantly higher thermal resistance compared to *Salmonella*. Tsai et al. (2019a) reported that the *D*-values of *E. faecium* in the cocoa powder were 59.9, 28.9, and 16.1 min at 70, 75 and 80°C, while the *D*-values of *Salmonella* in the cocoa powder were 46.2, 20.5 and 11.5 min at the same temperature. Similarly, *D*-values of *E. faecium* were determined to be more than two times higher than *D*-values of *Salmonella* in WMP at 85, 90 and 95°C (Wei et al., 2021). A valid pasteurization process usually required a more than 5 log reduction of *Salmonella* to be achieved (FDA, 2020). However, the milk powder would be overprocessed if the thermal process conditions were determined by aiming a more than 5 log reduction of *E. faecium*, which results in unnecessary quality loss and energy consumption. The use of a kill ratio based on the relationship between the microbial inactivation kinetics of *Salmonella* and *E. faecium* can improve the efficiency of the pasteurization process of dairy powder by reducing energy costs and quality deterioration. For example, > 5.5 log reduction of *Salmonella* was achieved after WMP was HARF heated to 95°C and held for 10 min, the corresponding *E. faecium* reduction was found to be 1.4 log. Therefore, when using a surrogate to conduct the process validation for pasteurization of WMP at 95°C, the process conditions that have been shown to provide > 1.4 log reduction of *E. faecium* reduction will indicate > 5 log reduction of *Salmonella* and can be considered as a valid pasteurization process for WMP.

4.5 Conclusions

In this study, HARF to preheat the milk powders to target pasteurization temperatures followed by holding in the hot air oven for the desired time has been shown to effectively pasteurize WMP and NFDM at a water activity of 0.10. More than 5 log reduction of *Salmonella* in WMP was achieved after HARF heated to 95°C and held for 10 min or HARF heated to 90°C and held for 30 min. As for NFDM, more than 5 log reduction of *Salmonella* would require HARF preheating to 95°C and then held for 15 min in the oven. *E. faecium* was found to be a suitable surrogate for *Salmonella* during the HARF heating combined convective oven processing of milk powders. The determined surrogate can be used for conducting the industrial-scale process validation.

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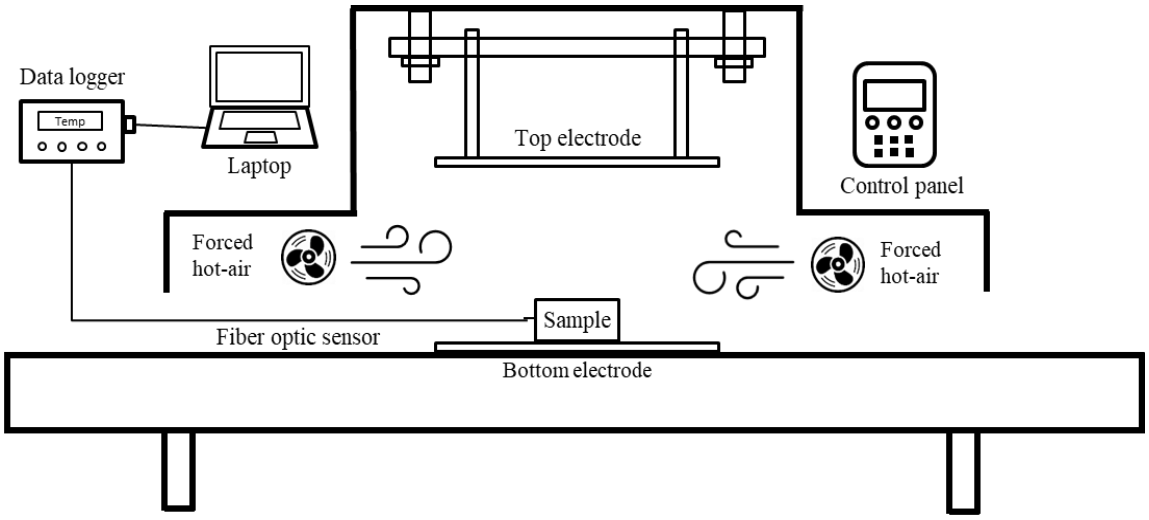
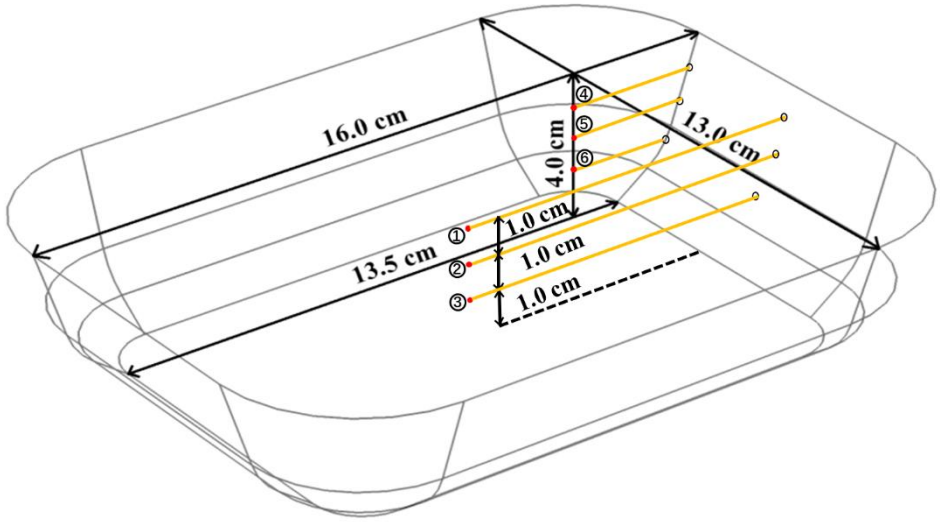
Table 4.1. Bacterial serotypes used for inoculating milk powders.

Bacterial name	Source
Salmonella Agona 447967	FDA, ORA Regional Lab (Jefferson, AR)
Salmonella Montevideo 488275	FDA, ORA Regional Lab (Jefferson, AR)
Salmonella Mbandaka 698538	FDA, ORA Regional Lab (Jefferson, AR)
Salmonella Reading Moff 180418	FDA Culture Collection (Bedford Park, IL)
Salmonella Tennessee K4643	University of Georgia (Athens, GA)
Enterococcus faecium NRRL B-2354	USDA, ARS (Peoria, IL)



(a)



(b)

Figure 4.1. (a) Schematic of 27.12 MHz, 6 kW radio frequency heating system for the milk powders, the electrode gas and power of radio frequency heating were adjusted by the control panel; (b) Dimensions for the laminated paper tray; locations of the six fiber optic sensors.

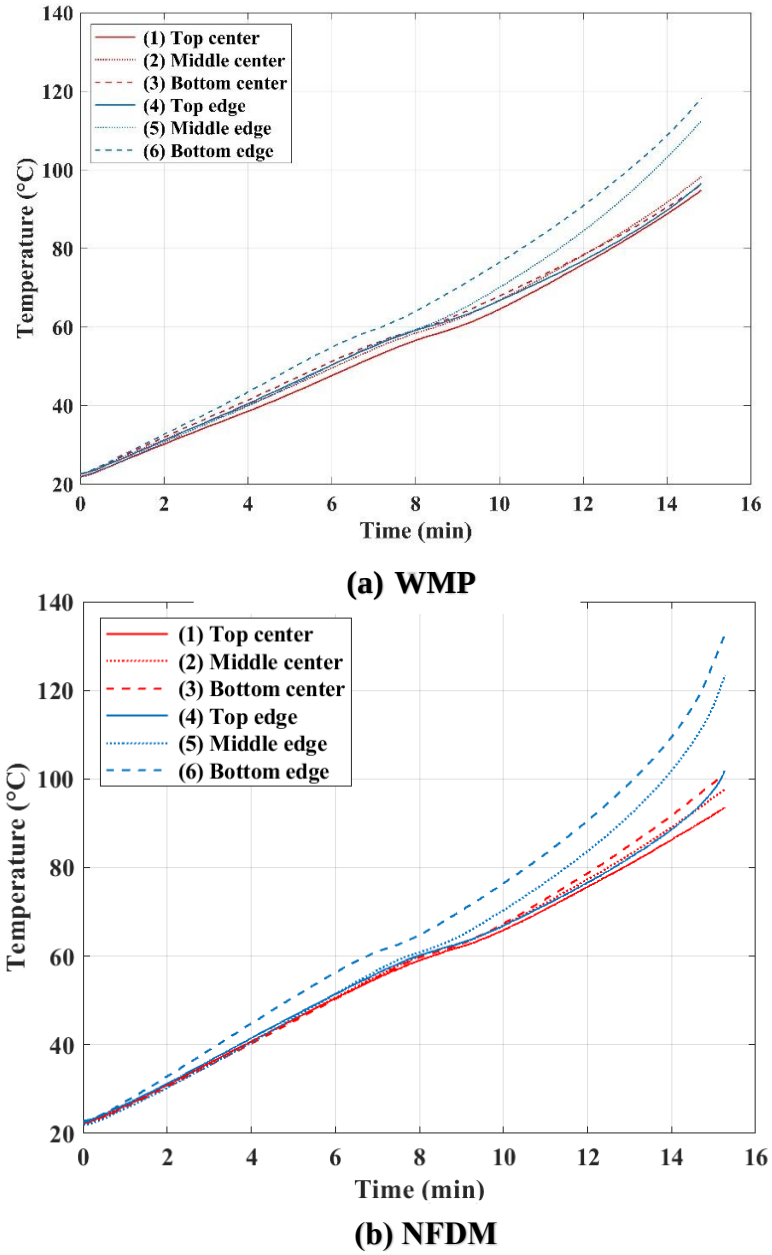


Figure 4.2. Temperature histories during hot-air assisted radio frequency heating of whole milk powder (WMP) and nonfat dry milk (NFDM).

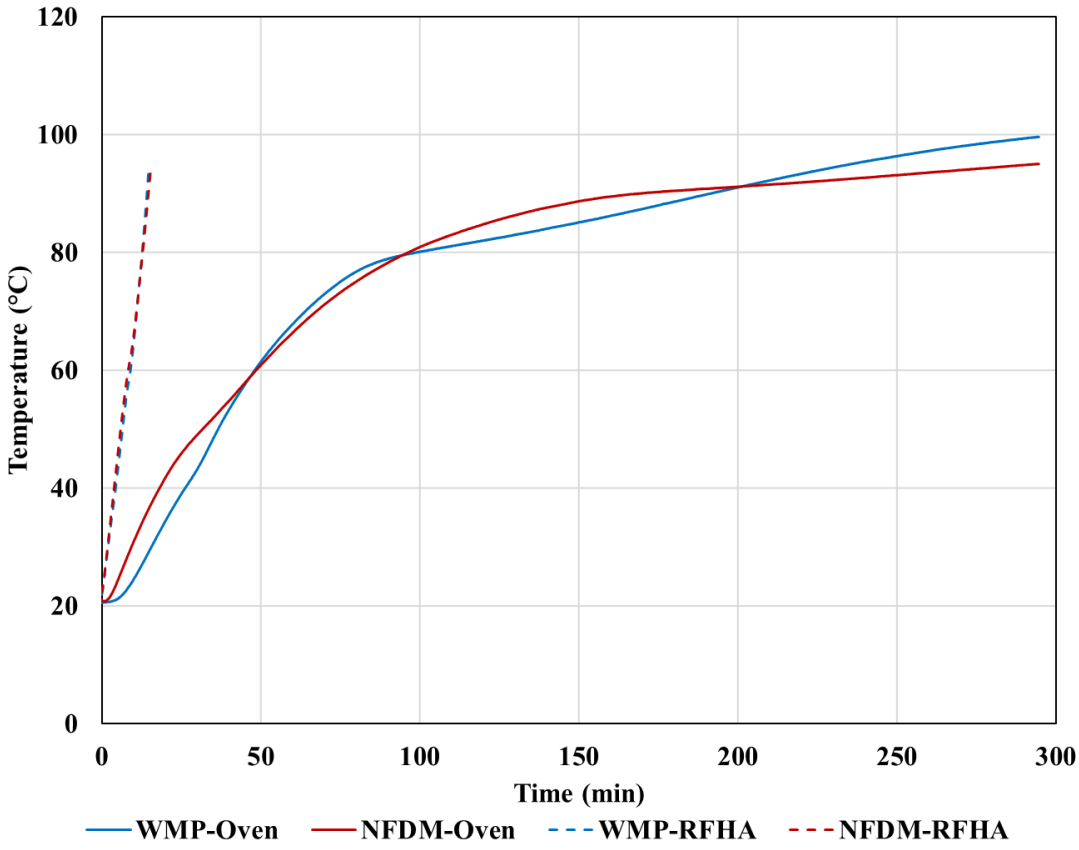


Figure 4.3. Temperature histories of cold spots under hot-air assisted radio frequency heating or oven heating (preheated to 105°C) of whole milk powder (WMP) and nonfat dry milk (NFDM).

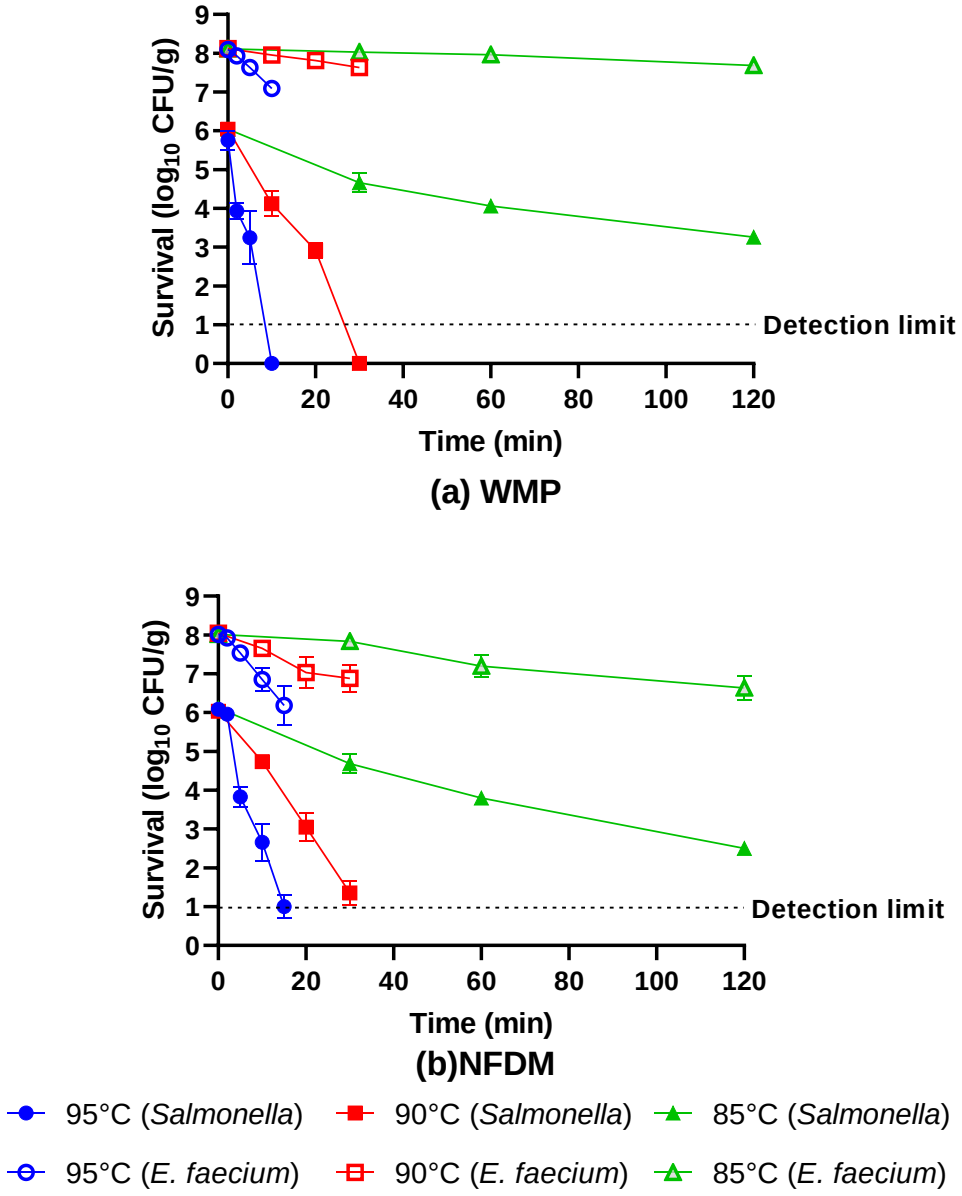


Figure 4.4. Survival curves of *Salmonella* and *E. faecium* in whole milk powder (WMP) and nonfat dry milk (NFDM) at a water activity of 0.10 that are pre-heated to 85, 90 and 95°C using hot-air assisted radio frequency heating following by holding at different times in the oven.

Chapter 5

A MICROBIAL CHALLENGE STUDY FOR VALIDATING CONTINUOUS RADIO-FREQUENCY ASSISTED THERMAL PROCESSING PASTEURIZATION OF EGG WHITE POWDER

5.1 Abstract

Spray dried egg white powder (EWP) is traditionally processed by hot room treatment for a prolonged period of time (67°C for 15 days) to enhance its functionality (foaming and gelling) and to microbial safety of EWP. Our prior research demonstrated that radio-frequency (RF) assisted thermal processing can considerably reduce the processing time, without compromising the functional properties of EWP. In this study, continuous RF processing was evaluated for pasteurization of EWP. EWP samples were inoculated with a 5-strain *Salmonella* cocktail or *Enterococcus faecium* NRRL B-2354 for the microbial challenge studies. To evaluate the inoculation method, stability and homogeneity tests were conducted for both *Salmonella* and *E. faecium* in EWP. Continuous RF heating of EWP was conducted in a 6-kW, 27.12 MHz pilot-scale parallel-plate RF heating system. RF-assisted thermal processing of EWP at 80°C for 2 h provided > 6.7 log reduction for *Salmonella*. *E. faecium* was found to be a suitable surrogate for *Salmonella* due to its higher resistance and similar inactivation kinetics during RF heating of EWP. The validated RF-assisted thermal process can be scaled up for use in the egg industry.

Keywords: Surrogate, low moisture food safety, dielectric heating, stability test, process validation.

5.2. Introduction

Egg white powder (EWP) has been used as the main ingredient in many food products because of its excellent gelling, water-holding and foaming properties (Lechevalier et al., 2007; Yang and Baldwin, 1995). Liquid egg white is very fragile, thus using EWP as a substitution usually offers several advantages, such as longer shelf life, easy storage, and less transport cost. In the past two decades, processed egg products have become increasingly popular, reaching up to 40% of the total egg consumption (American Egg Board, 2015; Hammershøj et al., 2004). Raw egg products have a history of being the most important vehicle of the *Salmonella* infection (Gantois et al., 2009). The traditional spray drying of liquid egg white does not inactivate all the pathogens (Baron et al., 1997; LiCari and Potter, 1970). As a typical low moisture food, the EWP has a water activity (a_w) of around 0.30 which limits the growth of *Salmonella* (Blessington et al., 2013). However, it has been reported that *Salmonella* can survive for a long period of time at low a_w storage conditions (Keller et al., 2013). Therefore, an appropriate pasteurization process needs to be developed for ensuring the food safety of EWP (Baron et al., 1999).

In the egg industry, EWP is usually pasteurized in a hot room by dry-heat at 67°C for 15 days (Baron et al., 2003). This process is also known for improving the gelling and foaming properties as well as the microbial safety of EWP (Hammershøj et al., 2004; Handa et al., 2001). However, this process is neither energy nor time efficient. It takes up to 1-2 days for the hot room method to raise the temperature at the coldest location of EWP package to a desired pasteurization temperature due to lower thermal conductivity (Boreddy, 2015). The slow heating rate may allow bacteria to build up heat resistance which results in significantly larger D-values (Chung et al., 2007; Zhang et al., 2018).

Therefore, this process requires a long processing time to achieve the desired microbial inactivation.

Radio-frequency (RF) heating is a form of dielectric heating (Boreddy and Subbiah, 2016; Chen et al., 2013) which heats up the food product volumetrically and rapidly (Chen et al., 2017, 2019; Lau et al., 2016; Wang et al., 2007a). Compared to the conventional heating method which heats the low moisture food very slowly due to their low thermal conductivity, RF heating has been shown to rapidly heat low moisture food (Li et al., 2017; Wang et al., 2014). Boreddy et al. (2016) showed that RF heating could significantly reduce the come-up time by 50 to 80 times compared to hot room method without compromising the functional properties of EWP. Due to the shortened processing time, RF heating can limit the time for bacteria to build up heat resistance, and thus be used as an alternative technology to the hot room method.

Risk-based preventive controls are important for ensuring food safety. The Food and Drug Administration (2018) requires the food industry to scientifically prove that their preventive controls will effectively limit or eliminate the hazards that are reasonably likely to occur. Therefore, a proper validation study must be performed by the food industry before applying a process technology. However, it is not feasible to directly introduce food pathogens like *Salmonella* into the facilities to evaluate the processing. Thus, it is imperative to use appropriate surrogates to conduct specific treatment validation studies (Food and Drug Administration, 2013). A proper surrogate should be non-pathogenic and have similar resistance or susceptibility to the technology in question and similar inactivation kinetics as the target microorganism, which can be used to predict behavior of the target pathogen when exposed to the inactivation process. *Enterococcus faecium* NRRL

B-2354 has been demonstrated as a non-pathogenic surrogate (Kopit et al., 2014) for *Salmonella* in various thermal processing of low a_w foods, such as extrusion of oat flour (Verma et al., 2018a) and carbohydrate-protein meal (Bianchini et al., 2014), RF heating of wheat flour (Villa-Rojas et al., 2017) and black pepper (Wei et al., 2018, 2019) and moist-air convection heating of almonds (Jeong et al., 2011).

The objectives of this study were to (i) demonstrate continuous RF heating as a proper pasteurization process for inactivation of a 5-strain *Salmonella enterica* subsp. *enterica* cocktail in EWP and (ii) evaluate *Enterococcus faecium* NRRL B-2354 as a suitable surrogate for *Salmonella enterica* subsp. *enterica* in RF heating of EWP.

5.3. Materials and methods

5.3.1 EWP Samples

Three batches of pasteurized EWP samples were obtained from three different production lots from Henningsen Foods, Inc. (Omaha, NE, USA) and stored at room temperature (25°C). Upon receiving the EWP samples, the moisture content was determined using a halogen moisture analyzer (HR73, Mettler Toledo Laboratory and Weighing Technologies, Greifensee, Switzerland) and a_w was measured by a dew point water activity meter (Aqualab Series 4TE, Decagon Devices Inc., Pullman, WA) at 25°C.

5.3.2 Background Microflora of EWP Samples

Aerobic plate counts of the pasteurized EWP samples were also quantified upon receiving to determine the background microflora of the samples. From each batch, three 10 g of samples were randomly collected and aseptically transferred to three sterile Whirl-Pak style bags. Then, 90 mL of 0.1% buffered peptone water (BPW; Becton, Dickinson and Company, Sparks, MD) was used to dilute the sample and the diluted sample was then

stomached for 1 min in an analog basic paddle mixer (Neutec Group Inc, NY, USA). The diluted sample was then tenfold serially diluted in 9 mL 0.1% BPW blanks, spread plated onto tryptic soy agar (TSA; Becton, Dickinson and Company, Sparks, MD) supplemented with 0.6% (w/v) yeast extract (YE; Becton, Dickinson and Company, Sparks, MD) (TSAYE) and incubated for 24 h at 37°C for enumeration.

5.3.3 Bacterial Strains and Sample Inoculation

Five different strains of *Salmonella enterica* subsp. *enterica* were selected for this study because of their association with various foodborne illness outbreaks associated with low moisture foods (Table 5.1). *E. faecium* NRRL B-2354 was selected as the non-pathogenic surrogate for the surrogate evaluation (Table 5.1). All bacterial cultures were kept in tryptic soy broth (TSB; Becton, Dickinson and Company, Sparks, MD) with 0.6% (w/v) yeast extract (TSBYE) supplemented with 40% glycerol and stored at -80°C.

The frozen culture (1 mL) of each bacterial strain was first aseptically transferred to 9 mL of TSBYE and was incubated for 24 h at 37°C. The overnight broth culture was spread plated (0.1 mL) onto a TSAYE plate and was incubated for 24 h at 37°C. The bacterial lawn on TSAYE was harvested with 3 mL of 0.1% BPW by agitating the cells into a suspension with a L-shaped spreader. The *Salmonella* cocktail was prepared by equally mixing the inoculum of each *Salmonella* strain and then vortexed for 10 s to achieve uniform distribution of cells. The initial population levels of the *Salmonella* cocktail and *E. faecium* inoculum were ca. 10^{10} CFU/mL and ca. 10^{9} CFU/mL, respectively.

A commercial mixer (stand mixer, No. W53294842, KitchenAid, Benton Harbor, MI) was used to prepare the inoculated EWP. EWP (1 kg ± 0.1 g) was aseptically transferred to a sanitized mixing bowl. Inoculum (10 mL of *Salmonella* cocktail or *E. faecium* NRRL B-

2354) was then sprayed onto the EWP sample, and then was mixed for 10 min at the lowest speed. The moisture content and a_w were elevated due to the addition of inoculum. To equilibrate the inoculated samples back to the original a_w, 200 g of the inoculated samples were aseptically transferred to a tray (230 x 300 x 15 mm) with a thickness of ca. 10 mm, and placed in a custom-designed humidity control chamber (Smith and Marks, 2015; Wei et al., 2018) and equilibrated for a minimum of five days. The relative humidity was set at 33.0 ± 0.3% inside the chamber to equilibrate the inoculated samples back to the original a_w.

5.3.4 Stability and Homogeneity Tests

To evaluate whether the inoculation method could provide a stable and homogenous population of bacteria, the stability and homogeneity tests were conducted for 15 days. On day 0, 1, 2, 3, 6, 9, 12, and 15th, three aliquots of EWP samples (3.0 ± 0.1 g) from each tray were randomly collected and packed in Whirl-Pak bags. The sample was then diluted by adding 27 mL 0.1% BPW, stomached for 1 min in an analog basic paddle mixer, duplicate-plated onto TSAYE supplemented with 0.05% (w/v) ammonium iron citrate (Sigma-Aldrich, Co., MO, USA), and 0.03% (w/v) sodium thiosulfate (Fisher Chemical, NJ, USA) (mTSA) for *Salmonella* or TSAYE supplemented with 0.05% (w/v) ammonium iron citrate, and 0.025% (w/v) esculin hydrate (Acros Organics, NJ, USA) (eTSA) for *E. faecium*, and incubated for 24 h at 37°C (Smith et al., 2015, 2016; Liu et al., 2018a). After incubation, colonies with a black center on mTSA were counted as *Salmonella* while black colonies on eTSA were counted as *E. faecium* NRRL B-2354. Two biological replications were conducted for the stability and homogeneity tests.

5.3.5 Continuous RF processing for EWP

5.3.5.1 EWP package and temperature measurement

As shown in Fig. 1, a cardboard box (140 x 103 x 132 mm^3) that is approximately $1/20^{th}$ the size of a commercial package was used to package the EWP samples (550 ± 0.1 g) to simulate the industrial hot room pasteurization package conditions of EWP. A custom-made polyethylene bag was first placed inside the cardboard box and then the EWP sample was filled into the polyethylene bag. The polyethylene bag was loosely closed at the top and the cardboard box was sealed with transparent tape.

To evaluate the real-time temperature distribution of EWP during RF heating, six fiber optic temperature probes (Neoptix, Inc., Quebec City, Quebec, Canada) with an accuracy of ± 0.6°C were inserted into the box through pre-drilled holes at the side wall of the cardboard box (Fig. 1). The temperature profile during RF heating was recorded by the probes every second. Probes 1, 2, 3 were inserted to the center (70 mm) and arranged from the top to the bottom vertically at 25 mm intervals and probes 4, 5, 6 were inserted close to both edges (20 mm) from the top to the bottom vertically with a 25 mm interval to each other.

5.3.5.2 RF Heating of EWP

RF heating was conducted in a 6 kW, 27.12 MHz pilot-scale parallel-plate RF heating system (Model SO-6B, Monga Strayfield Pvt. Ltd., Pune, India) as shown in Fig. 2. The RF heating system has a conveyor belt system, the speed of which is adjustable by a variable speed motor. The belt is made of polyester and the two ends of the belt were connected by a hook-and-loop fastener.

The packaged EWP sample was placed inside the RF heating chamber at the left

end of the bottom electrode and on the conveyor belt which would move the EWP sample from left to the right. During preliminary trials with a small gap between the top and bottom electrodes, there was moisture migration towards the bottom of the package due to the fast heating rate, resulting in caking of EWP. The heating rate could be adjusted by adjusting the electrode gap between the top and bottom electrodes. Therefore, the electrode gap was determined to be 250 mm by trial-and-error method to avoid caking of EWP (Boreddy, 2015).

According to previous studies (Boreddy et al., 2016, 2014), when packaged EWP was RF heated to 80°C and maintained at 80°C in a convection oven for 16 h, the foaming and gelling properties of EWP could be elevated to the same level as traditionally hot room processed EWP. Therefore, the time required by RF heating to raise the temperature of EWP to 80°C was determined for using in the microbial challenge studies. During RF heating of EWP, the temperatures at different locations were recorded and RF heating was stopped when the temperatures of cold spot reached at least 80°C. The temperature measurement was conducted for three times to determine come-up time. Based on the come-up time, the speed of the conveyor belt for continuous RF heating of EWP would be determined for further microbial challenge studies.

5.3.6 Continuous RF Processing for Inactivation of *Salmonella* and *E. faecium* NRRL B-2354

RF heating was used to rapidly heat EWP to the target temperature. However, the whole sample was not uniformly heated because of the fast heating rate (Al-Holy et al., 2005; Boreddy, 2015; Chen et al., 2016). Therefore, it is important to evaluate the thermal inactivation at different locations and especially the cold spot. To evaluate the thermal

inactivation at the cold spot, the inoculated pack method developed for RF heating process was used (Liu et al., 2018a; Wei et al., 2018). Briefly, the inoculated food product sample was packed in a small plastic bag which was transparent to RF waves and placed at the cold spot. In this method, the small sample pack was isolated from the bulk of the sample, thus requiring less amount of inoculated sample in addition to pinpointing the thermal inactivation to a specific location during the processing.

In this study, each 3.0 ± 0.1 g of EWP sample inoculated with *Salmonella* or *E. faecium* was packed in a polyethylene bag (40 x 30 x 2 mm). A total of 12 inoculated packs, 6 inoculated with *Salmonella* and 6 inoculated with *E. faecium*, were used to evaluate the thermal inactivation of both bacteria at different locations during RF heating of EWP. One pack of each was placed at locations 1 to 6 (Fig. 1), with the *Salmonella* inoculated pack stacked on top of the *E. faecium* inoculated pack. The inoculated samples were isolated by the packs, so samples in different packs would not cross-contaminate each other. Therefore, it was feasible to evaluate the microbial inactivation of different microorganisms at the same time. In most studies (Almond Board of California, 2014; Bianchini et al., 2014; Ceylan and Bautista, 2015; Verma et al., 2018b), surrogate evaluation was conducted using the same conditions as the target microorganism at different trials. However, the process variation at the same condition between different trials could contribute variations to the surrogate evaluation. The inoculated pack method could avoid the variation and compare surrogate to the target microorganism for the same process.

The packaged EWP with inoculated packs was RF-heated to 80°C using the conditions described in previously section on the moving conveyor belt. It was then immediately transferred to a mechanical convection oven (Model 28L, GCA Precision

Scientific Group, Chicago, IL) preheated to 80°C and was held for 0, 2, 8, and 16 h. For each time point, a new sample was prepared and was replicated three times using independent EWP batches.

Upon achieving the required holding period in the oven, the inoculated packs were taken out from the box and immersed into an ice water bath to prevent further thermal inactivation. Next, the contents of the inoculated packs were transferred to sterile Whirl-Pak bags, tenfold diluted using BPW and then stomached for 1 min in an analog basic paddle mixer. The stomached samples were then serially diluted in 9 mL of 0.1% BPW, spread plated onto mTSA for *Salmonella* or eTSA for *E. faecium* and incubated for 24 h at 37°C for enumeration.

5.4. Results and Discussion

5.4.1 Sample Inoculation, Stability and Homogeneity test

Moisture content could significantly affect the heating rate during RF heating of low moisture food (Jeong and Kang, 2014). Therefore, it is critical to control the moisture content of food samples before RF heating to have a repeatable process. *Salmonella* in low moisture food was shown to have elevated thermal resistance at a lower water activities (Smith et al., 2016). It is necessary to control the initial a_w of food sample before RF heating, which would significantly influence the microbial inactivation (Liu et al., 2018b; Villa-Rojas et al., 2017). In this study, the initial moisture and $a_{w,25°C}$ of EWP samples were 7.11 ± 0.11% (wet basis) and 0.31 ± 0.01, respectively. Once EWP samples were inoculated, the moisture and $a_{w,25°C}$ were elevated to 7.89 ± 0.13% and 0.40 ± 0.01, respectively. One day after equilibration in the humidity chamber, the moisture content and $a_{w,25°C}$ were equilibrated back to 6.96 ± 0.09% and 0.30 ± 0.01. Both moisture content and $a_{w,25°C}$ were

well-controlled during equilibration and before RF heating in this study.

Different inoculation methods have been shown to affect the survival of *Salmonella* in low moisture food (Bowman et al., 2015; Hildebrandt et al., 2017). A reliable inoculation method is required to prepare a stable contaminated product to be used in microbial challenge studies. The inoculation method used in this study has been shown to provide a stable microbial population in wheat flour prior to thermal treatment (Hildebrandt et al., 2016). The stability and homogeneity tests were conducted, and the results are shown in Fig. 3. High initial population level of both bacteria, 8.7 and 7.9 log CFU/g of *Salmonella* and *E. faecium*, respectively, were achieved by this inoculation method. After 15 days of equilibration, there was less than 1 log CFU/g reduction for *Salmonella* and less than 0.5 log CFU/g reduction for *E. faecium.* This indicated that the inoculation method used in this study allowed both the microorganisms to survive properly in the EWP samples. The error bars are the standard deviation from two biological replications which indicate high repeatability of this inoculation method. Within the same replication, samples from three random locations were enumerated and the maximum standard deviation was ± 0.18 CFU/g which demonstrated that the inoculated sample was mixed properly and had a good homogeneity. It has been reported that the thermal resistance of *Salmonella* would be enhanced under desiccated condition (Gruzdev et al., 2011). Therefore, it is important to allow bacteria to adapt to the desiccated condition before conducting challenge studies. Based on the stability test results, inoculated samples were equilibrated for at least five days before conducting RF heating of EWP. The maximum equilibration time was 10 days. Figure 5.3 showed that the population was relatively stable up to 15 days of equilibration.

5.4.2 Come-up Time of RF Heating of EWP

The temperature profiles of the six locations in the cardboard box during RF heating of EWP is shown in Fig. 4. The bottom edge (6), which took 55 min to reach 80°C, was determined to be the cold spot with an average heating rate of 1.47°C/s. Therefore, the come-up time of continuous RF heating of EWP was determined to be 55 min. Based on the come-up time, the speed of the conveyor belt for continuous RF heating of EWP was determined to be 0.8 m/h. The hottest spot was located at the center of the middle layer which was the geometric center. Among the six locations, the heating rate ranked from the fastest to the slowest are 2 > 1 > 5 > 3 > 4 > 6. The temperature difference between the hottest geometric center to the coldest bottom edge was 15°C. As shown in Fig. 4, the center locations always heated up faster than the edge locations at the same layer for all three layers. For both the center and edge locations, the fastest heating rate was achieved at the middle layer, followed by the top layer and the bottom layer. Hence, it is likely that the temperature difference between each location was mainly caused by thermal loss from the external surfaces of the box. The box was placed on a metal electrode plate, thus the bottom layer experienced considerable thermal loss due to the high thermal conductivity of the metal. Although air is a good thermal insulator, there was still some thermal loss from the top layer resulted in a slower heating rate than the middle layer. The heat was well conserved from escaping at the middle layer, which contributed to the fastest heating rate.

During RF heating of EWP, the heat was mainly generated within the package, and the surrounding air and bottom electrode plate were not heated up at all. The temperature difference between the environment and the food sample could affect the heating uniformity. Thus, the heating uniformity could be improved by maintaining the RF

applicator cavity at the target temperature. It has been shown that RF heating with hot-air assistance was able to improve heating uniformity and provide a more consistent heating pattern (Chen et al., 2017; Liu et al., 2013; Wang et al., 2014). In future studies, a hot-air assisted RF heating could be evaluated for EWP pasteurization processing.

5.4.3 Microbial Inactivation after RF Heating of EWP

RF heating of inoculated EWP was conducted and followed by different holding times. The microbial inactivation results are shown in Table 5.2. During the come-up time, the microbial inactivation among the six locations were considerably different from each other which might result from the non-uniform heating. The magnitude of the microbial inactivation followed a similar trend as the rank of heating rate. For example, Location 2 with the fastest heating rate achieved 3.97 log reduction of *Salmonella* at the end of come-up time, before transferring to oven. Location 6, which was the cold spot, had only 0.58 log reduction of *Salmonella* due to its slowest heating rate. The inactivation of *E. faecium* also showed a similar trend as *Salmonella*. These results agreed well with the temperature measurement which indicated that the inoculated packs were placed at the correct locations. Using inoculated packs to evaluate the microbial inactivation at the cold spot has also been shown in other studies (Liu et al., 2018a; Wei et al., 2018). This method isolated inoculated samples from uninoculated samples, which could evaluate the microbial inactivation at specific locations with higher precision. Because only partial samples were inoculated, this method minimized the amount of inoculated sample preparation for validation studies. Therefore, for industrial validation of RF heating, the inoculated pack method can be used to reduce the required amount of inoculated samples while allowing for more efficient evaluation of different spots.

After holding for 1 h in the convection oven, more than 5 log reduction was achieved at most of the locations except location 6 which had the slowest heating rate. The variation of microbial reduction at different locations was also decreased, possibly due to improvement of heating uniformity in the hot air oven. At the end of the RF heating process, the inner temperature of the box was slightly higher than the outer temperature of the box during the RF heating. After the box was transferred to the oven, the outer temperature of the box would have increased first due to convection from the hot air in the oven and diffusion from the hotter geometric center of the sample. The decreased difference of microbial inactivation between the hot spot and cold spot from come-up time to 1 h holding time indicated that the temperature difference declined after transferring to the oven. Table 5.2 shows that the bacterial counts were under detection limit (< 10 CFU/g) for all six locations after 2 h of holding in the oven. With a more than 5 log reduction of *Salmonella* population, this continuous RF heating met the 5-log pathogen reduction performance standard (Food and Drug Administration, 2015). Therefore, EWP could be pasteurized by the process of RF heating to 80°C followed by holding at 80°C for 2 h. Compared to the traditional hot room method, RF heating considerably reduced the process time for EWP pasteurization. Boreddy (2015) reported that RF heating combined with 16 h of holding in the oven was required for improvement in functionality of EWP and therefore microbial challenge studies were also conducted for RF heating followed by 16 h of holding in the oven. As expected, the microbial counts at all six locations were below the detection limit (Table 5.2).

At both 0 and 1 h holding time, reduction of *E. faecium* was either significantly lower ($p < 0.05$) or not different compared to *Salmonella* at all locations which indicated

that *E. faecium* has a similar or higher thermal resistance than *Salmonella* during RF heating. Therefore, *E. faecium* can be considered as a suitable surrogate for *Salmonella* during RF heating of EWP. The Food Safety and Modernization Act requires food producers to validate their critical processing steps (Food and Drug Administration, 2018). One of the most effective form of validation is to introduce a non-pathogenic surrogate into the processing facilities and conduct an in-plant validation. Therefore, it is critical to identify a proper surrogate for specific processes and products. Although *E. faecium* has been evaluated as a good surrogate for *Salmonella* during some thermal processing of low moisture foods, it is not always a proper surrogate for *Salmonella* (Rachon et al., 2016). The results presented here show that *E. faecium* could be a suitable surrogate for *Salmonella* for RF-assisted heating of EWP at 80 °C, though its suitability should be validated for other process conditions.

In this study, RF assisted thermal processing has been shown to effectively inactivate *Salmonella* in EWP with considerably shorter processing time. *E. faecium* has been found to be a suitable surrogate for *Salmonella,* which could be used in larger scale validations. In practice, it is important to scale-up the lab-scale experiment to an industrial application. Several studies have shown that continuous RF process is usually a way to scale-up the lab-scale stationary RF heating to an industrial-scale RF treatments (Jiao et al., 2012; Piyasena et al., 2003; Wang et al., 2007a, 2007b). The continuous RF heating would be able to process larger amount of food products in a short time compared to the stationary RF heating. In the industrial application, it is necessary to minimize the non-uniform heating during RF process. It has been reported that heating uniformity of continuous RF process could be improved by hot air assistance, intermittent stirring or electrode

modification (Al-Holy et al., 2005; Chen et al., 2016, 2015; Jiao et al., 2015).

5.5. Conclusions

In this study, continuous RF-assisted thermal processing was validated as an alternative pasteurization process for EWP for the egg industry. The geometric center was found to be the hot spot during the RF heating, which improved heating uniformity when the samples were moved to the hot air oven. The inoculation method was shown to provide stable inoculated samples with high microbial load for microbial challenge studies. RF heating for 55 min followed by 2 h of holding in hot air oven was able to pasteurize EWP with > 6 log reduction of *Salmonella*. *E. faecium* was found to be a suitable surrogate for *Salmonella* for RF-assisted heating of EWP at 80 °C, therefore, can be used for the validation of industrial-scale thermal pasteurization process. In future studies, the heating uniformity of RF heating of EWP could be further improved by mixing or vibration or supplying hot air in the RF oven.

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Table 5.1. Bacterial strain names and related foodborne outbreaks.

Strain name	Related outbreak	Source	Reference
Salmonella Agona 447967	Oats cereal	FDA, ORA Regional Lab (Jefferson, AR)	(Centers for Disease Control, 1998)
Salmonella Montevideo 488275	Black and red pepper	FDA, ORA Regional Lab (Jefferson, AR)	(Centers for Disease Control, 2010)
Salmonella Mbandaka 698538	Sprouts	FDA, ORA Regional Lab (Jefferson, AR)	(Jackson et al., 2013)
Salmonella Reading Moff 180418	Alfalfa sprout	FDA Culture Collection (Bedford Park, IL)	(Centers for Disease Control, 2016)
Salmonella Tennessee K4643	Peanut butter	University of Georgia (Athens, GA)	(Centers for Disease Control, 2007)
Enterococcus faecium NRRL B-2354	--	USDA, ARS (Peoria, IL)	--

Table 5.2. Microbial inactivation at different locations with initial RF heating to 80°C followed by different holding times.

Location	Bacteria	Microbial Reduction (log CFU/g)			
		Holding time in the oven after RF preheating (h)			
		0	1	2	16
1	*Salmonella*	3.8 ± 0.4	6.1 ± 1.1	> 6.7	> 6.7
	E. faecium	2.1 ± 0.9*	5.1 ± 1.5	> 6.8	> 6.8
2	*Salmonella*	4.0 ± 0.4	6.7 ± 0.4	> 6.7	> 6.7
	E. faecium	2.3 ± 0.6	6.1 ± 1.2	> 6.8	> 6.8
3	*Salmonella*	0.8 ± 0.2	5.2 ± 1.0	> 6.7	> 6.7
	E. faecium	0.4 ± 0.2	4.2 ± 0.8	> 6.8	> 6.8
4	*Salmonella*	1.5 ± 0.3	6.1 ± 0.2	> 6.7	> 6.7
	E. faecium	0.9 ± 0.7	3.7 ± 0.20*	> 6.8	> 6.8
5	*Salmonella*	1.8 ± 0.2*	5.4 ± 0.6	> 6.7	> 6.7
	E. faecium	0.4 ± 0.1	4.0 ± 1.0	> 6.8	> 6.8
6	*Salmonella*	0.6 ± 0.3	4.7 ± 0.9	> 6.7	> 6.7
	E. faecium	0.3 ± 0.2	3.7 ± 0.6	> 6.8	> 6.8

* Indicates that log reductions of *E. faecium* is significantly lower than that of *Salmonella* at the same location ($p < 0.05$).

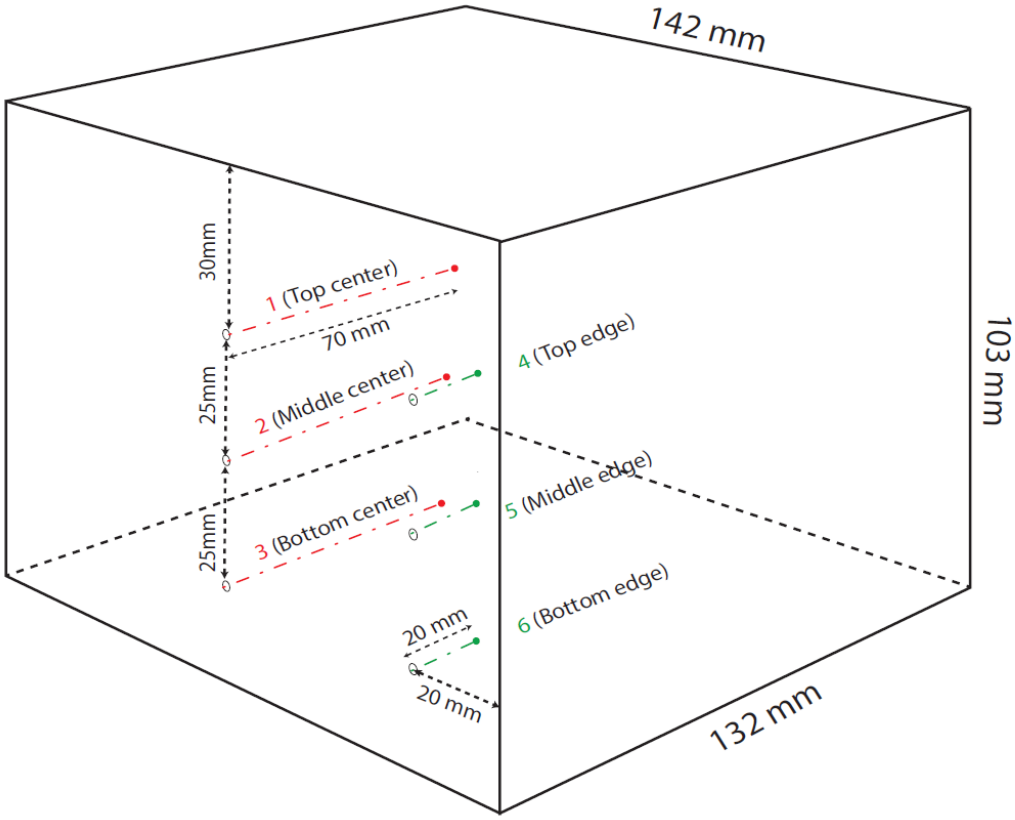

Figure 5.1. Dimensions of the cardboard box. Dot-dash lines indicated the locations of the six fiber optic sensors. The dot at the end of each fiber optic sensors indicate the location of each inoculated pack.

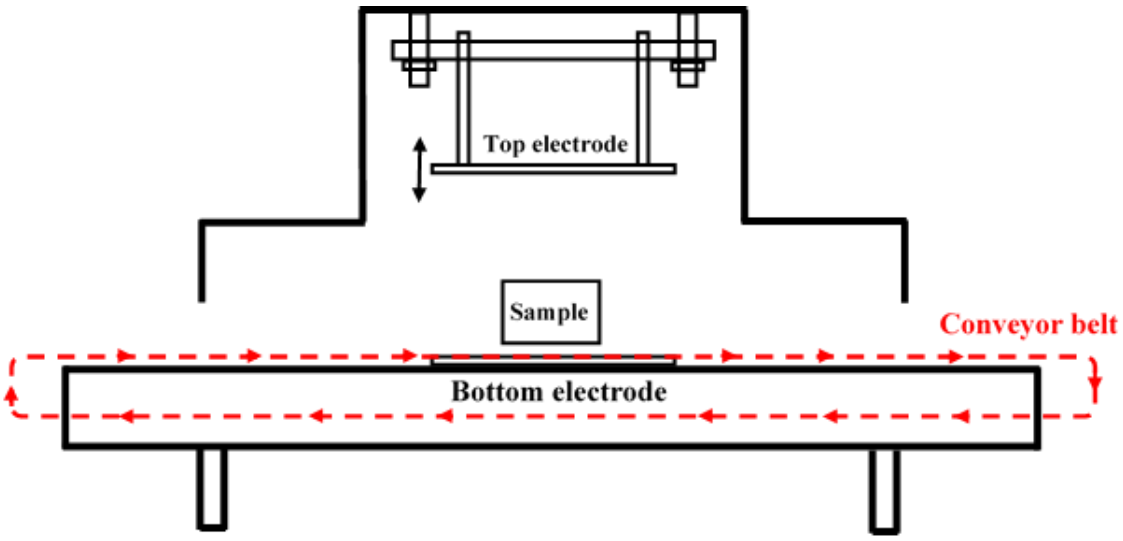

Figure 5.2. Schematic diagram of 27.12 MHz, 6 kW radio frequency heating system for egg white powder. The speed of conveyor belt was 0.8 m/h.

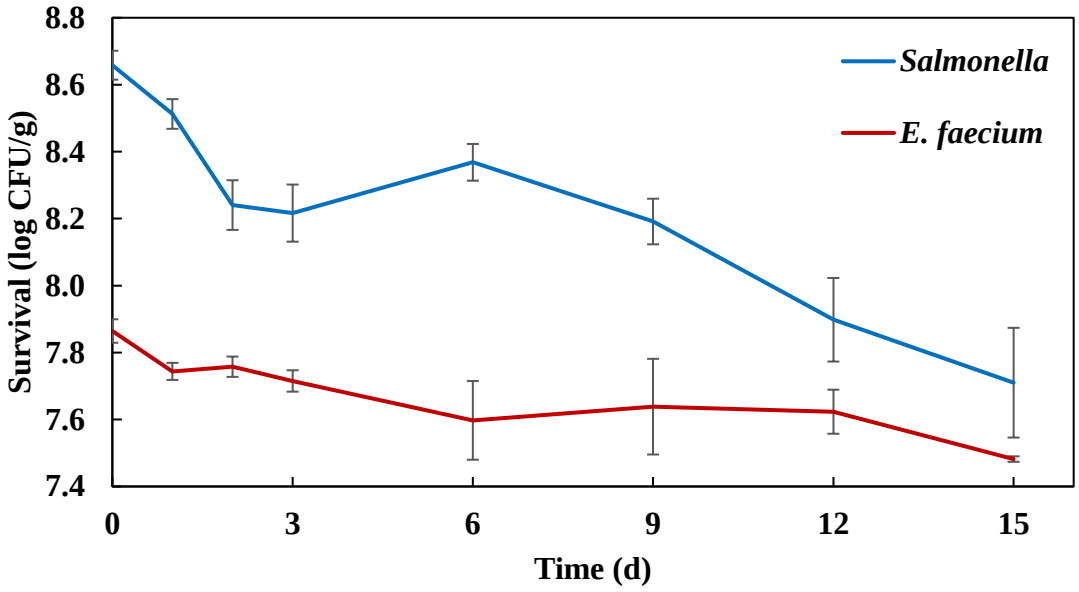

Figure 5.3. Stability and homogeneity tests of *Salmonella* and *E. faecium* over 18 days in the equilibration chamber. Error bars represent standard deviations of two biological replications. In each biological replication, three subsamples were tested.

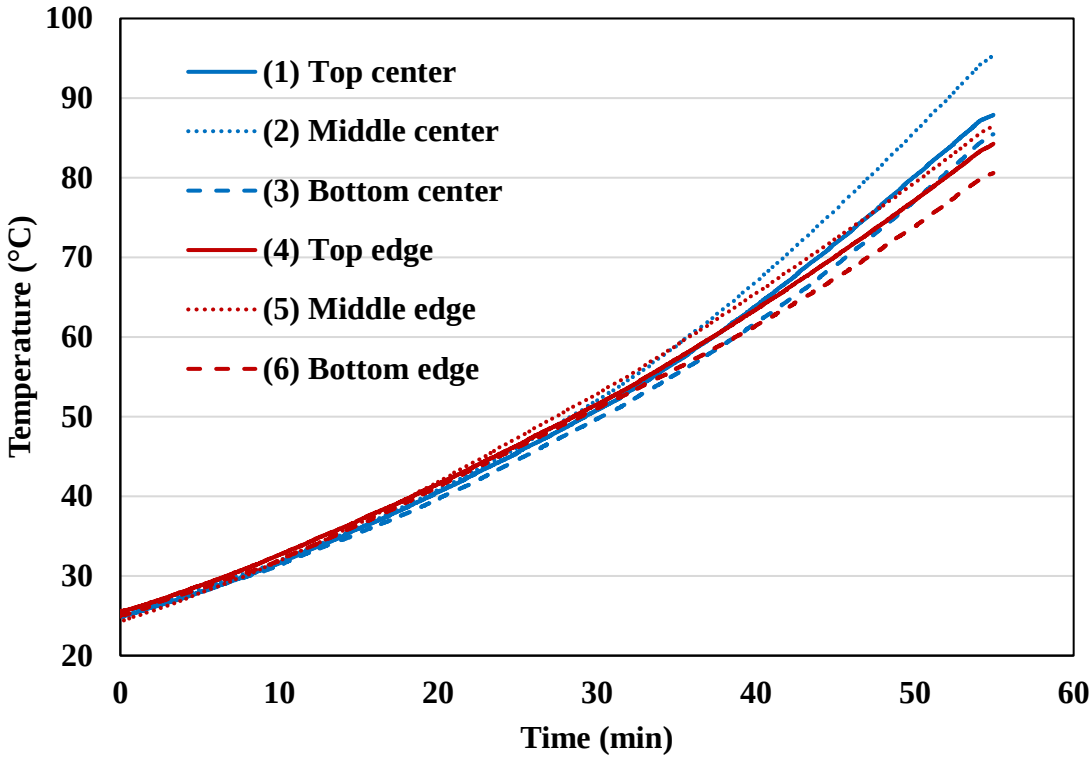

Figure 5.4. Comparison of temperature profiles of different locations during RF heating of EWP.

Chapter 6

MODELING THE EFFECT OF TEMPERATURE AND RELATIVE HUMIDITY ON THE ETHYLENE OXIDE FUMIGATION OF *SALMONELLA* AND *ENTEROCOCCUS FAECIUM* IN WHOLE BLACK PEPPERCORN

6.1 Abstract

This research investigated the effect of temperature and relative humidity (RH) conditions during ethylene oxide (EtO) fumigation for inactivation of *Salmonella* and *Enterococcus faecium* NRRL B-2354 on whole black peppercorns. Black peppercorn samples were inoculated with a five-strain *Salmonella* cocktail or *E. faecium* inoculum. EtO (735.3 mg/L) fumigation was conducted at different treatment temperatures (46, 53, and 60°C) and different RH levels (30,40, and 50%) for the inactivation of inoculated black peppercorn samples with different exposure times (2 to 180 min). The temperature, RH, and exposure time exhibit significant linear effects on microbial inactivation. Bacterial inactivation during EtO fumigation was described by the Weibull model with the $R^2 > 0.70$ and RMSE < 0.20 log CFU/g at all conditions. The concave upward trend of the Weibull model indicated a tailing effect. The inactivation data were also used to develop the response surface model as a function of temperature, RH, and exposure time to predict the reduction of *Salmonella* or *E. faecium* on whole black peppercorn during EtO fumigation. The developed models predicted log reductions with RMSE of 0.48 and 0.45 log CFU/g for *Salmonella* and *E. faecium*, respectively. Technical information for developing and validating EtO fumigation for whole black peppercorn could be estimated based on the developed model.

Keywords: Surrogate, Weibull model, response surface model, low moisture food safety, *Salmonella*, *E. faecium*

6.2 Introduction

Ethylene oxide (EtO) fumigation is a dry and low-temperature sterilization process, which has been demonstrated to considerably reduce the microbial load in different food commodities as well as on medical devices (Gimeno et al., 2018; Leistritz, 1997; Schweiggert et al., 2007). The main inactivation mechanism of EtO is an alkylation reaction which adds alkyl groups to sulfhydryl, hydroxyl, amino, and carboxyl groups to denature functional proteins, DNA, and RNA structures of microorganisms (Mendes et al., 2007). Ethylene oxide fumigation has been conventionally used to reduce the bacterial load and inactivate pathogens by the spice industry (Gilbert et al., 1964; Tateo & Bononi, 2006; Vajdi & Pereira, 1973). According to the American Spice Trade Association, around 40 to 85% of spices imported into the U.S. are treated with EtO annually (ATSA, 2020).

Salmonella contamination of black pepper has caused several multistate foodborne outbreaks in the United States (Centers for Disease Control, 2010; McKee, 1995). Because black pepper is usually served as ready-to-eat food which does not require a further cooking step before consumption, the *Salmonella*-contaminated black pepper could cause severe food safety issues. The Preventive Control Rule of the Food Safety Modernization Act (FSMA) requires all processors to identify possible risks and to outline and implement strategies that control or eliminate consequences of contamination events (FDA, 2018). Several decontamination methods that currently being used to pasteurize black pepper have some limitations, such as insufficient inactivation in ozone treatment, quality deterioration in steam treatment and poor consumer acceptance in gamma-ray irradiation (Adler, 1965;

Newkirk et al., 2018; Zhao & Cranston, 1995). Ethylene oxide fumigation is reported as an effective decontamination method for spices, without significant degradation to appearance and flavor (ATSA, 2020; Duncan et al., 2017). However, a recent study (Newkirk, 2016) has reported large variations on *Salmonella* inactivation on spices treated at two different commercial EtO fumigation facilities, which indicated that different protocols could be followed to operate the EtO fumigation in different facilities. Inappropriate application of EtO fumigation could result in severe food safety issues, such as insufficient inactivation or residue problems in the EtO treated product.

The Food Quality Protection Act Tolerance Reassessment Decision Document was developed by the EPA and includes the tolerances for EtO and its reaction product, ethylene chlorohydrin (ECH) residues, which are 7 and 940 ppm on spices, respectively (EPA, 2006). All commercial processors of spices must use process parameters during EtO fumigation that will not result in exceeding the residual limits set by the EPA. Since 2008, any spice treated by EtO in the United States must follow the directions on the EtO label. Despite this requirement, commercial processing facilities use a variety of concentrations of EtO, chamber temperature, and relative humidity of the environment.

Although EtO fumigation is a non-thermal process, treatment temperature has been reported to be a critical parameter that affects the efficacy of microbial inactivation during the process (Heider et al., 2002). Also, the environmental relative humidity (RH) has been found to considerably affect the microbial inactivation of EtO fumigation (Gilbert et al., 1964; Mendes et al., 2007). However, most studies evaluated the effect of temperature or RH independently, while the cross effect of temperature and RH has not been investigated. Therefore, a comprehensive study should be conducted to identify the optimal conditions

and provide guidance for EtO fumigation processors to ensure food safety.

Process validation has been required by the U.S. Food and Drug Administration (FDA, 2018) to demonstrate that effective control of microbial hazards has been applied properly. However, it is extremely risky to directly introduce foodborne pathogens, such as *Salmonella enterica*, *Escherichia coli* O157:H7*,* or *Listeria monocytogenes* into the process validation in industrial facilities. Therefore, the use of a non-pathogenic surrogate for process validation is recommended by the FDA (2013) to minimize the risk. *Enterococcus faecium* NRRL B-2354 has been demonstrated to be a suitable surrogate for *Salmonella* in many low moisture food matrices, such as nuts (Abd et al., 2012; Ahmad et al., 2019; Almond Board of California, 2007), wheat and oat flours (Liu et al., 2018; Verma et al., 2018b; Villa-Rojas et al., 2017), spices (Chen et al., 2019, 2020; Newkirk et al., 2018; Wei et al., 2018, 2019) and dry powders (Liu et al., 2019; Wei et al., 2020).

The objectives of this study were to evaluate the effects of temperature, relative humidity, and exposure time on the efficacy of EtO fumigation of whole black peppercorn inoculated with *Salmonella* or *E. faecium* and investigate the suitability of *E. faecium* as the surrogate for *Salmonella* during this process.

6.3 Materials and Methods

6.3.1 Whole Black Peppercorn Samples

Three batches of commercially steam-sterilized whole black peppercorns from three different production lots were received from McCormick & Company, Inc (Sparks, MD, USA). The black peppercorn samples were packed in plastic bags and stored in a walk-in cooler at -12°C prior to inoculation.

The water activity (a_w) of black peppercorn samples were controlled and measured

before each EtO fumigation treatment. A water activity meter (4TE, METER Group, Pullman, WA) was used to measure the a_w of black peppercorn samples at room temperature (25°C). Upon receiving the samples, the initial a_w of black peppercorn samples was determined to be 0.58. Approximately 3 g of sample was placed on a disposable sample container and loaded into the test chamber of water activity meter to determine the sample water activity at 25°C.

6.3.2 Bacterial Strains and Inoculum Preparation

A *Salmonella* cocktail was prepared by mixing five strains of *Salmonella enterica* which were selected based on their previous association with the low-moisture product outbreaks or recalls (CDC, 1993, 2004, 2013, 2016). *Salmonella* Agona 447967, *Salmonella* Montevideo 488275, and *Salmonella* Mbandaka 698538 were obtained from the U.S. Food and Drug Administration ORA Arkansas Regional Lab (Jefferson, AR). *Salmonella* Tennessee K4643 was obtained from Dr. Larry Beuchat at the University of Georgia, and *Salmonella* Reading Moff 180418 was obtained from the FDA Culture Collection in Bedford Park, IL. *E. faecium* NRRL B-2354, which served as the surrogate in this study, was obtained from the National Center for Agricultural Utilization Research in Peoria, Illinois. All isolates were stored at −80 °C as frozen stocks in 40% glycerol before inoculum preparation.

The inoculum was prepared using the agar-lawn method of Hildebrandt et al., (2016), which has been shown to provide a stable bacterial population in low moisture foods. Each vial of the frozen stock (1 mL) was thawed at room temperature for 5 min, then subjected to two sequential transfers (24 h at 37°C) in 10 mL tryptic soy broth (211825, Becton, Dickinson and Company, Sparks, MD) supplemented with 0.6 % (w/w) yeast

extract (TSBYE) (212720, Becton, Dickinson and Company, Sparks, MD). The overnight incubated culture was then streaked onto plates of tryptic soy agar (236920, Becton, Dickinson and Company, Franklin Lakes, NJ) supplemented with 0.6 % (w/w) yeast extract (TSAYE) and incubated for 24 h at 37°C to prepare the working plates. The working plates were sealed with parafilm and stored at 4°C for up to one month. For each experimental replicate, new working cultures and different batch samples were used allowing for biological replication.

A single colony from each working plate was transferred to 10 mL TSBYE and incubated for 24 h. Then, 0.1 mL of the overnight culture was spread onto a TSAYE plate and incubated at 37°C for 24 h to produce the bacterial lawn. Then, 3 mL of 0.1 % (w/w) buffered peptone water (BPW, 218103, Becton, Dickinson and Company, Sparks, MD) was used to harvest the grown lawns by using a sterile L-shape spreader to agitate lawns from the agar plate. To prepare the *Salmonella* cocktail, 2 mL of the bacterial lawns of each *Salmonella* strain was mixed in a sterile 15 mL centrifuge tube (339650, Thermo Fisher Scientific, Rochester, NY) and vortexed for 30 s to achieve uniform distribution of cells. The prepared *Salmonella* cocktail and *E. faecium* inoculum contained the bacterial population of ca. 10.5 and 9.5 log CFU/mL, respectively, and were used to inoculate samples within 2 h of preparation.

6.3.3 Sample Inoculation

One day before inoculation, black peppercorn samples were taken out from the cooler and equilibrated to room temperature (23°C). For each batch, 200 ± 0.1 g of the black peppercorn samples were weighed and placed in a resealable plastic bag. The cap of the centrifuge tube containing inoculum was replaced with a finger-operated spray head

(ps20-410-natural, Midwest Bottles LLC, Garrison, KY); 4 mL of the inoculum was sprayed onto the black peppercorn samples and then the inoculated samples were hand-massaged for 5 min. The sample inoculation was performed within a biosafety level 2 cabinet. The inoculated samples were then transferred to a relative humidity chamber (Smith et al., 2016) to equilibrate the moisture content and a_w back to its original level. The inoculated samples were equilibrated at room temperature (23°C) with an environmental RH level of 58% (natural water activity of black peppercorn) for at least five days to allow the bacterial cells adapt to the low moisture environment before inactivation treatments, and for a maximum of 14 days to maintain a stable initial bacterial population level based on the stability tests conducted by Wei et al., (2018).

6.3.4 EtO Fumigation of Black Peppercorn

Following inoculation and equilibration, a single layer of whole black peppercorn samples (3 ± 0.1 g) inoculated with *Salmonella or E. faecium* were placed on a petri dish (100 mm of diameter) for EtO fumigation. EtO fumigation of inoculated black peppercorn samples was conducted using a gas sterilizer (Steri-Vac 5XL, 3M Company, Saint Paul, MN) with 100% ETO gas. The sterilizer uses the EtO cartridge (4-100, 3M Company, Saint Paul, MN) that provides a fixed gas concentration of 735.3 mg/L based on the chamber size. Three temperature levels (46, 53, and 60°C) and three levels of RH (30, 40, and 50%) were selected to evaluate the effect of treatment temperature and RH on the antimicrobial efficacy of EtO fumigation on whole black peppercorns. For most of the treatment conditions, five gas exposure times ranging from 2 to 180 min were applied to obtain the bacterial inactivation curve. At the highest temperature and RH level (60°C and 50% RH), only two exposure times (2 and 5 min) were applied due to the rapid inactivation. At the

end of the EtO fumigation, gas aeration occurred under a vacuum in the locked chamber for a minimum of 8 h to completely remove EtO gas.

6.3.5 Bacterial Enumeration

After the EtO fumigation treatment, samples were transferred into a sterile Whirl-Pak bag (Nasco, Fort Atkinson, WI), 10-fold diluted with 0.1% BPW, placed in a paddle mixer (9000471, Neutec Group Inc, Farmingdale, NY) and mixed for 1 min. The homogenized sample was then serially diluting in 0.1 % BPW, spread plated onto TSAYE supplemented with 0.05% (w/v) ammonium iron citrate (Sigma-Aldrich, Co., MO, USA), and 0.03% (w/v) sodium thiosulfate (Fisher Chemical, NJ, USA) (mTSA) for *Salmonella* or TSAYE supplemented with 0.05% (w/v) ammonium iron citrate, and 0.025% (w/v) esculin hydrate (Acros Organics, NJ, USA) (eTSA) for *E. faecium* and incubated for 24 h at 37°C (Liu et al., 2018; Smith et al., 2016). After the incubation, colonies with a black center were counted as *Salmonella* and black colonies were counted as *E. faecium*, respectively.

6.3.6 Model Analysis

All EtO fumigation treatments were performed in triplicate. For each treatment condition, the Weibull-type model was used to fit the survival data:

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

where N and N_0 (CFU/g) are the *Salmonella* or *E. faecium* survivors at time t and 0, t is the exposure time (min), δ (min) is the scale parameter and β is the shape parameter of the Weibull inactivation curve, where $\beta > 1$ indicates a concave downward trend, $\beta < 1$ indicates a concave upward trend of the inactivation curve and $\beta = 1$ indicates a linear

trend. The bacterial survival data at each treatment condition were fitted to Weibull model using the Levenberg–Marquardt algorithm for non-linear least squares regression as implemented in the curve_fit function of the Python (version 3.7) SciPy library (Newville et al., 2015).

The effects of three factors (temperature, RH, and exposure time) on the inactivation of *Salmonella* or *E. faecium* were studied using the response surface model. The second-order polynomial equation was used to develop a predictive model for the bacterial inactivation as below:

$$\log_{10}\left(\frac{N}{N_0}\right) = \beta_0 + \beta_1 * T + \beta_2 * RH + \beta_3 * t + \beta_{12} * T * RH + \beta_{13} * T * t + \beta_{23} * RH * t + \beta_{11} * T^2 + \beta_{22} * RH^2 + \beta_{33} * t^2$$

where N and N_0 (CFU/g) are the *Salmonella* or *E. faecium* survivors at exposure time t and 0, β_i are constant regression coefficients, T is the treatment temperature (°C), RH is the relative humidity (%), and t is the exposure time (min). The parameters of this model were estimated using response surface method functions in the open-source statistical software, R (version 4.0.2, Lenth, 2009).

To evaluate the fitness of the model, the goodness of fit was quantified by the root mean square error (RMSE) in (log CFU/g):

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

where $log_{10}(N)_{observed,i}$ is the observed log reduction and $log_{10}(N)_{predicted,i}$ is the predicted log reduction from the model.

The corrected Akaike information criterion (AICc) also was used for the evaluation of each model:

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

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 (Motulsky & Christopoulos, 2003). Lower RMSE and AIC_c values indicate a higher likelihood that the model is correct for the prediction of the observation data.

6.4 Results and Discussion

6.4.1 Bacterial Inactivation

Whole black peppercorn with a moisture content of 11.9 ± 0.2% (wet basis) and water activity of 0.58 ± 0.01 was used to evaluate the efficacy of EtO fumigation at different temperature and RH levels. The background microorganisms in the steam-sterilized whole black peppercorn samples were < 10 CFU/g (below detection limit), which would have negligible impact on the inoculated bacterial enumeration. The black peppercorn samples were then inoculated with *Salmonella* cocktail or *E. faecium* with a bacterial population of 7.51 ± 0.31 or 7.63 ± 0.25 log CFU/g, respectively.

The inactivation of *Salmonella* and *E. faecium* in black peppercorn after treatment with EtO gas at 30% RH and 46, 53, 60°C for 2 to 180 min is shown in Figure 6.1. After 2 min of exposure at 30% RH, 1.15 ± 0.19, 1.76 ± 0.09, and 2.48 ± 0.26 log CFU/g reductions of *Salmonella* were achieved at 46, 53, and 60°C, respectively. While 3.23 ± 0.27, 3.81 ± 0.08, 4.05 ± 0.21 log CFU/g reductions of *Salmonella* were achieved at 46, 53, and 60°C, respectively, with an exposure time of 180 min at 30% RH. A greater reduction of *Salmonella* was achieved at a higher treatment temperature with the same exposure time. The increasing efficacy of EtO fumigation at higher treatment temperature observed in this

study is in agreement with other studies (Cotton & Roark, 1928; Heider et al., 2002). Heider et al. (2002) reported that a 10°C increase of the treatment temperature would double the efficacy of 600 mg/L EtO with 60% RH for the inactivation of *Bacillus subtilis* spores.

The inactivation of *Salmonella* and *E. faecium* in black peppercorn after treatment with EtO gas at 30%, 40%, 50% RH and 53°C for 2 to 180 min is shown in Figure 6.2. After 2 min of exposure, 1.76 ± 0.09, 2.57 ± 0.10, and 2.87 ± 0.26 log CFU/g reductions of *Salmonella* were achieved at 30, 40, and 50% RH, respectively. At 30 and 40% RH, 3.81 ± 0.08 and 4.74 ± 0.20 log CFU/g reductions of *Salmonella* were achieved with 180 min of exposure time, respectively, while 4.92 ± 0.13 log CFU/g reductions of *Salmonella* were achieved with 20 min of exposure time at 50% RH. Higher reductions of both bacteria were observed at a higher level of RH at the same exposure time. Ethylene oxide fumigation is considered as a dry sterilization process with no liquid water involved, while it is not carried out under completely anhydrous conditions as moisture vapor is present around the material being processed. RH level has been shown to considerably affect the efficacy of EtO fumigation of *Bacillus subtilis* (Gilbert et al., 1964). Gilbert et al. (1964) also reported that a high RH level could overcome the resistance that *Bacillus subtilis* spores built up during the dehydration.

At all three temperature and RH levels, it can be observed that the inactivation rate of both bacteria leveled off sharply with the increasing exposure time. Close to 40% of the total *Salmonella* reduction was achieved within the first 2 min of exposure time. Less than 1 log reduction of *Salmonella* was achieved in the last 120 min of exposure time. The reduced inactivation rate for both bacteria indicates a tailing effect meaning that a portion of the bacterial population could gain resistance to EtO toxicity. The similar tailing effect

was also observed in EtO inactivation of *Bacillus subtilis* spores (Gilbert et al., 1964).

6.4.2 Weibull Model Fitting

The survival of *Salmonella* and *E. faecium* on whole black peppercorns after EtO fumigation at different temperatures and RH conditions were plotted and the resulting curve fit using the Weibull model was determined (Table 6.1). In general, the R^2 was found to be higher than 0.70 with the RMSE < 0.20 log CFU/g at all conditions. At all conditions, the β values of Weibull models were found to be < 1, which indicated that the inactivation curves have concave upward trends. Thus, the developed Weibull model was able to describe the leveling off of the inactivation rate during EtO fumigation.

Weibull model has also been used to describe the inactivation model for several other gaseous decontamination technologies. For example, Mahmoud et al., (2008) reported that the Weibull model was a good fit for modeling the inactivation of *E. coli* O157: H7, *L. monocytogenes* and *S.* Poona on the whole cantaloupe by chlorine dioxide gas. Non-linear survivor curves of inactivation of *L. monocytogenes* within a biofilm matrix by gaseous chlorine dioxide were fitted using the Weibull model (Vaid et al., 2010). Weibull model has also been used to describe the inactivation curve of *E. coli* O157: H7, *S.* Typhimurium, and *L. monocytogenes* in apple juice with gaseous ozone treatment (Choi et al., 2012).

The Weibull models of the bacterial inactivation for different gaseous technologies have all been shown to present concave upward trends, which usually suggest that the remaining bacterial population can adapt to applied stress (Mahmoud et al., 2008). One possible reason to explain the tailing effect found in this study is that weaker or more EtO sensitive bacteria are first destroyed at a rapid death rate while leaving behind survivors of

stronger or higher resistance to EtO. Another reason to explain this tailing effect could be that the bacterial cells are first surrounded by EtO molecules with high kinetic energy to trigger the inactivation, then the kinetic energy of molecules decreases with the exposure time, which results in the slower inactivation rate (Mahmoud et al., 2008). As the inactivation rate decreases with increasing exposure time, a more severe treatment (higher temperature, EtO gas concentration and RH level) is recommended, which could provide a sufficient inactivation of *Salmonella* with shorter exposure time.

6.4.3 Surrogate Evaluation

According to the FDA (2000), a proper surrogate for process validation should be a non-pathogenic bacterium that behaves similarly or is more resistant to the applied process compared to the pathogen of concern. *E. faecium* has been found to be suitable surrogate for *Salmonella* in many low moisture foods during the thermal processing (Newkirk et al., 2018; Villa-Rojas et al., 2017; Wei et al., 2020). In addition, *E. faecium* was found to have comparable reductions to a cocktail of multiple *Salmonella* spp. inoculated on cashews and macadamia nuts treated with the antimicrobial gas propylene oxide (Saunders et al., 2018).

The comparison of reductions of *Salmonella* and *E. faecium* during EtO fumigation of whole black peppercorn is shown in Figure 6.3. In general, the better survival of *E. faecium* was observed when compared to *Salmonella*, which indicates that *E. faecium* has a higher resistance to EtO fumigation than *Salmonella*. Also, the inactivation curves of *E. faecium* are similar to that of *Salmonella* such as tailing effect. Therefore, *E. faecium* may be acceptable as a surrogate for *Salmonella* for EtO fumigation on whole black peppercorns at the tested temperatures, RH levels and EtO concentration.

6.4.4 Response Surface Modeling

As both temperature and RH were found to be critical process parameters for EtO fumigation of black peppercorn, the response surface model was used to fit the bacterial inactivation at different temperature and RH levels to evaluate the effect of temperature and RH on EtO fumigation simultaneously. The estimated response surface model parameters for prediction of the reductions of both *Salmonella* and *E. faecium* during EtO fumigation have been summarized in Table 6.2. In the response surface models, temperature, RH, and exposure time have shown significant linear effects ($P < 0.05$) on EtO fumigation of black peppercorn. The response surface model has been widely used as a tool to predict bacterial inactivation during complicated treatment conditions. López-Romero et al. (2018) evaluated the effects of gallic acid, eugenol, and temperature on inactivation of *Salmonella* in ground chicken; Verma et al. (2018a) evaluated the effect of moisture, fat content, temperature and screw speed (residence time) on in inactivation of *Salmonella* in oat flour; and Huang et al. (2009) evaluated the effect of surfactin and iturin on sterilization of *Salmonella* Enteritidis in meat.

The comparison of experimental and response surface model predicted reduction of *Salmonella and E. faecium* was shown in Figure 6.4. The response surface models provide favorable predictions for both *Salmonella* and *E. faecium* with an RMSE of 0.48 and 0.45 log CFU/g, respectively. From the predicted reduction curves, it can also be observed that larger inactivation is achieved at higher temperature and RH levels. As shown in Figure 6.3, a high RH level is necessary to achieve more than 5 log reduction of *Salmonella*. For example, at the high treatment temperature (60°C), less than 4 log reduction of *Salmonella* was achieved after exposure to EtO for 180 min at 30% RH. While

more than 5 log reduction of *Salmonella* was achieved at low treatment temperature (46°C) with 20 min exposure time at 50% RH. However, the treatment temperature still plays an important role at the medium RH level (40%), as only 4.02 and 4.75 log reductions of *Salmonella* were achieved at 46 and 53°C with 180 min of exposure time, respectively, while 5.39 log reductions of *Salmonella* were achieved at 60°C after exposing to EtO for only 60 min.

The contour plots in Figure 6.5 give the prediction of reductions of *Salmonella* and *E. faecium* as a function of time and temperature at different RH levels. At 30% RH, because of the leveling off of the inactivation rate after 120 min of exposure time, the models do not perform well and show decreasing in reduction at a longer exposure time. At the higher RH levels (40 and 50%), the models can predict the reductions for both *Salmonella* and *E. faecium* well. From the contour plots, a synergistic effect was observed between temperature and RH. To achieve the same reduction, increasing treatment temperature would require a lower RH level and less exposure time, or lower temperature and less exposure time are needed for a higher RH level.

According to the FDA (2020), a proper pasteurization process should provide 5 log reduction of the pathogen of concern. Based on the contour plots, the pink or red zone indicated a more than 5 log reduction of *Salmonella* during EtO fumigation. A more than 5 log reduction of *Salmonella* in black peppercorn could be achieved with at least 40% RH or higher, while less than 4.5 log reduction of *Salmonella* was achieved at 30% RH even at the maximum exposure time (180 min) used in this study. At 40% RH, a minimum treatment temperature of 56°C is required to achieve more than 5 reduction of *Salmonella* with 180 min of exposure time. Considerably shorter exposure time is required at 50% RH,

as more than 5 log reduction of *Salmonella* can be achieved with only 20 min of exposure time at 58°C. The response surface models developed in this study will help the food industry understand the interactive effect of temperature and RH on the EtO fumigation of *Salmonella* and *E. faecium* in black peppercorn and identify the proper temperature, RH and exposure time combinations for the standardization of EtO fumigation as a pasteurization process to ensure food safety.

6.5 Conclusions

Weibull models were developed to describe the bacterial inactivation kinetics of EtO fumigation at different treatment conditions. A tailing effect was observed on the inactivation curves. *E. faecium* was found to be a suitable surrogate for *Salmonella* during EtO fumigation of black peppercorn. Response surface models were developed to predict bacterial reduction based on temperature, RH, and exposure time. Temperature and RH levels have been found to significantly ($P < 0.05$) affect EtO fumigation of black peppercorn. At the highest RH (50%) and temperature (60°C) used in this study, more than 5 log reductions of *Salmonella* can be obtained within 20 min of exposure time. Processors may be able to use these models for identifying the relative humidity and temperatures that may improve inactivation during EtO processing, or predicting reductions from similar EtO processes, but results should not be extrapolated to other types of spices or other types of thermal processing, as it may result in an unsafe product. In-plant validations with an appropriate surrogate to confirm model predictions are highly recommended.

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6.7 References

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Table 6.1. Model parameters of Weibull models for inactivation of *Salmonella* or *E. faecium* by ethylene oxide fumigation at gas concentration of 735.3 mg/L at different temperature and relative humidity levels.

Bacteria	Temperature (°C)	RH	δ (min)	β	R^2	RMSE (log CFU/g)	AIC_C
Salmonella	46	30	1.0	0.2	0.92	0.1	-244
		40	0.002	0.1	0.86	0.1	-217
		50	0.04	0.2	0.76	0.2	-172
	53	30	0.05	0.2	0.90	0.1	-238
		40	0.001	0.1	0.89	0.1	-211
		50	0.08	0.3	0.87	0.2	-190
	60	30	0.001	0.1	0.82	0.1	-222
		40	0.01	0.2	0.84	0.2	-170
		50	0.0001	0.2	0.88	0.1	-327
E. faecium	46	30	0.07	0.1	0.75	0.1	-289
		40	0.0001	0.1	0.78	0.1	-278
		50	0.03	0.2	0.77	0.1	-210
	53	30	0.1	0.1	0.81	0.1	-268
		40	0.4	0.2	0.73	0.2	-169
		50	0.2	0.3	0.87	0.2	-200
	60	30	1.7	0.2	0.88	0.1	-223
		40	0.1	0.2	0.81	0.2	-192
		50	0.0001	0.1	0.74	0.1	-328

Table 6.2. Model parameters (standard error) of response surface models for inactivation of *Salmonella* or *E. faecium* in whole black peppercorn by ethylene oxide fumigation at gas concentration of 735.3 mg/L.

Regression coefficients	*Salmonella*	*E. faecium*
β_0	22 (7.1)	52 (6.0)
β_1	-0.72 (0.222	-1.3 (0.19)
β_2	-0.23 (0.13)	-0.76 (0.11)
β_3	0.0050 (0.013)	-0.036 (0.011)
β_{12}	0.0028 (0.0012)	0.0086 (0.0010)
β_{13}	0.00016 (0.000017)	0.00061 (0.00014)
β_{23}	0.00040 (0.00018)	0.00063 (0.00015)
β_{11}	0.0065 (0.0019)	0.011 (0.0017)
β_{22}	0.0024 (0.0012)	0.0051 (0.0010)
β_{33}	-0.000091 (0.000017)	-0.000054 (0.000014)
Adjusted R^2	0.76	0.80
RMSE (log CFU/g)	0.5	0.5
AIC_C	196	153

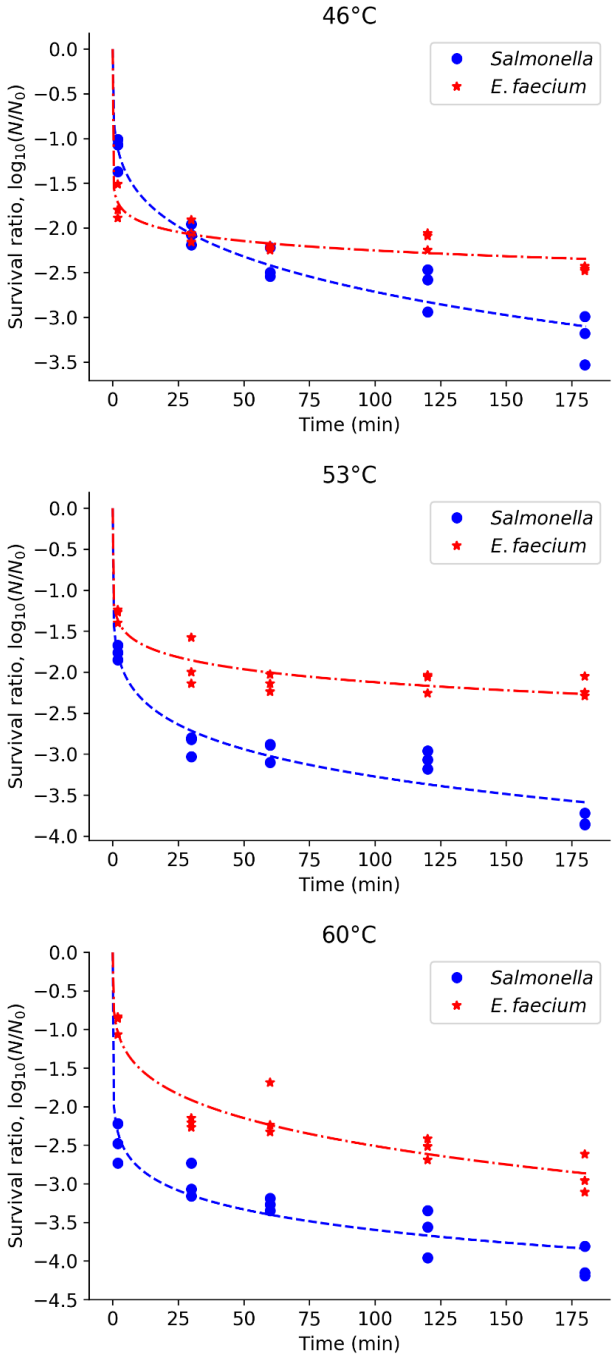

Figure 6.1. Experimental and Weibull model predicted reduction of *Salmonella* (●, dashed lines) and *E. faecium* (★, dash-dot line) in whole black peppercorn treated by ethylene oxide fumigation (735.3 mg/L) at 30% RH.

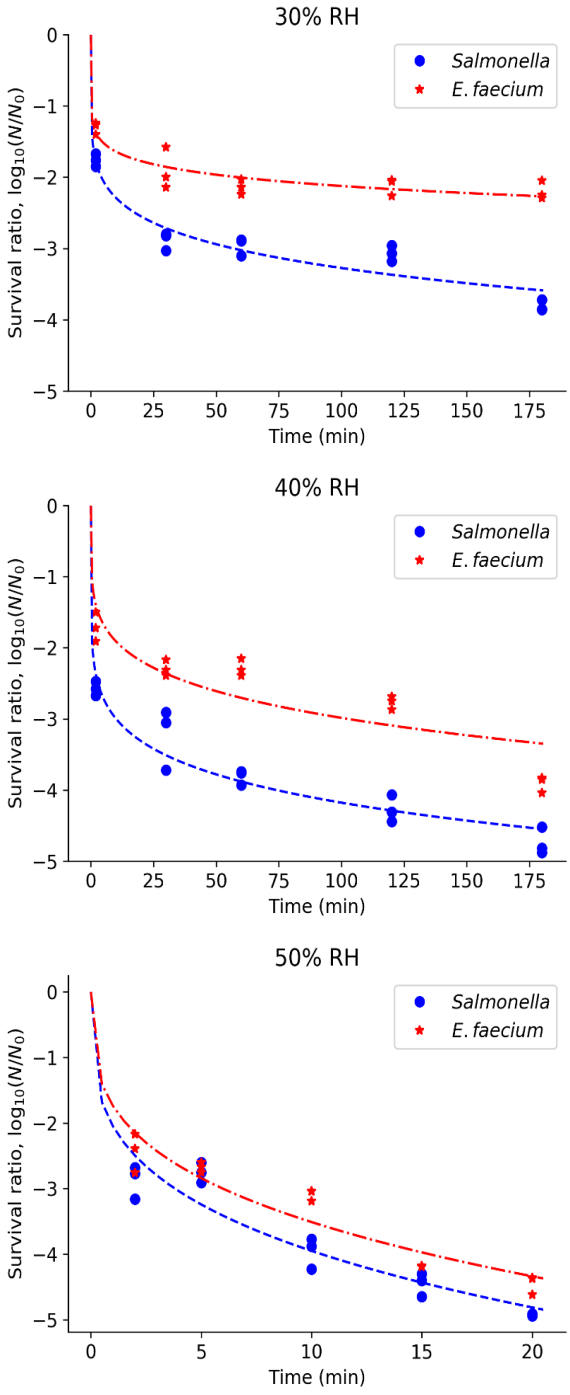

Figure 6.2. Experimental and Weibull model predicted reduction of *Salmonella* (●, dashed lines) *and E. faecium* (★, dash-dot line) in whole black peppercorn treated by ethylene oxide fumigation (735.3 mg/L) at 53°C.

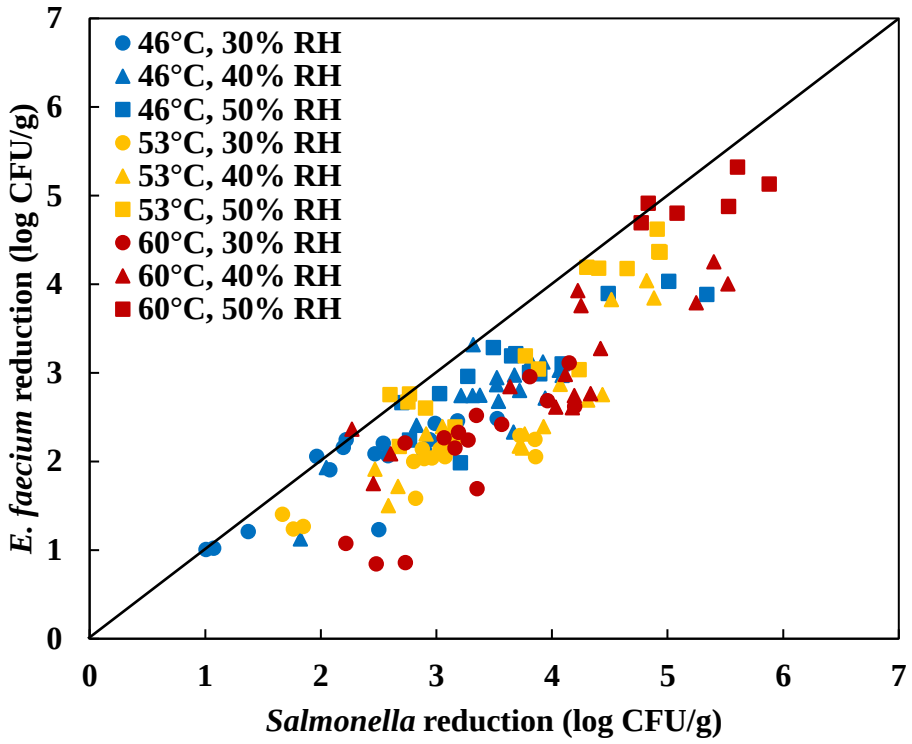

Figure 6.3. Comparison of reductions of *Salmonella* and *E. faecium* during ethylene oxide fumigation (735.3 mg/L) of whole black peppercorn. Each point represents an individual ethylene fumigation oxide run.

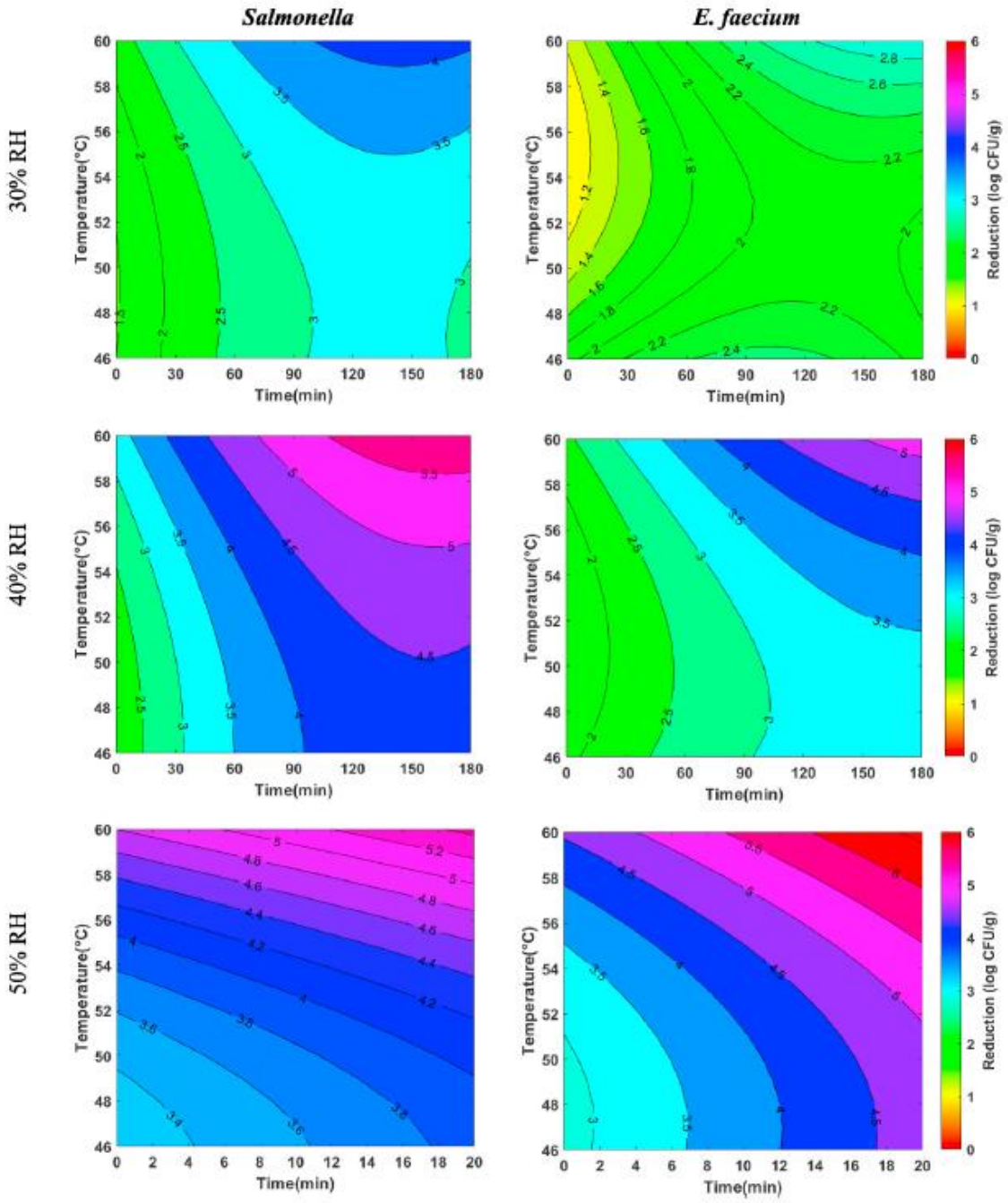

Figure 6.4. Experimental and response surface model predicted reduction of *Salmonella* and *E. faecium* in whole black peppercorn treated by ethylene oxide fumigation (735.3 mg/L) at different temperature and RH levels.

Chapter 7

GASEOUS CHLORINE DIOXIDE TECHNOLOGY FOR IMPROVING THE MICROBIAL SAFETY OF SPICES

7.1 Abstract

The consistent occurrence of *Salmonella* in spices has become a concern to the food industry. The thermal technology such as steam pasteurization is commonly used to pasteurize spices; however loss of volatiles are reported. The objective of this study is to evaluate gaseous chlorine dioxide (ClO_2) as a non-thermal antimicrobial technology for inactivating pathogens in spices with minimal quality deterioration. In this study, black peppercorn and cumin seed samples were inoculated with a five-serotype cocktail of *Salmonella* or *Enterococcus faecium* NRRL B-2354. The inoculated samples were treated by ClO_2 gas at different gas concentrations (5, 10, and 15 mg/L) and relative humidities (60, 70, and 80%) at room temperature (25°C) at five gas exposure times (60, 120, 180, 240, 300 min). More than 5 log reduction of *Salmonella* was achieved in both spices with 300 min of ClO_2 gas treatment at 15 mg/L and 80% RH. The corresponding values for *E. faecium* were 4.36 and 4.17 log CFU/g in black peppercorn and cumin seed, respectively. The ClO_2 gas inactivation kinetics were determined at each gas concentration and RH using the log-linear model. Modified Bigelow models were developed to evaluate the effect of gas concentration and RH on the inactivation kinetics of *Salmonella* during ClO_2 gas treatment. Additionally, the reduction of *Salmonella* was evaluated as a function of gas concentration, RH, and exposure time using the response surface model. Gas concentration, RH, and exposure time showed significantly positive linear effects on *Salmonella*

inactivation during ClO_2 gas treatment. *E. faecium* was found to be a suitable surrogate for *Salmonella* for this non-thermal gaseous treatment and can be used for future process validation. This study provides guidelines on the implementation of gaseous ClO_2 for enhancing microbial safety of spices.

Keyword: *Salmonella*, *E. faecium*, surrogate, black peppercorn, cumin seed, non-thermal lethality

7.2 Introduction

Spices are aromatic vegetable products that have been widely used as food flavorings or seasonings for more than two thousand years (Embuscado, 2015). Spices such as black pepper and cumin are usually grown in a tropical and humid environment, where they are often contaminated with high levels of microorganisms, including *Salmonella*, *Escherichia coli*, *Bacillus*, and *Staphylococcus* (Gryczka et al., 2020; Pafumi, 1986). Over the past decade, several foodborne outbreaks have been associated with the consumption of contaminated spices, such as curry powder (FDA, 2013), spice blend (FDA, 2013), white pepper (FDA, 2013), cumin (Ozturk et al., 2018), and garlic powder (Banerjee & Sarkar, 2003). According to Vij et al. (2006), 95% of the U.S. food recalls associated with spices have been caused by *Salmonella* contamination. In 2010, salami products made with *Salmonella* contaminated black and red pepper had caused a foodborne outbreak which led to 272 laboratory-confirmed cases of salmonellosis in 44 states (CDC, 2010). Several recent studies have shown that *Salmonella* can survive in spices for extended storage time and has high thermal resistance (Chen et al., 2019; Keller et al., 2013; Verma, Chaves, Howell, et al., 2021; Wei, Vasquez, et al., 2021).

In 2013, a risk profile on pathogens and filth in spices was issued by FDA (2013)

and called for the identification of process control to reduce the public health risk posed by the consumption of contaminated spices. Therefore, it is necessary to evaluate an adequate antimicrobial technology for improving the microbial safety of spices. Currently, there are several pasteurization technologies, such as ethylene oxide fumigation, steam treatment, and irradiation that have been commercially used for the decontamination of spices (Chen et al., 2021; Farkas & Andrassy, 1988; Heider et al., 2002; Schneider, 1993; Song et al., 2014; Wei et al., 2021). Although these interventions can effectively reduce the microbial load in spices, there are some disadvantages associated with them. For instance, previous studies reported quality deteriorations such as loss of color and flavor in steam-treated or irradiation treated spices (Abdel-Khalek, 2008; Schweiggert et al., 2007; Toofanian, 1986; Vajdi & Pereira, 1973; Waje et al., 2008). Ethylene oxide is a cancerogenic gas that has been banned in the European Union (Bononi et al., 2014). Ethylene oxide can also rapidly react with chloride in spice products to yield the more stable carcinogenic byproduct like ethylene chlorohydrin (Fowles et al., 2001).

Chlorine dioxide (ClO_2) is a strong oxidizing agent, which has a broad and high biocidal activity (Benarde et al., 1965; Trinetta et al., 2011). Chlorine dioxide has been used as an antimicrobial intervention in the beverage industry for improving the microbial safety of drinking water since the mid-1990s (Benarde et al., 1965; Golden et al., 2019). ClO_2 is generally recognized as safe (GRAS) and has been approved for using as an additive in fruits and vegetables to enhance their microbial safety (FDA, 2017; Rane et al., 2020). Gaseous ClO_2 has been shown to inactivate effectively pathogens in fresh produces, such as lettuce, cantaloupe, blueberries, strawberries, and raspberries (Bridges & Wu, 2018; Chai et al., 2020; Mahmoud et al., 2008; Mahmoud & Linton, 2008; Pao et al., 2007; Sy et

al., 2005; V. Trinetta et al., 2010). The use of gaseous ClO_2 for pasteurization of low moisture foods has not been well studied and the relevant research is limited. Thus, this study aimed to evaluate the efficacy of gaseous ClO_2 for the pasteurization of spices.

Previous studies used the sachet method by mixing two dry precursors (sodium chlorite and an activating acid) to generate gaseous ClO_2 for inactivation of *Salmonella* on almonds and black peppercorn; thus the gas concentration was not stable and experienced a dynamic change during the treatment (Rane et al., 2020; Wang et al., 2019). Also, the relative humidity (RH) of the environment during ClO_2 treatment was not controlled or evaluated even though others have found it to be a critical for microbial inactivation (Park et al., 2018; Wei et al., 2021), was not well controlled and evaluated. Therefore, a systematic study of gaseous ClO_2 for inactivation of *Salmonella* in spices at different gas concentrations and RH is necessary to guide the spice industry on effectively applying the gaseous ClO_2 technology.

Food producers are required by the Food Safety Modernization Act (FDA, 2013) to show that the established preventive controls could ensure food safety by validating their "kill step" process. The U.S. FDA (2013) allows and recommends for the use of a suitable surrogate to replace the target food pathogen in process validation. *Enterococcus faecium* NRRL B-2354 has been found to be a suitable for *Salmonella* during thermal processing of low moisture foods, such as radio frequency heating of black pepper (Wei et al., 2019), cumin seeds (Chen et al., 2020), basil leaves (Verma, et al., 2021) and extrusion of oat flour (Bianchini et al., 2014; Verma et al., 2018). However, the suitability of *E. faecium* as a surrogate for *Salmonella* during ClO_2 treatment has not been well investigated.

The objectives of this study were to investigate 1) the effects of gas concentration,

RH, exposure time on inactivation of *Salmonella* in spices during gaseous ClO_2 treatment and 2) evaluate the suitability of *E. faecium* as a surrogate for *Salmonella* during antimicrobial gaseous treatment.

7.3 Materials and Methods

7.3.1 Spice Samples Procurement and Storage

Three production batches of commercially steam-sterilized black peppercorn and cumin seed samples were supplied by McCormick & Company, Inc (Sparks, MD, USA). The spice samples were packed in sterile plastic bags and stored in a walk-in cooler at -12°C for long term storage. Also upon receipt, the water activity (a_w) at room temperature (25 °C) and aerobic plate count of the samples were tested using a dewpoint a_w meter (Model 4TE, Meter Group, Pullman, WA) using a dewpoint water activity meter (Model 4TE, Meter Group, Pullman, WA) and enumeration procedures described in Wei et al. (2019), respectively. Briefly, three 10-g subsamples were randomly taken from each sample, diluted with 90 mL of 0.1% buffered peptone water (BPW, Becton, Dickinson and Company, Sparks, MD), and plated onto tryptic soy agar supplemented with 0.6% (w/w) yeast extract (TSAYE, Becton, Dickinson and Company) with incubation at 37°C for 24±2 h.

7.3.2 Bacterial Strains and Inoculum Preparation

Five serotypes of *Salmonella enterica* cultures were obtained from the FDA ORA Arkansas Regional Lab (Jefferson, AR) and the FDA Culture Collection (Bedford Park, IL) and stored in 40% (v/v) glycerol at -80°C. Selected serotypes of *S. enterica* used in this study were isolated from low moisture food-assoiated outbreaks: *S.* Agona 447967, isolated from roasted oat cereal-associated outbreak (CDC, 1998); *S.* Montevideo 488275, isolated

from black and red pepper-associated outbreak (CDC, 2010); *S.* Mbandaka 698538, isolated from sprout-associated outbreak (CDC, 2016); *S.* Tennessee K4643, isolated from peanut butter-associated outbreak (CDC, 2012); and *S.* Reading Moff 180418, isolated from cumin seed-associated outbreak (FDA, 2013). A non-pathogenic bacterium, *Enterococcus faecium* NRRL B-2354, was used in this study.

A frozen vial (1 mL) of each bacterial strain (*Salmonella* or *E. faecium*) was thawed at 37°C for 5 min, aseptically transferred to 10 mL of tryptic soy broth supplemented with 0.6% (w/w) yeast extract (TSBYE; Becton, Dickinson and Company), and incubated at 37°C for 24±2 h. A loopful (~10 μL) of the incubated bacterial culture was streaked onto TSAYE agar plates with incubation at 37°C for 24±2 h to obtain isolated colonies. The incubated plate (working plate) was wrapped with parafilm wrapping film (PM-999; Bemis, MO) and stored in a refrigerator at 4°C for up to 30 d.

The agar-lawn method was used to prepare the inoculum, which has been shown to yield a stable bacterial population in low moisture foods (Hildebrandt et al., 2016). For each strain of *Salmonella* or *E. faecium*, a sterile loop was used to transfer one isolated colony from each working plate to 10 mL of TSBYE tube and incubated at 37°C for 24±2 h. The incubated bacterial broth (0.1 mL) was then spread plated onto TSAYE plate and incubated at 37°C for 24±2 h. Afterwards, 3 mL of 0.1% BPW was dispensed onto the plate to help agitate the bacterial cells on the agar-lawn using a sterile L-shaped spreader. A five-serotype *Salmonella* cocktail (10 mL) was prepared by transferring 2 mL of harvested cells of each strain and mixing equally mixing 2 mL of different serotypes of *Salmonella* lawns in a sterile 15 mL centrifuge tube. The collected *E. faeicum* lawns was used as *E. faecium* inoculum.

7.3.3 Sample Inoculation and Equilibration

The same procedures for inoculating black peppercorn and cumin seeds described by Wei et al. (2019) and Chen et al. (2019), respectively, were followed in this study. These procedures have been shown to provide stable and homogeneous *Salmonella* and *E. faecium* populations. Black peppercorn or cumin (150 ± 0.1 g) were weighed and transferred into a sterile whirl-pak bag, sprayed with 3 mL of *Salmonella* cocktail or *E. faecium* inoculum, sealed, and hand messaged for 5 min to homogenize the inoculum. Subsequently, the inoculated samples were transferred to sanitized aluminum trays (230 x 300 x 15 mm) and placed into a custom environmental RH chamber (Lau & Subbiah, 2020) to restore the inoculated spices to its original a_w within 24 h. The inoculated sample was acclimated for five days post inoculation, which allowed the bacteria to adapt to the low moisture environment of the spices prior to ClO_2 gas treatment.

7.3.4 ClO_2 Gas Production and Treatment

The setup of ClO_2 gas treatment chamber is shown in Figure 7.1. A ClO_2 gas generator (Model Minidox-M, ClorDiSys Solutions, Inc., Lebanon, NJ) was used to produce ClO_2 gas by passing 2% chlorine gas and 98% nitrogen gas (Matheson Tri-Gas, Irving, TX) through three sodium chlorite cartridges in series (Clordisys Solutions, Inc). The generated ClO_2 gas was injected into a custom design treatment chamber (735 x 443 x 685 mm^3) at 20 L/min. The ClO_2 gas concentration was monitored and controlled by an internal photometer (Model CDT-1, Clordisys Solutions, Inc) and a programmable logic controller (Model DL-06, Automation Direct, Cumming, GA), respectively. An RH and temperature sensor (Model 6621, Testo, Titisee-Neustadt, Germany) was used to measure the environmental RH and temperature in the treatment chamber. Based on the sensor

readings, RH in the treatment chamber was controlled by automactially turning ON a relay switch circuit (Clordisys Solutions, Inc) to activate an ultrasonic cool water mist humidifier (EE-5301, Crane, Itasca, IL) until the target RH was reached. Two electrical fans were placed inside the treatment chamber to circulate ClO_2 gas and water mist.

In this study, the bacterial inactivation was evaluated at three different gas concentrations (5, 10, and 15 mg/L), three RH levels (60, 70, and 80%), and five gas exposure or treatment times (60, 120, 180, 240, and 300 min) at room temperature (25°C). For each treatment combination, an inoculated sample (3 g) was placed on a petri dish and placed in the treatment chamber. Chlorine dioxide gas was generated and injected into the chamber simultaneously as the chamber was being humidified until the target gas concentration was reached. The sample was exposed to the gas and constant RH for a predetermined exposure time. Afterwards, the ClO_2 gas was exhausted and scrubbed (Clordisys Solutions, Inc). Residual ClO_2 gas was monitored using a handheld gas detector (Model D16 Portasens III; Analytical Technology, Inc.) to ensure complete gas removal prior to opening the chamber to retrieve the treated sample. All ClO_2 gas treatments were performed in triplicate.

7.3.5 Bacterial Enumeration

The bacterial enumeration of treated sample was conducted within 30 min after ClO_2 gas treatment. The ClO_2 gas treated sample (3 g) was first transferred to a sterile filter bag and diluted with 27 mL of neutralizing buffer (NB, 42.5 mg of monopotassium phosphate, and 0.16 g of sodium thiosulfate per 1 L deionized water) to quench the remaining ClO_2 residues in the sample (Park et al., 2018). The diluted sample was homogenized in a paddle mixer (9000471, Neutec Group Inc, Farmingdale, NY) for 1 min,

serially diluted in NB, spread plated onto TSAYE supplemented with 0.05% (w/v) ammonium iron citrate (Sigma-Aldrich, Co., MO, USA) and 0.03% (w/v) sodium thiosulfate (Fisher Chemical, NJ, USA) for *Salmonella* inoculated samples or 0.025% (w/v) esculin hydrate (Acros Organics, NJ, USA) for *E. faecium* inoculated samples and incubated at 37°C for 24±2 h. The grown yellow colonies with a black center were counted as *Salmonella* survivors and black colonies were counted as *E. faecium*.

7.3.6 Gaseous ClO_2 Microbial Inactivation Kinetics

At each gas concentration and RH combination, the log-linear model (1) was fit the bacterial survival data:

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

where N (CFU/g) is the number of survivors at time t, N_0 (CFU/g) is the initial number of survivors at time 0, t (min) is the ClO_2 gas treatment time, D (min) is the time required to achieve a 10-fold reduction of the bacteria at specific gas concentration and RH.

To evaluate the effect of gas concentration and RH on the D-values of *Salmonella* in black peppercorn and cumin seed, a modified version of a Bigelow-type model (2) was used (Gaillard et al., 1998):

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

where D_{ref} is the time (min) needed to achieve a ten-fold reduction in the population at C_{ref} and RH_{ref}, C is the gas concentration (mg/L), C_{ref} and RH_{ref} are the optimized gas concentration and treatment RH (Dolan et al., 2013), z_C or z_{RH} is the gas concentration or treatment RH increment needed to decrease the D-values by 10-fold. All parameters for the modified Bigelow-type model were estimated using OLS minimization with nlinfit in

MATLAB 2018 (MathWorks Inc, MA).

The effects of three factors (gas concentration, RH, and exposure time) on the inactivation of *Salmonella* in black peppercorn and cumin seed were evaluated using the second-order response surface model (3) as below:

Response surface model: $\log_{10}\left(\frac{N}{N_0}\right) = \beta_0 + \beta_1 * C + \beta_2 * RH + \beta_3 * t + \beta_{12} * C * RH + \beta_{13} * C * t + \beta_{23} * RH * t + \beta_{11} * C^2 + \beta_{22} * RH^2 + \beta_{33} * t^2$ (3)

where N and N_0 (CFU/g) are the *Salmonella* or *E. faecium* survivors at exposure time t and 0, β_i are constant regression coefficients, and t is the gas exposure time (min). The parameters of this model were estimated using response surface method functions in the open-source statistical software, R (Lenth, 2009).

7.3.7 Scanning Electron Microscopy (SEM) Analysis

A modified method described in Wang et al. (2020) was followed in this study. The inoculated black peppercorn samples were first fixed with 2.5% glutaraldehyde for 2 h at room temperature and then processed for dehydration through an ethanol series (30 to 100%). Then, samples were mounted onto the aluminum specimen stubs, placed in a 42°C vacuum oven overnight, and sputter-coated with a thin layer of chromium with a sputter coater (Desk V, Denton Vacuum, Moorestown, NJ). A field-emission scanning electron microscope (S-4700, Hitachi, Tokyo, Japan) was used to collect the image of coated samples at magnification ranging from 500 to 20000 X and at voltage of 5 kV.

7.4 Results and Discussion

7.4.1 Bacterial Inactivation of Gaseous ClO_2

The survival of inoculated *Salmonella* and *E. faecium* in black peppercorn and cumin seed during gaseous ClO_2 treatment at gas concentration of 5, 10, and 15 mg/L and

RH of 60, 70, and 80% for 0-300 min is shown in Figures 7.2 and 7.3. As expected, the bacterial survival decreased with increasing ClO_2 exposure time and RH. At 5mg/L of ClO_2, 1.5, 2.5, and 3.4 log CFU/g reductions of *Salmonella* in black peppercorn were achieved at 60, 70, and 80% RH, respectively, at 300 min of exposure time. The corresponding values for cumin seed were 1.7, 2.1, and 3.3 log CFU/g. With the increased gas concentration, a higher *Salmonella* inactivation was observed at the same RH level. For example, with an exposure time of 300 min and 80% RH, 3.4, 4.7, and 5.4 log CFU/g reductions of *Salmonella* were achieved at gas concentration of 5, 10, and 15 mg/L in black peppercorn. The corresponding values for cumin seed were 3.3, 4.5, and 5.4 log CFU/g. In this study, more than 5 log reduction of *Salmonella* was only observed at the most extreme condition (15 mg/L, 80% RH, and 300 min of exposure time) for both spices. Rane et al. (2020) reported that 3.7 log CFU/g reduction of *Salmonella* was achieved in black peppercorn after being exposed to ClO_2 treatment (0.4 mg ClO_2/g black peppercorn) at 80% RH for 240 min, while only 2.3 log CFU/g reduction of *Salmonella* was achieved at 45% RH. Similarly, a 2.2 log CFU/g reduction of *Salmonella* was achieved after inoculated black peppercorn exposed to ClO_2 treatment (0.4 mg ClO_2/g black peppercorn) for one day (Golden et al., 2019). However, the major drawback in those two studies was that the gas concentration was not constant (the highest gaseous ClO_2 concentration during the exposure was less 1.5 mg/L) during the treatment and the RH was not controlled. Thus, a consistent high gas concentration (10-15 mg/L), high RH, and long exposure time would be necessary for an effective ClO_2 treatment to achieve more than 5 log reduction of *Salmonella* in spices.

Several studies have evaluated the efficacy of gaseous ClO_2 for pasteurization of

the fresh produce and fruit. Compared to low moisture foods, it usually requires a shorter treatment time to achieve the desired inactivation of target pathogens in high moisture foods. For instance, more than 5 log reduction of *Salmonella* was achieved with 5 mg/L ClO_2 gas in 19 min for lettuce (Mahmoud & Linton, 2008) and with 0.5 mg/L ClO_2 gas for 10 min for tomatoes (Bhagat, 2010). Mahmoud et al. (2007) also reported that strawberries treated with ClO_2 gas (5 mg/L) for 13.5 min could achieve pasteurization (5 log reduction of *Salmonella, E.coli* O157:H7, and *L. monocytogenes*). The use of high RH (90-95%, temperature no reported) during the treatment could be potentially responsible for the high efficacy of ClO_2 gas for pasteurization of high moisture foods (Valentina Trinetta et al., 2011).

Log-linear models and the estimated *D*-values for *Salmonella* in the two spices are shown in Table 7.1. The *D*-values of both spices decreased with increasing gas concentration and RH, which indicated that higher efficacy of bacterial inactivation was obtained at higher gas concentration and RH. The microbial inactivation kinetics have not been systematically investigated in low moisture foods, most studies only reported the bacterial reduction at selected treatment conditions (Golden et al., 2019; Rane et al., 2020, 2021). However, several high moisture foods related studies have determined the microbial inactivation kinetics of ClO_2 gas and estimated the *D*-values at different treatment conditions. The *D*-values of *Salmonella* were determined to be 2.7 and 3.8 min on lettuce (Mahmoud et al., 2008) and strawberries (Mahmoud et al., 2007) at 5 mg/L ClO_2 gas. In this study, the *D*-values of *Salmonella* were found to be 103.4 and 94.4 min in black peppercorn and cumin seed. From the *D*-values of *Salmonella*, it can also be observed that ClO_2 gas was more effective for bacterial inactivation on high moisture foods than low

moisture foods tested in this study.

The determination of microbial inactivation kinetics and *D*-values are usually conducted for estimation of thermal processing conditions (temperature and treatment time) but are less commonly investigated for gaseous treatment. The *D*-value can be a useful tool to estimate bacterial reduction at selected treatment conditions and identify adequate process conditions for pasteurization. To better understand the effect of gaseous treatment conditions on the *D*-value, a modified version of a Bigelow-type model was employed, and the estimated parameters are shown in Table 7.2. Models and kinetic parameters can be used for the development of the food pasteurization process to improve safety and to understand the mechanism of microbial inactivation (Parish, 2006). The corresponding *D*-values at different treatment conditions (gas concentration and RH) estimated by the model for both spices were shown in Figure 7.4. The generated contour plots show all estimated *D*-values within the range of tested conditions (5-15 mg/L gas concentration and 60-80% RH), which can be useful for interpolation of *D*-values at within the range of treatment conditions tested in this study. The modified Bigelow-type model has been shown to provide a better prediction for thermal inactivation of *Salmonella* compared response surface model in wheat flour (Smith et al., 2016), milk powder (Wei et al., 2020), basil leaves (Verma, Chaves, Howell, et al., 2021), and *L. monocytogenes* in potato slices (Valdramidis et al., 2006). Besides, unlike the response surface model, the parameters of the modified Bigelow model contain the *D* and *z*-values estimated from the bacterial survival, which provides a degree of phenomenological meaning (Smith et al., 2016).

7.4.2 Effects of Gas Concentration and RH

To evaluate the effect of multiple treatment conditions on bacterial inactivation, the

response surface model has been widely implemented. For instance, Verma, et al. (2018) evaluate the effects of moisture, fat content, temperature, and screw speed on *Salmonella* inactivation in oat flour during extrusion; López-Romero et al. (2018) evaluate the effects of gallic acid, eugenol, and temperature on *Salmonella* inactivation in ground chicken; Chen et al. (2021) evaluate the effects of temperature and RH on *Salmonella* inactivation during ethylene fumigation of cumin seed. The gas concentration, RH, and exposure time have been shown to be critical process parameters for ClO_2 gas treatment of spices. Thus, the response surface model was used to fit the bacterial inactivation and evaluate the effects of gas concentration, RH, and exposure time on the bacterial inactivation during ClO_2 gas treatment of spices. The estimated parameters of response surface models for both spices are shown in Table 7.3. The gas concentration, RH, and exposure time have shown significant positive linear effects ($P < 0.05$), which was in agreement with the increased bacterial inactivation at higher gas concentration, RH, and longer exposure time. Several studies have shown that the RH has a significant effect on the efficacy of ClO_2 gas treatment and higher RH increases the effectiveness of bacterial inactivation (Park et al., 2018; Rane et al., 2020; Wang et al., 2019). Under high RH level, bacteria could become more sensitive to ClO_2 gas due to the hydration and accumulation of moisture on their surface (Wang et al., 2019). Also, the moisture coated on the surface of microorganisms could absorb ClO_2 gas and increase the efficacy of oxidation of ClO_2 (Mahmoud et al., 2008). After exposed to ClO_2 gas at 60, 70 and 80% RH for 300 min, the increased moisture contents (wet basis) were found to be 1.1, 1.7 and 2.6% in black peppercorn and 0.2,0.8 and 1.4% in cumin seed.

The contour plots in Figure 7.5 provide the estimation of *Salmonella* reduction as

functions of gas concentration, RH, and exposure time. It can be observed that at 60% RH, less than 3 log reduction of *Salmonella* was achieved in both spices even at the highest gas concentration and longest exposure time tested in this study. Greater than 5 log reduction was only achieved at a gas concentration of 15 mg/L and 80% RH with 300 min of exposure time. Several studies have reported that ClO_2 gas treatment has low efficacy on microbial inactivation in low moisture foods such as almond (Rane et al., 2020; Wang et al., 2019) and spices (Golden et al., 2019). Although Wang et al. (2019) showed that the combination of ClO_2 gas and mild heat would have an interaction effect and increase the efficacy on microbial inactivation, however, the heat treatment for long time could cause quality deterioration such as loss of volatile compounds in spices. According to the FDA (2013), studies have shown that contamination of *Salmonella* in spices is usually below 3 log MPN/g. As *Salmonella* does not grow in low moisture foods, 3 log reduction may be sufficient. To achieve 3 log reduction, ClO_2 gas treatment at 15 mg/L and 70% RH for 300 min would be sufficient for both spices. Also, a shorter exposure time (120 min) would be required to achieve a similar reduction at 15 mg/L and 80% RH. Based on the contour plots, the proper treatment conditions can be estimated for ClO_2 gas treatment to achieve the desired *Salmonella* reduction. Thus, ClO_2 gas can be an alternative technology for spice processors for improving the microbial while minimizing the quality loss compared to thermal technologies.

7.4.3 SEM Analysis of *Salmonella* in Black Peppercorn

The morphological changes of *Salmonella* on the surface of black peppercorn during ClO_2 gas treatment were evaluated using SEM analysis and are shown in Figure 7.6. Gaseous ClO_2 is highly water-soluble and behaves similarly to aqueous ClO_2 in inactivating

microorganisms (Linton et al., 2006). The inactivation mechanism of aqueous ClO_2 is to first react with bacterial membrane proteins and lipids to increase the permeability of the outer membrane and disrupt protein synthesis (Benarde et al., 1967). The oxidative damage to the outer membrane leads to the destruction of the trans-membrane ionic gradient which could be the primary lethal event at the physiological level (Berg et al., 1986). In Figures 6a and 6b, it can be observed that untreated *Salmonella* cells had smooth cell membranes with high integrity. Also, a few aggregates of cells were wrapped together by an extracellular matrix, and the formation of a rudimentary biofilm can be observed. In Figures 6c and 6d, the SEM images revealed a rougher surface morphology and shrinkage of the cell after ClO_2 treatment when compared to the untreated samples. The loss of membrane integrity and damaged cell surface supports the idea that the inactivation by ClO_2 gas is through the disruption of the cell membrane, which mitigates cell growth.

7.4.4 Surrogate for Process Validation

To evaluate the efficacy of an antimicrobial intervention, the most effective way is to conduct an in-plant validation (Acuff et al., 2020). Usually, the introduction of the human pathogen to the food processing plant for process validation should be avoided and the use of surrogate is recommended by FDA (2013). *E. faecium* NRRL B-2354 have been found to be suitable for *Salmonella* in several thermal processing of low moisture foods (Chen et al., 2019; Liu et al., 2018; Verma, et al., 2021; Verma, et al 2018; Wei, et al., 2021). However, the suitability of *E. faecium* as a surrogate for *Salmonella* during gaseous treatment of low moisture foods has not been well investigated. In this study, *Salmonella* and *E. faecium* were inoculated on spice samples for the same ClO_2 gas, which allowed direct comparison between their responses to the gaseous treatment. The comparisons of

reductions of *Salmonella* and *E. faecium* during ClO_2 gas of both spices are shown in Figure 7.7. In Figure 7.7a, *E. faecium* had less reduction compared to *Salmonella* in black peppercorn at most of treatment conditions. In cumin seed (Figure 7.7b), although *E. faecium* was shown to has higher reduction compared to *Salmonella* in some treatment conditions, the *Salmonella* reduction was higher than *E. faecium* at all treatment conditions that achieved a more than 4 log reduction of *Salmonella*. Therefore, when *E. faecium* had at least 4 log reduction, *Salmonella* had higher than 4 log reduction indicating that *E. faecium* can be used as a suitable surrogate for *Salmonella* for conducting the process validation of ClO_2 gas treatment.

7.4 Conclusions

The non-thermal gaseous ClO_2 treatment has been shown to effectively inactivate *Salmonella* in spices at high gas concentration and RH. More than 5 log reduction of *Salmonella* was achieved on both spices after exposure to ClO_2 gas treatment at 15 mg/L and 80% RH for 300 min. The log-linear model was fitted to the bacterial survival data and to determine microbial inactivation kinetics during the ClO_2 treatment. The developed modified Bigelow model can estimate the *D*-value of *Salmonella* on both spices at different gas concentrations and RH. *Salmonella* reduction as a function of gas concentration, RH, and the exposure time was estimated using the response surface model, which reveals positive effects of gas concentration, RH, and exposure time on *Salmonella* inactivation during the ClO_2 treatment. The SEM analysis was used to evaluate the morphological changes of *Salmonella* on the surface of black peppercorn during ClO_2 gas treatment, which supported bactericidal mechanism of ClO_2 gas. *E. faecium* was found to be a suitable surrogate for *Salmonella* during the ClO_2 treatment. Further studies of ClO_2 gaseous

technology can be performed to determine changes to the quality and sensory properties of spices.

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Table 7.1. Inactivation kinetics of inoculated *Salmonella* in black peppercorn and cumin seed during ClO_2 gas treatment at different gas concentrations and RH.

Spice	Gas concentration (mg/L)	RH (%)	*D*-value (min) (95% confidence interval)	RMSE (log CFU/g)	Adjusted R^2
Black pepper	5	60	210.4 (172.9 to 268.6)	0.2	0.85
		70	144.0 (116.9 to 187.6)	0.3	0.83
		80	103.4 (84.9 to 132.2)	0.4	0.85
	10	60	180.6 (157.4 to 211.7)	0.2	0.92
		70	132.2 (160.8 to 173.7)	0.4	0.82
		80	69.6 (60.2 to 82.5)	0.4	0.92
	15	60	146.3 (121.4 to 183.9)	0.3	0.86
		70	89.2 (74.7 to 110.6)	0.4	0.87
		80	60.3 (51.5 to 72.8)	0.5	0.90
Cumin seed	5	60	169.8 (152.1 to 192.2)	0.1	0.95
		70	148.8 (128.6 to 176.6)	0.2	0.91
		80	94.4 (85.3 to 105.7)	0.2	0.96
	10	60	165.9 (147.8 to 190.2)	0.2	0.94
		70	134.5 (115.2 to 161.5)	0.3	0.91
		80	67.1 (61.2 to 74.3)	0.3	0.97
	15	60	130.1 (112.0 to 155.2)	0.3	0.91
		70	105.93 (94.3 to 120.9)	0.2	0.95
		80	58.7 (52.2 to 66.9)	0.4	0.95

Table 7.2. Estimated model parameters of modified Bigelow models for both black peppercorn and cumin seed.

Model parameter	Black peppercorn	Cumin seed
D_{ref} (min) (95% confidence interval)	118.3 (110.4 to 126.2)	114.9 (101.5 to 128.3)
$\text{Concenteration}_{ref}$ (mg/ L)	10	10
$z_{\text{concentration}}$ (95% confidence interval)	57.1 (38.0 to 76.2)	75.4 (63.5 to 87.3)
RH_{ref} (%)	70	70
z_{RH} (95% confidence interval)	57.3 (46.5 to 68.0)	71.3 (61.4 to 81.1)
Adjusted R^2	0.96	0.89
RMSE (min)	8.7	15.3

Table 7.3. Model parameters of response surface models for inactivation of *Salmonella* in black peppercorn and cumin seed by ClO_2 gas treatment.

Model parameter	Black peppercorn	Cumin seed
β_0	10	23
β_1	-0.26	-0.37
β_2	-0.24	-0.62
β_3	-0.022	-0.018
β_{12}	0.0033	0.0054
β_{13}	0.00038	0.00026
β_{23}	0.00032	0.00035
β_{11}	0.0026	0.0010
β_{22}	0.0017	0.0041
β_{33}	0.0000079	0.0000012
Adjusted R^2	0.95	0.96
RMSE (log CFU/g)	0.23	0.22

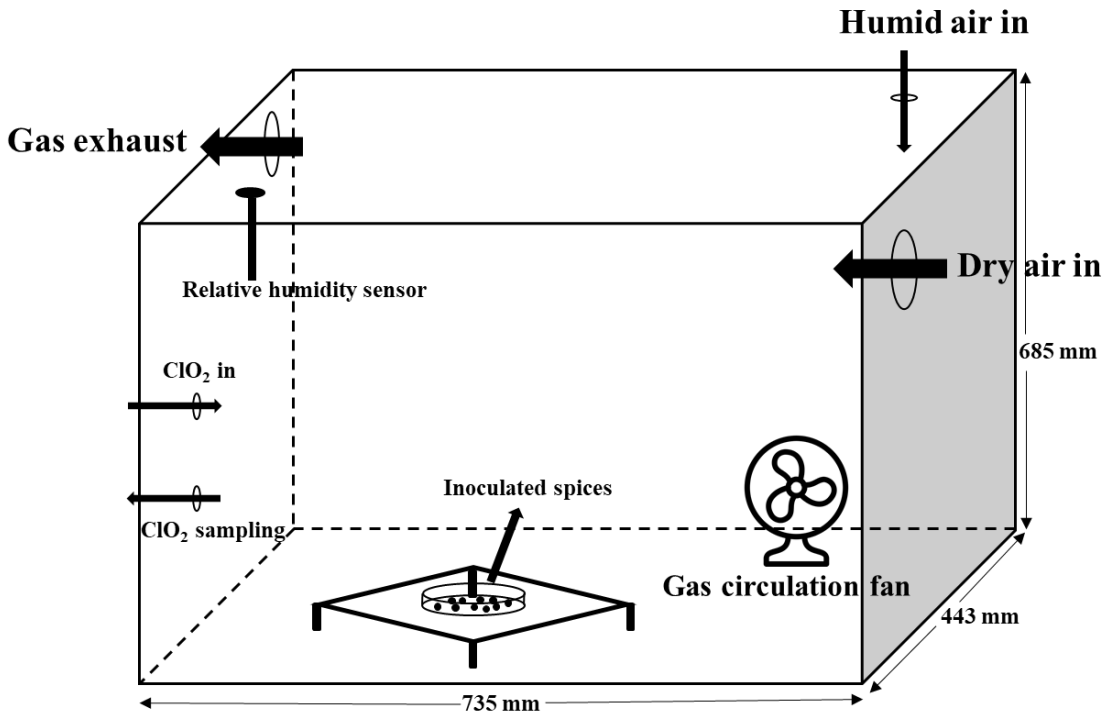


Figure 7.1. Schematic of ClO_2 gas treatment chamber setup.

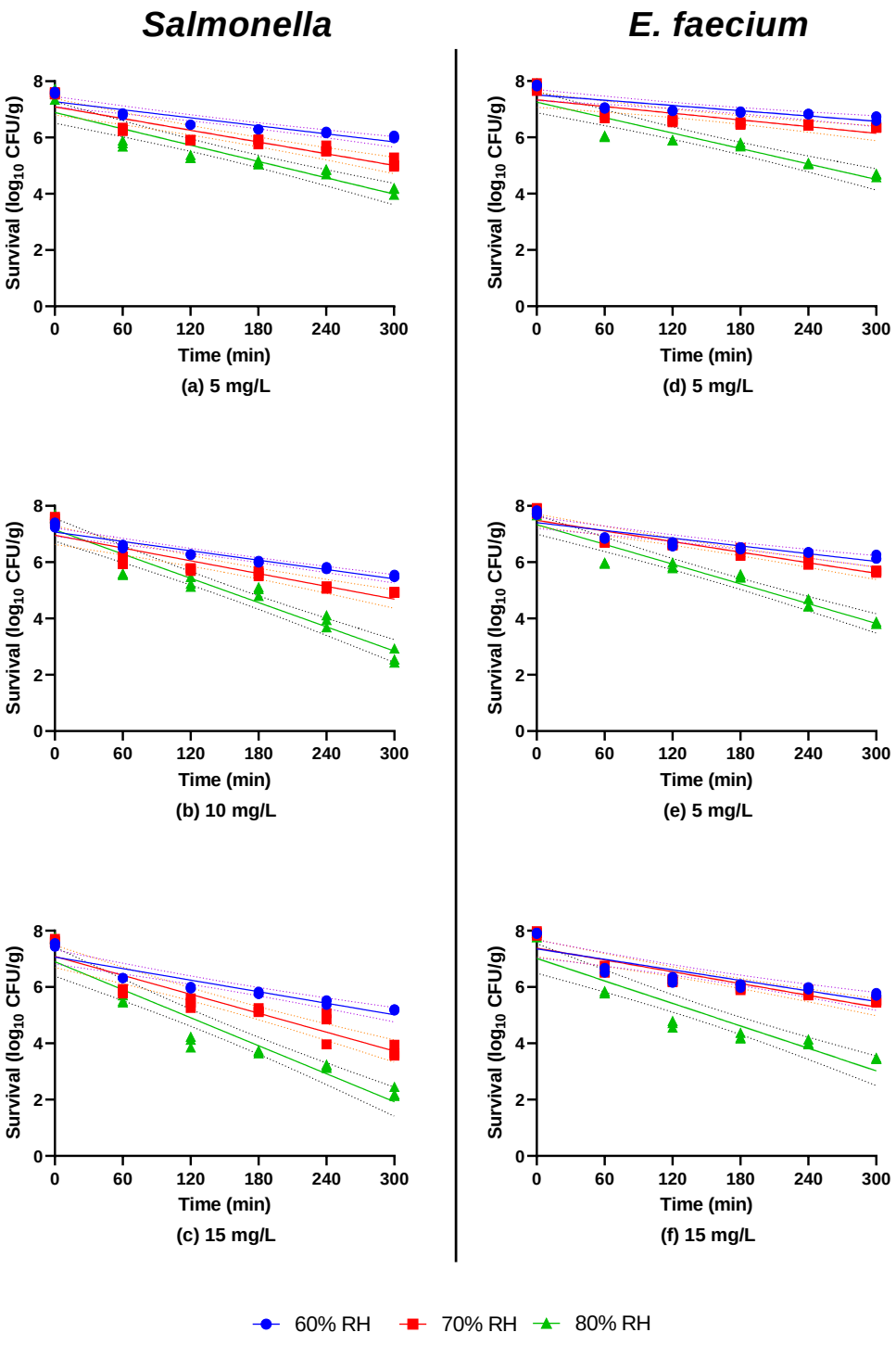


Figure 7.2. ClO_2 gas inactivation kinetics of *Salmonella* and *E. faecium* in black peppercorn at different gas concentrations (5, 10 and 15 mg/L) and RH (60, 70 and 80%). The dotted lines for each survival curve indicate the 95% confidence interval.

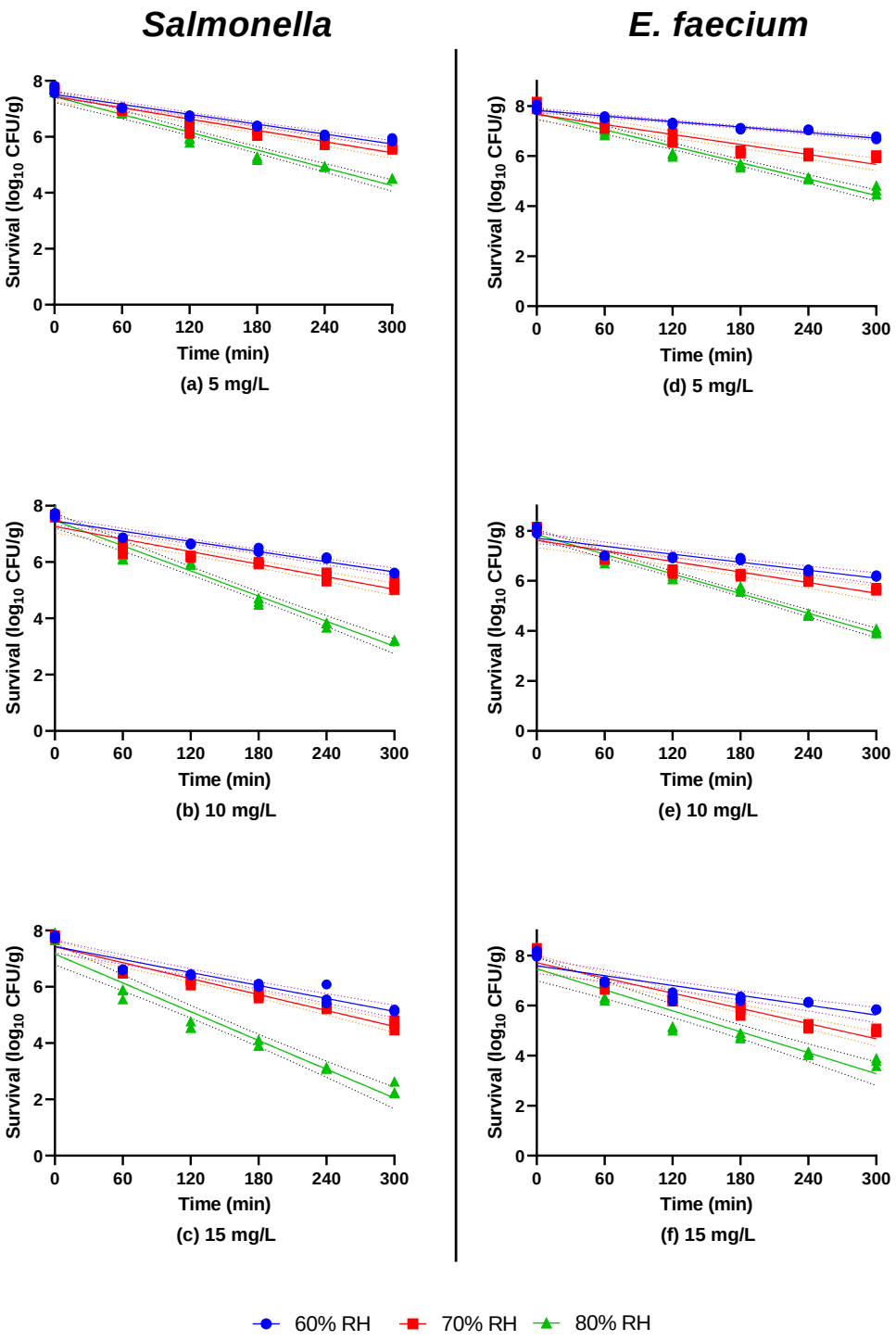
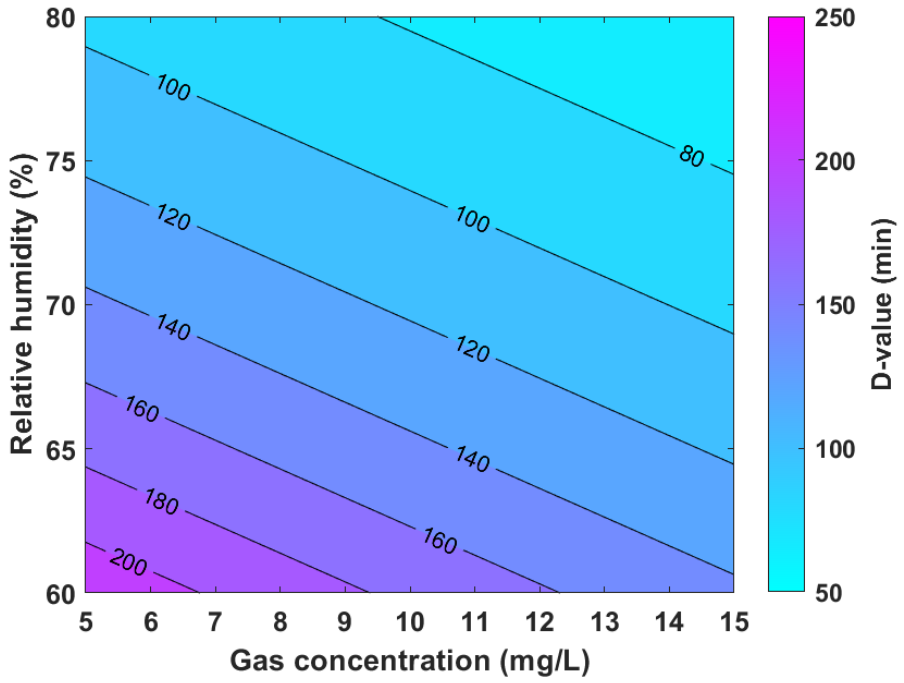
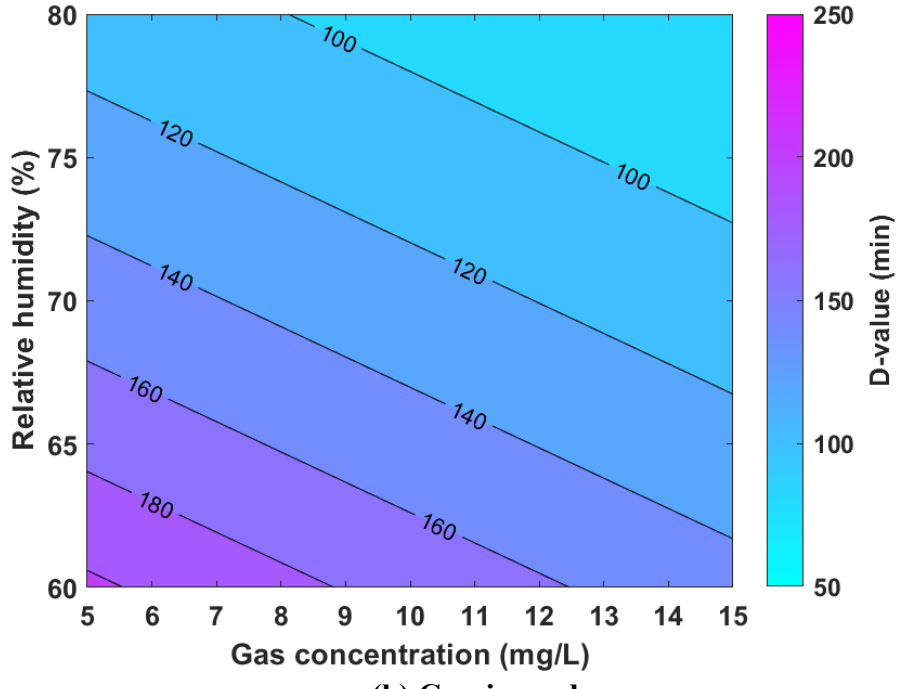


Figure 7.3. ClO_2 gas inactivation kinetics of *Salmonella* and *E. faecium* on cumin seed at different gas concentrations (5, 10 and 15 mg/L) and RH (60, 70 and 80%). The dotted lines for each survival curve indicate the 95% confidence interval.



(a) Black peppercorn



(b) Cumin seed

Figure 7.4. Contour surface plots of estimated *D*-values (min) of *Salmonella* in black peppercorn and cumin seed as a function of gas concentration and RH by modified Bigelow model.

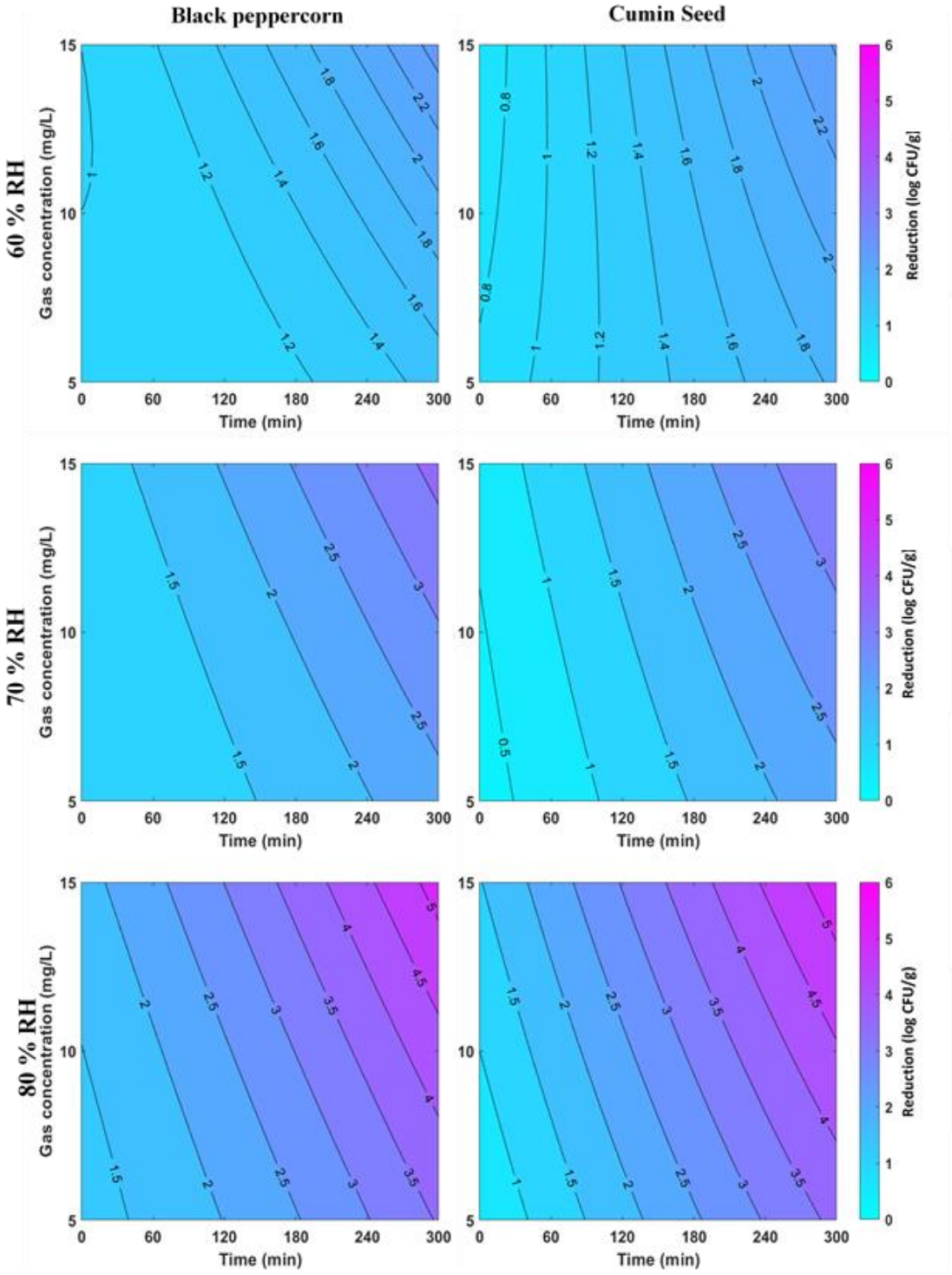


Figure 7.5. Contour surface plots of response surface model estimated *Salmonella* reduction in black peppercorn and cumin seed during ClO_2 gas treatment.

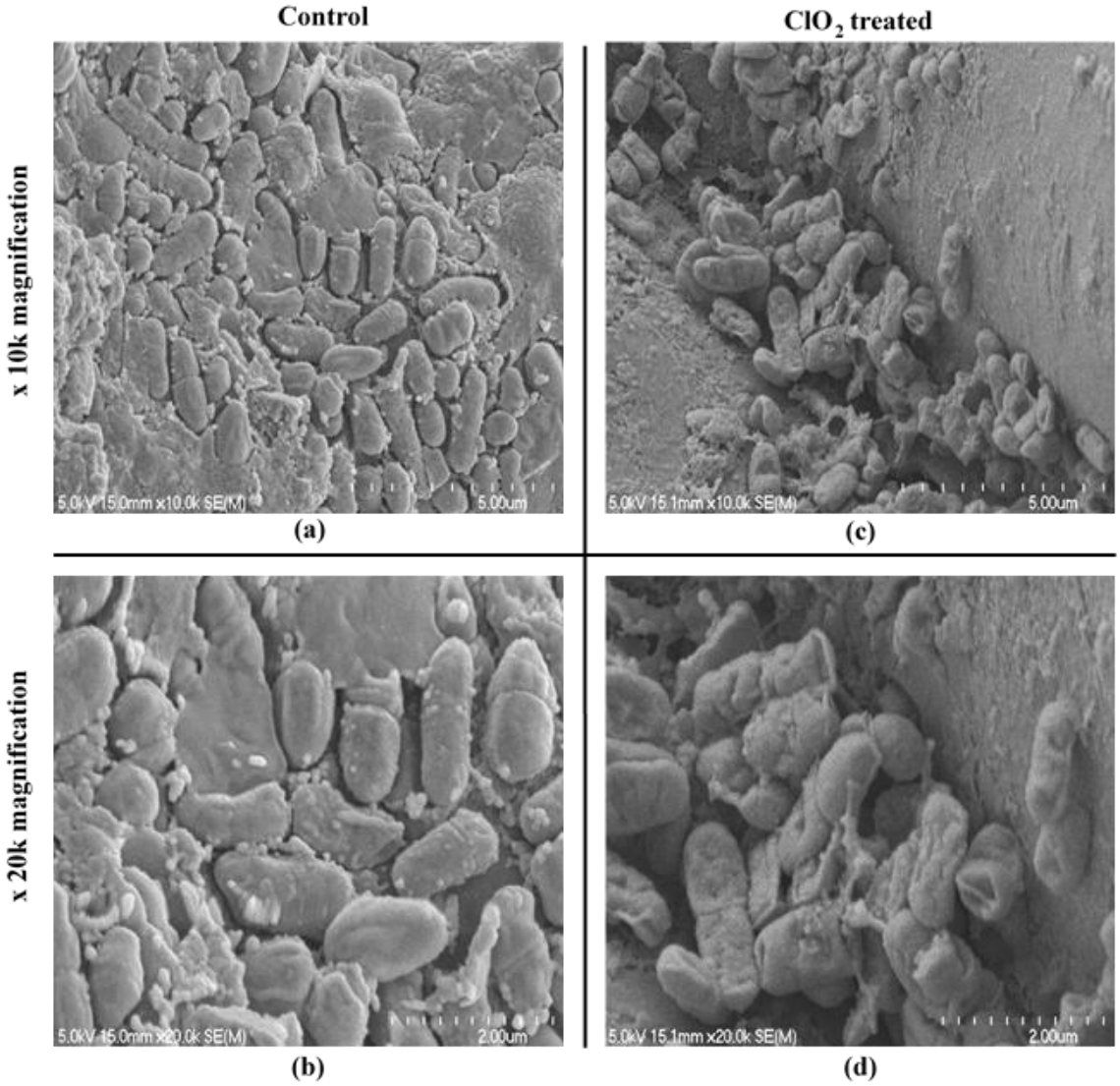
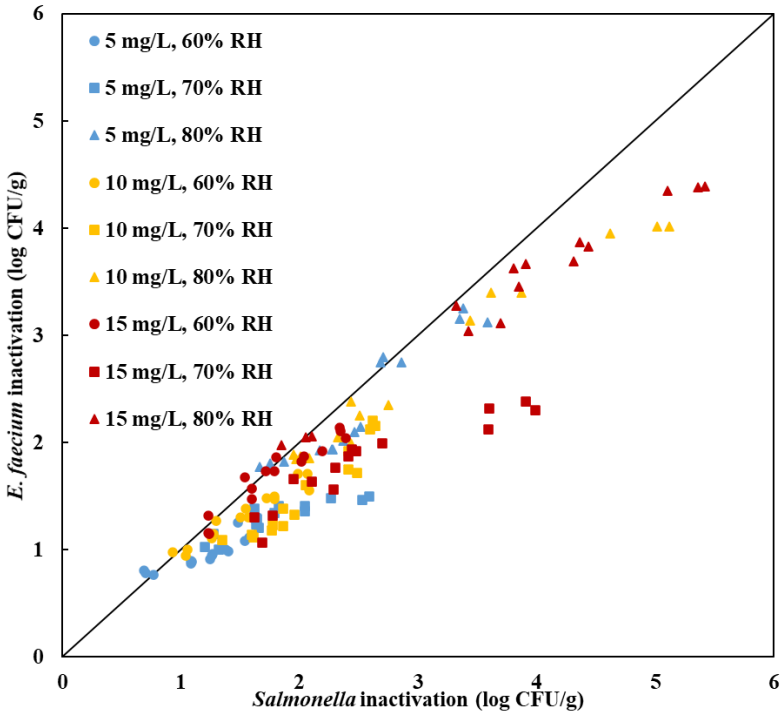
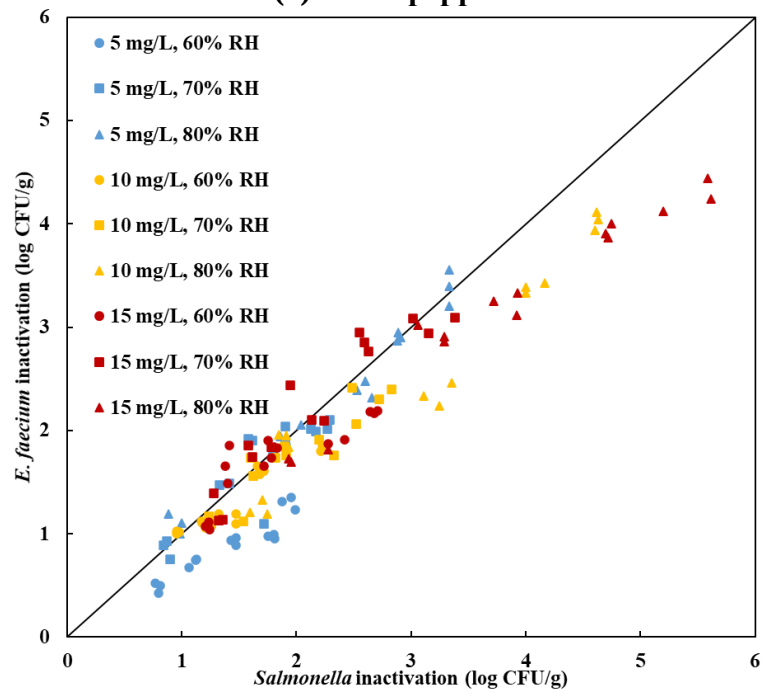


Figure 7.6. Scanning electron microscope images of *Salmonella* inoculated black peppercorn and ClO_2 gas treated black peppercorn. (a) surface of *Salmonella* inoculated black peppercorn – magnification x10k; (b) surface of *Salmonella* inoculated black peppercorn – magnification x20k; (c) surface of ClO_2 gas treated black peppercorn – magnification at10k; (d) surface of ClO_2 gas treated black peppercorn – magnification at 20k.



(a) Black peppercorn



(b) Cumin seed

Figure 7.7. Comparison of reductions of *Salmonella* and *E. faecium* during ClO_2 gas treatment of black peppercorn and cumin seeds. Each point represents an individual treatment.

Chapter 8

CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE RESEARCH

The persistence of *Salmonella* in low moisture foods (LMF) results requires an effective pasteurization process for ensuring food safety. In this dissertation, both thermal and non-thermal technologies were investigated for improving the microbial safety of dairy powders or spices.

8.1 Conclusions

8.1.1 Objective 1. Validation of thermal processing technology (RF heating) for enhancing the microbial safety of milk and egg white powders

The microbial inactivation kinetics of *Salmonella* in milk powders at different a_w were determined by conducting the isothermal treatment at different temperatures using the thermal-death-time (TDT) sandwich system. At low a_w, the thermal resistance of *Salmonella* increased, which increases the difficulty of reducing *the Salmonella* population in milk powders. During thermal processing of milk powders, a_w decreases due to moisture evaporation, so microbial inactivation kinetics at different a_w levels was critical to estimate the bacterial reduction for the thermal process. Two mathematical models were developed to fit the *Salmonella* inactivation data in whole milk powder (WMP) and nonfat dry milk (NFDM). As there were no significant difference in thermal resistance of *Salmonella* in WMP and NFDM, a universal model was developed for both milk powders. The modified Bigelow-type model provided better fit than the response surface model at the tested temperature and a_w range. The developed models provide technical information for the development of the thermal pasteurization process for improving microbial safety of milk powders and help the dairy industry identifying the proper thermal treatment conditions to

conduct the process validation. It presented a starting pointing for the subsequent establishment of the novel thermal process – radio frequency (RF) heating for improving microbial safety of milk powders.

Enterococcus faecium was found to be a suitable surrogate for *Salmonella* in WMP and NFDM at different temperatures and storage times. Stable population levels of *Salmonella* and *E. faecium* were observed in WMP and NFDM. Storage time of up to 90 d showed no effect on the thermal resistance of *Salmonella*. Hence, a proper pasteurization process will deliver the same level of *Salmonella* inactivation regardless of the period of storage.

The hot-air assisted RF heating (HARF) of milk powders could heat milk powders to 95°C within 16 min, while the hot-air oven took around 5 h. The HARF could minimize total treatment time by rapidly heating the milk powder to a high temperature followed by a short holding period in the oven, thus potentially minimizing quality deterioration. Results of the microbial challenge study showed that after HARF heating to 95 °C, followed by 10 and 15 min of holding in the oven, resulted in more than a 5-log reduction of *Salmonella* in WMP and NFDM, respectively. Thus, HARF heating was an effective way to ensure the microbial safety of milk powders. Also, *E. faecium* was evaluated as a suitable surrogate that can replace *Salmonella* for conducting the in-plant process validation in the dairy industry.

A continuous RF heating process was developed to improve the microbial safety and quality of egg white powder. RF heating to 80 °C and holding for 2 h resulted in more than a 6-log reduction of *Salmonella* in egg white powder, which was a great improvement over the traditional hot-room method (i.e., holding at 65 °C for two weeks) used for

pasteurizing egg white powder. The developed continuous RF heating process could shorten the time to less than a day. *E. faecium* was found to be a suitable surrogate for *Salmonella* during RF heating of egg white powder.

8.1.2 Objective 2. Validation of non-thermal gaseous technologies (EtO and ClO_2) for enhancing the microbial safety of spices (black peppercorn and cumin)

Comprehensive studies were conducted to investigate antimicrobial efficacy of ethylene oxide (EtO) fumigation and gaseous chlorine dioxide (ClO_2). EtO fumigation effectively inactivated *Salmonella* in black peppercorn. At high relative humidity (50 % RH), more than a 5-log reduction of *Salmonella* was achieved after exposing the sample to EtO gas for 20 min at 46 °C. When the temperature was increased to 60°C, more than a 5-log reduction of *Salmonella* was achieved within 5 min of exposure time at 50% RH. Temperature and RH were found to significantly affect microbial inactivation during EtO fumigation. A response surface model was developed to guide users on selecting proper combinations of temperature, RH, and exposure time that would yield the desired level of inactivation of *Salmonella*.

In ClO_2 treatment, more than 5 log reduction of *Salmonella* were achieved in both black peppercorn and cumin seed after 300 min gas exposure at 15 mg/L and 80% RH at room temperature. Gas concentration and RH significantly affected the microbial inactivation during ClO_2 treatment of spices. The *D* and *z*-values were estimated using log-linear model and could provide technical information for identifying proper treatment conditions for process validation.

E. faecium has been demonstrated to be a suitable surrogate for *Salmonella* in thermal process validation studies, but not in gaseous treatments of LMF. This dissertation

demonstrated that *E. faecium* is a suitable surrogate for *Salmonella* during EtO fumigation of black peppercorn.

8.2 Recommendations for Future Research

In this dissertation, several process technologies have been evaluated to improve the microbial safety of LMF. Future research could focus on optimizing and scaling up the current processing technologies. Continuous RF heating of milk powders could be further studied, which will be more applicable in the dairy industry. More thermal inactivation kinetics data should be collected at a low a_w range, as moisture evaporates during heating. Computer simulation could be used to optimize the RF heating of milk powders to improve the heating uniformity. Also, based on the collected thermal inactivation kinetics and temperature history data, computer simulation could predict microbial inactivation, which will be a valuable tool for identifying the proper treatment conditions for conducting the industrial-scale process validation. The use of kill-ratio between *Salmonella* and *E. faecium* could be further explored, which would improve considerably the efficiency of the pasteurization process of milk powders by reducing energy costs and quality deterioration.

RF heating has been well studied for improving the microbial safety of multiple LMF. However, practical use of RF heating in the food industry has been limited, likely due to the lack of guidance on how to scale-up this technology. Many studies only showed that RF heating provides good inactivation of target food pathogens in some special scenarios, which will be difficult for food industries to repeat similar procedures. The framework of the development of RF heating for improving microbial of milk powders presented in this dissertation could be extended to other food products. Not only the effective microbial inactivation of RF has been shown, but also the microbial inactivation

kinetics data were provided for further application of this technology. So, the food industry will have more options when identifying the proper pasteurization process conditions based on their specific requirements.

RF heating of egg white powder has been shown to improve its quality, such as gelling and foaming properties. Therefore, the continuous RF heating of egg white powder could be further optimized to improve the product quality while ensuring food safety. Similarly, the heating uniformity can be improved to avoid caking of powder and allow a faster heating rate, which could shorten the processing time. The mechanism of increased gelling and foaming properties by RF heating compared to traditional hot-room heating could be studied at molecular level by understanding protein denaturization. The caking of dairy powders usually happens during RF heating, which results from moisture migration or reaching lactose stickiness temperature. Therefore, a mechanical crack process should be established and included after RF heating of dairy powders.

Two gaseous technologies (EtO and ClO_2) have been systematically investigated for the inactivation of *Salmonella* in spices. However, only a small amount of samples (~3 g) were used in the study to identify the microbial inactivation kinetics of each gaseous technology. The 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 package; 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. Process validation of the gaseous technologies could be conducted to simulate the industrial production line, which would

provide a better demonstration for the application. For example, spice products could be first packed in different commercial containers and then send for the gaseous treatment. The ideal solution will be that the spice products will be pasteurized while being packed in their final retail package, like bottles, cans, and bags, which will minimize the risk of further cross-contamination.

Once the proper process conditions for gaseous treatment have been identified to ensure the microbial safety of spices, the gaseous residuals and their corresponding byproducts should be evaluated to assure the chemical safety of spices. EtO is a carcinogenic gas and it will form ethylene chlorohydrin when EtO is used as a postharvest fumigant in spices. According to the Code of Federal Regulations (40 CFR § and 180.151), the EtO gas residues and ethylene chlorohydrin should be less than 7 and 940 ppm, respectively, in spices like black pepper and cumin. When the EtO fumigation has been validated for ensuring microbial safety, the gas residues and byproducts should also be evaluated at the corresponding treatment conditions. ClO_2 is a strong oxidizing and unstable gas. After oxidation reactions with spices, it will rapidly break down to chlorate (ClO^{3-}) and chlorite (ClO^{2-}) ions, which can further convert to chloride (Cl^{-}). The chlorinated byproducts need to be determined in the ClO_2 treated spices.

There are many novel pasteurization technologies, such as electron beam, cold plasma, pulse electric field, hydrogen peroxide, ozone, pulse UV light, and pulse light emitting diode which could be explored for improving microbial of LMF. Although some of these technologies have limited effectiveness in the inactivation of food pathogens, improving the implementation of current technologies or combining different technologies could provide potential solutions for enhancing the microbial safety of LMF. Although *E.*

faecium was found to be a suitable surrogate for *Salmonella*, its suitability as a surrogate for other food pathogens should be further explored.