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PROCESS INTERVENTIONS FOR IMPROVING THE MICROBIOLOGICAL SAFETY OF LOW MOISTURE FOOD INGREDIENTS

by

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A DISSERTATION

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In Partial Fulfillment of Requirements

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Major: Food Science and Technology

Under the Supervision of Professor Jeyamkondan Subbiah and Terry Howell Jr.

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PROCESS INTERVENTIONS FOR IMPROVING THE MICROBIOLOGICAL SAFETY OF LOW MOISTURE FOOD INGREDIENTS

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University of Nebraska, 2021

Advisors: Drs. Jeyamkondan Subbiah & Terry Howell Jr.

The recurrence of *Salmonella* in low moisture foods and the implementation of the FSMA rule requires a need to validate legacy and novel processing technologies. In this dissertation, a legacy thermal (extrusion), a novel thermal (radiofrequency (RF) heating), and a non-thermal (chlorine dioxide) technology, were evaluated as intervention technologies for *Salmonella* in low moisture foods.

The twin-screw extruder was performed at different levels of screw speeds, temperatures, moisture contents, and fat contents to understand the impact of processing conditions on *Salmonella* inactivation in oat flour. At temperature >65°C, the *Salmonella* population was below the detection limit. At 55°C, *Salmonella* reduction ranged from 0.0 to 9.0 log CFU/g. A response surface model developed for mean residence time (MRT) showed that temperature had no significant effect, whereas screw speed, moisture content, and fat content had a significant linear effect on the MRT. On replacing the screw speed with MRT in the previously developed microbial inactivation models, the R^2 value for *Salmonella* and *Enterococcus faecium* NRRL B-2354 increased slightly from 0.83 to 0.85 and 0.84 to 0.89, respectively. As MRT is challenging to

measure, a slight improvement in the accuracy of the models does not warrant the use of MRT in the inactivation models.

The thermal inactivation kinetics of *Salmonella* and *E. faecium* was determined in dried basil leaves using a dry heating method. As the water activity and temperature increased, the *D*-value of both microorganisms decreased. During RF heating, the inoculated sample was placed in the identified cold spot and was heated for 45, 55, 65 s. Both microorganisms were below the detection limit at 65 s without significantly affecting the quality of dried basil leaves. The results from the chlorine dioxide gas treatment showed that as the gas concentration and relative humidity increases, the *D*-value of both microorganisms decreases. The results presented in this dissertation can help the food industries in planning in-plant validation studies for improving the microbial safety of low moisture foods.

To my Family & Friends

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PREFACE

Chapter III in this dissertation has been published in LWT:

- Verma, T., & Subbiah, J. (2019). Conical twin-screw extrusion is an effective inactivation process for *Salmonella* in low-moisture foods at temperatures above 65°C. *LWT*, *114*, 108369.

Chapter IV in this dissertation has been published in Food Control:

- Verma, T., & Subbiah, J. (2020). Use of residence time versus screw speed in the response surface model for microbial inactivation during single-screw extrusion of low-moisture food. *Food Control*, *115*, 107293.

Chapter V in this dissertation has been published in Food Microbiology:

- Verma, T., Chaves, B. D., Howell Jr, T., & Subbiah, J. (2021). Thermal inactivation kinetics of *Salmonella* and *Enterococcus faecium* NRRL B-2354 on dried basil leaves. *Food Microbiology*, *96*, 103710.

Chapter VI in this dissertation has been published in Food Control:

- Verma, T., Chaves, B. D., Irmak, S., & Subbiah, J. (2021). Pasteurization of dried basil leaves using radio frequency heating: A microbial challenge study and quality analysis. *Food Control*, 107932.

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Chapter I: Introduction

1.1 Background

With the ever-increasing world population reaching an estimated eight billion by 2022, the demand for food products, particularly safer and more attractive ones, also increases. However, food safety is still a significant challenge for both consumers and manufacturers. Foodborne illness is an issue of great concern in the United States and all over the world. Foodborne diseases due to pathogens are estimated to cause 9.4 million illnesses each year in the United States (Scallan et al., 2011). According to the annual report by the Centers for Disease Control and Prevention (CDC), in 2017, 841 foodborne disease outbreaks were reported, resulting in 14,481 illnesses, 827 hospitalizations, 20 deaths, and 14 food product recalls (CDC, 2019a).

The Food and Agriculture Organization defines low moisture foods as food items with water activity lower than 0.85 (Batz et al., 2014). These foods can either be naturally low in moisture or high moisture that undergoes an additional drying or dehydration process (GMA, 2009). Throughout history, low moisture food products like flour, spices, nuts, and herbs have been considered as low-risk commodities for microbial contamination because of the low water activity of these foods, which acts as a natural barrier for bacterial growth. However, recently low moisture foods have been implicated in numerous recalls and outbreaks. According to the CDC, between 2006 to 2020, 26 foodborne outbreaks associated with *Salmonella* were reported to be linked to

low moisture foods like puffed cereals (CDC, 1998), peanut butter (Medus et al., 2009), nuts (CDC, 2009; CDC, 2014; CDC, 2016), spices (CDC, 2019b), and pet food (CDC, 2008). Therefore, *Salmonella* is an emerging issue in low moisture foods, as reflected by different recalls and outbreaks.

The microbial safety of low moisture foods can no longer be assumed simply because it does not support the growth of pathogenic bacteria like *Salmonella* (GMA, 2009). Although the bacteria cannot multiply under desiccated conditions, the low water activity does not affect the survivability of pathogenic bacteria that may already be present in the food. According to the FDA Reportable Food Registry, between 2009 to 2014, 65 recalls were associated with spices and seasonings, 47 recalls with nuts and nut products, 6 recalls with whole and milled grains and flours, and 2 recalls with breakfast cereals because of their contamination with *Salmonella*. It has been seen that even a lower infectious dose (as low as one cell) of *Salmonella* poses a significant health risk to the consumers (GMA, 2009; Lehmacher et al., 1995; Zink, 2008). Therefore, it is critical that the food safety issues related to pathogenic contamination of low moisture foods need to be addressed effectively.

In this context, the Food Safety Modernization Act (FSMA) was passed in 2011, which mandates all food processors to focus on implementing and validating their process controls as interventions to prevent and control biological hazards identified in their processes. The implementation of processing interventions assists in reducing the

risk of biological hazard in low moisture foods and ensures that the food products are microbiologically safe to consume (Brackett et al., 2014).

1.2 Selection of process technologies

Because of the aforementioned hazards associated with low moisture foods, the best approach for improving their microbial safety is to use processing interventions. Thermal processing is the most widely used method, which has been shown to effectively reduce microbial hazards in both high and low water activity foods.

Extrusion is a thermal process that transforms the raw ingredients into the final food product due to the involvement of high temperature and high moisture (Harper, 1994). Therefore, traditionally, thermal extrusion was assumed to be a process that pasteurizes the food product due to high temperatures. However, extruded food products such as toasted oat cereals, pet food, and snacks remain as one of the highly recalled low moisture foods (CDC, 1998; CDC, 2008). The majority of the microbial challenge studies conducted on extrusion processing have been performed on a single-screw extruder which suggests that temperature above 85°C is sufficient in reducing the microbial load from the food product (Anderson et al., 2017; Bianchini et al., 2012; Crane et al., 1973; Li et al., 1993; Likimani et al., 1990; Verma et al., 2018). The food industry prefers to use a twin-screw extruder over a single-screw extruder because it offers consistent product quality, process flexibility, easy raw material processing, and easy cleaning (Riaz, 2000). There is currently a lack of studies that evaluates the efficacy of twin-screw extrusion on *Salmonella* inactivation. Therefore, it is imperative to address this research gap,

considering that the industry use of twin-screw extruders is more prominent than the single-screw extruders.

Radio frequency (RF) heating is a novel thermal processing method that has been explored by various researchers for pasteurization of low moisture foods. The rapid volumetric heating, better heating uniformity, and higher penetration depth make RF heating a better option than the conventional heating method (Jiao, Tang, and Wang, 2014). However, the industrial applications of RF heating are limited either due to the higher initial cost or is still not validated for microbial decontamination. It is essential to explore the efficacy of RF heating in different food matrices to allow the food industry to fully adopt this technology. For spices such as whole black peppercorn (Wei et al., 2018), fine ground black pepper (Wei et al., 2019), cumin seeds (Chen et al., 2019), and paprika (Ozturk et al., 2020), RF heating has been very effective in reducing the microbial load to below the detection limit without affecting the final food quality. There are no studies available that evaluate the effect of RF heating for pasteurization of various herbs such as dried basil leaves, oregano, parsley, and rosemary. Historically, spices and herbs have been used as ready-to-eat seasonings on various cooked foods without an additional cooking step. The presence of any pathogen in these spices and herbs would pose a severe public health threat. Since RF heating has shown promising results in spices, the efficacy of this technology should be explored in herbs as well. This will allow the spice and herb industry to utilize RF heating for the pasteurization of their food products.

Thermal methods are the most commonly used for pathogen reduction in low moisture foods. However, the involvement of heat sometimes impacts the quality of the final food product. For example, the heat-sensitive components present in spices and herbs might be affected during the thermal treatment. Also, pathogens such as *Salmonella* develop thermal resistance in the desiccated environment, which poses a significant challenge while working with low moisture foods (Podolak et al., 2010). Therefore, gaseous technologies such as chlorine dioxide, ozone, vaporized hydrogen peroxide can be potentially used as non-thermal methods against pathogens in low moisture foods. Gases have the ability to diffuse through air spaces and pores due to a higher diffusion coefficient, allowing them to perform well on irregularly-shaped granular foods. However, in contrast to the abundant research available on the use of gaseous technologies in high moisture foods, the current state-of-the-art knowledge of these gases in low moisture foods is minimal. Due to the scarcity of research in low moisture foods, food processors have no reference point, affecting the adoption of gaseous technologies in the food industry. Therefore, a systematic investigation of the effects of process parameters, gas types, and different forms of low moisture foods is required, which will provide the food industry with a reliable starting point for implementing gaseous technologies as part of their food safety plan.

1.3 Goal and objectives

The goal of this dissertation is to evaluate the efficacy of various process interventions (thermal and non-thermal) on reducing the microbial population in

different low moisture foods like flour (oat flour) and herb (dried basil leaves). There are three main objectives to achieve the overall goal:

Objective 1: Validation of extrusion processing for inactivation of *Salmonella* in oat flour

a. Develop a response surface model for inactivation of *Salmonella* during conical twin screw extrusion of oat flour.
b. Evaluate the use of mean residence time instead of screw speed on the accuracy of response surface model for inactivation of *Salmonella* and *Enterococcus faecium* NRRL B-2354 during single-screw extrusion of oat flour.

Objective 2: Validation of radio frequency heating for pasteurization of dried basil leaves

a. Evaluate the effect of water activity on thermal inactivation of *Salmonella* and *Enterococcus faecium* NRRL B-2354 in dried basil leaves.
b. Investigate the efficacy of radio frequency heating for the reduction of *Salmonella* on dried basil leaves.
c. Assess the quality of dried basil leaves post radio frequency treatment.

Objective 3: Validation of non-thermal antimicrobial gas for inactivation of *Salmonella* in dried basil leaves

a. Evaluate the efficacy of chlorine dioxide gas treatment on the inactivation of *Salmonella* on dried basil leaves.
b. Evaluate *Enterococcus faecium* NRRL B-2354 as a surrogate for *Salmonella*.

c. Conduct the quality analysis of dried basil leaves post chlorine dioxide treatment for byproduct formation.

1.4 Dissertation organization

Chapter II includes the literature review about the various processing technologies, thermal and non-thermal, that have been employed for improving the microbial safety of different food products.

Chapter III evaluates the efficacy of conical twin-screw extrusion on the inactivation of *Salmonella* in oat flour. A response surface model was developed to estimate *Salmonella* inactivation as a function of screw speed, moisture content, and fat content.

Chapter IV compares the use of screw speed versus mean residence time in a response surface model to estimate the *Salmonella* inactivation during single screw extrusion of oat flour. Firstly, a response surface model was developed for mean residence time as a function of moisture content, fat content, temperature, and screw speed. The developed model for mean residence time was used to replace screw speed in a previously developed model for microbial inactivation, and the improvement in accuracy of response surface models was determined.

Chapter V investigates the effect of water activity on the thermal inactivation of *Salmonella* on dried basil leaves. Two secondary models were developed to estimate the effect of water activity and temperature on the *D*-value of *Salmonella* on dried basil

leaves. Also, the suitability of *Enterococcus faecium* NRRL B-2354 as a surrogate for *Salmonella* on dried basil leaves was evaluated.

Chapter VI demonstrated the efficacy of radio frequency heating as potential process technology for drying and pasteurization of dried basil leaves. The suitability of *Enterococcus faecium* NRRL B-2354 as a potential surrogate for *Salmonella* was evaluated. The effect of radio frequency heating on the quality of dried basil leaves was also assessed.

Chapter VII evaluated the antimicrobial efficacy of non-thermal gaseous technology, chlorine dioxide, on inactivation of *Salmonella* and *Enterococcus faecium* NRRL B-2354 on dried basil leaves. The formation of byproducts in the dried basil leaves post chlorine dioxide treatment was also evaluated.

Chapter VIII summarizes the results from Chapter III to VIII and presents the recommendations for future work.

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Chapter II: Literature Review

2.1 Introduction

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

Table 2.1 summarizes the outbreaks pertinent to low moisture foods from 1973-2019. It is evident from the Table that the number of outbreaks reported in low moisture foods have seen reasonable increase over the decades. In the last decade alone about 18 incidences have been recorded infecting more than 1300 individuals. About 80% of the foodborne illnesses was due to *Salmonella* contamination, followed by *E. coli* (16%) and *Listeria* (4%). Among the various foods associated with *Salmonella*

contamination, butter products such as peanut butter, nut butter, and soynut butter were more frequently reported probably due to its higher fat content. Children were found to be the most susceptible group to foodborne illnesses in low moisture foods. For instance, 3000 infections were reported for *Salmonella* contamination in infant milk formula in Trinidad in 1973. Interestingly, all the outbreaks reported have been in western countries or developed countries, except for an outbreak in Trinidad. Potentially, there might have been a large number of outbreaks across the world that are not being tracked or reported. In addition, it is also equally important to consider the variation in susceptibility to pathogenic infection between the people in developed and other developing or under-developed countries. These outbreak incidents and recalls necessitate the urgent need for the development and validation of process controls to assure the microbial safety of the finished product.

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

2.2 Process technologies for microbial inactivation in low moisture foods

2.2.1 Thermal processes

Although several food preservation methods exist, the most widely used method is thermal processing. The use of heat to eliminate microbial hazards is usually more efficient in products with a high-water activity ($a_w > 0.85$) (Bialka and Demirci, 2007; Nieto, Castro, and Alzamora, 2001). While thermal treatments will eliminate or reduce the microbial load, one needs to carefully evaluate the quality of final products in selecting the right processing method and parameters. Most of the technologies evaluated for microbial safety in low moisture foods are also commercially available. In this section, we briefly review different thermal methods and their efficacy to reduce microorganisms in low moisture foods.

a) Extrusion

Extrusion cooking is a traditional process that has been widely used by the food industry for several decades. The extrusion process simultaneously combines various unit operations like cooking, mixing, kneading, shaping, and forming to create a variety of low-density, puffed cereals, and snack foods (Riaz, 2000). While the new designs and applications have been developed, the basic science behind extrusion has not undergone a drastic change in over 60 years. In an extrusion process, the raw materials are added to the extruder barrel, and the screws inside the barrel convey the food forward. During this time, the food material undergoes kneading with the help of screws

and is exposed to high temperature and high pressure. Finally, the food material exits the barrel under pressure through a die where the food material usually puffs due to moisture evaporation (Riaz, 2000). A variety of food products like breakfast cereals, corn puffs, pet foods, snacks can be produced using the extrusion process.

The extrusion process reduces the microbial load on food commodities due to the high temperature, pressure, and shear exerted on the product. Extrusion of products such as maize was reported to degrade the product DNA into many smaller fragments (Murray et al. 2007). Ukuku et al. (2012) suggested that the shear stress created by the extrusion process on the bacterial cell is the primary factor responsible for their inactivation. Furthermore, the effect of shear stress on microbial inactivation was positively correlated with the temperature.

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

required to be validated as a kill step such that it will effectively control the identified hazard.

According to the FSMA, validation is defined as the collection of scientific and technical evidence of the preventive control measure, if adequately implemented, can control the identified hazard. Table 2.2 summarizes the validation studies related to extrusion processing in different food products. Results from these studies showed that performing the extrusion process above 85˚C is effective in reducing the microbial load during the extrusion process. A majority of these studies were conducted on a single-screw extruder with a limited number of parameters (temperature and moisture content) evaluated on the microbial reduction. The results from these studies suggested that temperature and moisture content have a positive effect on the pathogen inactivation. Anderson et al. (2017) conducted a pilot-scale extrusion validation study where the effect of temperature and water activity on *Salmonella* reduction was evaluated. The results showed that temperature above 82°C and a_w of 0.89 (20% moisture content) was sufficient to achieve 5-log reduction of *Salmonella* in oat flour. Although temperature and moisture content are critical parameters to monitor, it is equally important to evaluate other process variables such as screw speed, screw configuration, fat content, and their effect on microbial reduction.

Verma et al. (2018a) conducted a similar but more comprehensive study on a lab-scale single-screw extruder where a response surface model was developed to describe the effect of moisture content, fat content, screw speed, and temperature on the

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

Even though the twin-screw extruder involves a higher capital and maintenance cost, the food industry still prefers to use a twin-screw extruder over a single screw because it offers many advantages. These included consistent product quality, easy processing of a wide range of raw materials, lower energy consumption, a higher level of process flexibility, better control of process parameters, and easy to use and clean (Riaz, 2000). Verma and Subbiah (2019) evaluated the effect of various product and process parameters on *Salmonella* inactivation during twin-screw extrusion of oat flour. The results showed that the *Salmonella* population was below the detection limit (<10 CFU/g) at a temperature ≥65°C. Overall, their study demonstrated that the twin-screw extrusion was more effective in reducing the microbial load from the product than the single-screw extrusion.

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

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

conducting the extrusion validation studies as it is easier and convenient for the food processors to control.

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

Overall, the extrusion process is a promising thermal technology that has the capability to reduce or eliminate the biological hazard from the food. Numerous combinations of extruder barrel screw and nozzle dies are used in the food industry to customize their products. Those factors will significantly impact the process conditions and therefore the microbial inactivation. The developed response surface models or

optimal conditions for bacterial reduction are identified in the lab-scale extruder which may not work on an industrial-scale extruder. A multiphysics model can be developed to predict the process conditions (temperature, moisture content, shear) along the barrel screw. However, the validation of such a multiphysics model is challenging, as it is hard to measure those parameters at various points along the screw.

b) Radio frequency

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

The use of RF treatment for pasteurization of food products have been reported to exhibit thermal effect in inactivating microorganisms. Thermal effects are mostly regarded as the possible reason for the inactivation, as the heat generation attributes the cell death of microorganisms (Awuah et al., 2005; Hamoud-Agha et al., 2014; Kou et al., 2018; Shazman et al., 2007). On the other hand, fewer studies have reported a high degree of microbial inactivation at lower product temperatures due to non-thermal

effects (Saadi et al., 2014). Non-thermal effects are highly unlikely, as low electric field intensity involved in RF heating cannot penetrate the cell membrane to achieve microbial inactivation.

According to the U.S. Federal Communications Commission, only three frequencies, i.e., 13.56, 27.12, and 40.68 MHz, have been authorized for industrial, scientific, and medical applications (Piyasena et al., 2003). These frequencies are selected to avoid any interference with the communication system. RF heating has been commercially used for post-baking of cookies and thawing of meat (Dag, Singh, and Kong, 2020). Several studies have used the RF heating process for inactivating pathogenic bacteria in various low moisture foods. Table 2.3 summarizes the studies using RF heating for pasteurization of low moisture foods. Although RF heating proved to be a promising technique to inactivate pathogens, its industrial applications are limited due to the higher initial cost or is still under development for microbial decontamination.

In traditional thermal processing, the temperature gradient drives the heat transfer in the food product. In low moisture food, the heat transfer rate is low due to the lower thermal conductivity. This results in overheating of the edges due to which food products such as spices lose volatiles leading to quality deterioration (Boreddy et al., 2016). However, with RF heating, the temperature gradient is not required as RF volumetrically heats up the food product. RF process heats the food product rapidly to a high temperature within a short time which not only reduces the microbial load but also

minimizes the quality deterioration. RF heating has also been reported to enhance the quality of low moisture foods compared to the traditional thermal processing. For example, Boreddy et al. (2016) reported that the foaming and gelling properties of egg white powder were significantly increased post RF-assisted thermal processing.

One of the disadvantages associated with the RF technique is the non-uniform heating of the food product. The non-uniform heating may lead to the overheating of some parts of the food product while the other part may still be cold, thereby causing the food safety issues in the product and deteriorate its quality (Jiao et al., 2014; Piyasena et al., 2003; Tiwari et al., 2011). The heating non-uniformity in the food product could occur due to several factors such as container geometry, moisture content, thermal properties, and dielectric properties of food. To reduce the non-uniform heating problem, Liu et al. (2013) suggested the use of hot air during the RF treatment of the sample. Similarly, Wang et al. (2006) reported that the mixing of the sample during RF treatment would improve its heating uniformity. However, it a challenge to build a mixer that does not interact with the RF electric field and therefore, no successful mixing mechanisms have been developed or reported so far. The best method is to intermittently mix the samples without the presence of RF electric field. Therefore, it is critical to identify the cold spots and hot spots generated during non-uniform heating and should be used to evaluate the microbial inactivation in the food product.

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

During RF heating, a considerable amount of moisture is lost from the food product due to steam generation. This not only affects the final food quality but also its shelf life. The American Spice Trade Association (ASTA) stipulates that moisture content of black pepper during storage should be less than 10.5% (American Spice Trade Association, 2011). Black pepper will be susceptible to fungal attack if its moisture content is >10.5%. To avoid the reduction in mass due to moisture loss, the spice industry recommends moisture of black pepper close to the maximum safe moisture content, 10.5%. To

achieve close to 10.5% moisture content after RF heating, several validation studies have suggested increasing the moisture content of the sample before the treatment. This not only contributes towards inactivating the microorganisms easily but also brings the final moisture content of the sample close to its native level post treatment. For instance, Wei et al. (2019) increased the moisture content of ground black pepper to 12.8% before RF heating. The moisture content of the sample after RF heating reached 10.5%, meeting the ASTA storage guidelines. In addition, Wei et al. (2019) and Chen et al. (2019) reported that covering the sample container with a vented plastic film helps in releasing the excess steam from the treated sample and enhances the heating uniformity.

The use of RF heating for decontamination purposes for food industry applications is still under development. With FSMA regulations for process validation, a suitable non-pathogenic surrogate need to be evaluated for the food industry to adopt by validating RF heating at the industrial level. Several researchers have investigated the use of *E. faecium* as a suitable surrogate for *Salmonella* in various low moisture foods (Table 2.3). These study results suggested *E. faecium* as an acceptable surrogate owing to its higher thermal resistance than *Salmonella*.

Overall, RF heating is a promising thermal technique that offers some advantages over conventional methods to decontaminate food products. FSMA requires that the appropriate surrogate for target pathogen needs to be validated for different food

matrices. Therefore, the identification of a surrogate for different food products is imperative to help the food industry conduct in-plant validation studies. Modeling RF heating, adjusting the electrode gap, and configuring packages would help in improving the heating uniformity. If the food product is RF heated in a package, the moisture condensation may lead to caking and lumping of the final food product. The use of steam vent packages will help in releasing the excess steam during RF treatment and improve the final food quality.

c) Steam

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

The mechanism for microbial inactivation of steam pasteurization is similar to thermal process, as the steam process increases the temperature of food. These high temperatures are detrimental to the structure of proteins, nucleic acids, and lipids. It leads to the denaturation of proteins and nucleic acids, thus disrupting the cell metabolism. Lipids become too fluid within the cell membrane to continue to maintain the cellular content, thus, leading to cell lysis and inactivation of the microorganism. Table 2.4 summarizes the studies applying steam treatment for inactivation of various pathogens in low moisture foods.

Among different steam methods, wet steam is most commonly used to treat spices in the United States (ASTA, 2011). Although the wet steam method has proved to be effective in reducing the microbial load, the major disadvantage of using this method is the significant increase in the moisture content of the treated sample, which can result in a reduction of shelf life of the final product. Lee et al. (2006) used steam pasteurization method to achieve >4.0 log reduction of *Salmonella* Enteritidis from the surface of raw shelled almonds within 65 s of treatment. However, the wet treatment led to a significant quality loss as the almonds became puffy and skin peeled off very easily due to the high moisture content (Lee et al., 2006). Therefore, an additional drying step is necessary following the steam treatment to remove the excess moisture prior to storage.

To control the condensation on the product, a dry steam process, controlled condensation steam (CSS), has also been used as an alternative to steam pasteurization. In a typical CSS process, the temperature of the system is usually raised to just above the saturation temperature to avoid condensation on the product (Gurtler et al., 2014). However, the efficacy of the CSS system for bacterial reduction in a food product depends on the temperature, exposure time, and pressure. Schweiggert et al. (2007) reported that the use of high temperatures during CSS treatment had a negative impact on the quality of low moisture foods such as spices and herbs (Schweiggert, Carle, and Schieber, 2007). Therefore, researchers investigated the use of CSS in combination with vacuum for reducing microbial load in various food products. Shah et al. (2017)

evaluated the efficacy of vacuum steam pasteurization on the inactivation of foodborne pathogens in various low moisture foods. It was concluded that maintaining the temperature at 95°C for 120 s resulted in reduction of pathogens below the detection limit (<10 CFU/g). Further, a lower processing temperature helped in retaining the quality of low moisture foods.

Super-heated steam (SHS) pasteurization is another dry steam method reported in the literature that has recently attracted a lot of attention for its various advantages such as efficient heat transfer, prevention of nutrient oxidation in food, and energy efficiency (Sook Yun, Zzaman, and Yang, 2015). SHS is typically produced by providing additional heat to the steam to raise the steam temperature above the saturation temperature. A drop in temperature will not result in condensation unless the temperature is decreased to below the saturation temperature point (Cenkowski et al., 2007). SHS has been long known as a safe, non-polluting technology with low energy consumption, if the steam is recycled (Chou and Chua, 2001). Ban and Kang (2016) reported that the application of SHS treatment at 200°C for 15 and 30 s resulted in >5.0 log reduction of foodborne pathogens in almonds and pistachio without altering their quality. Another study reported that the SHS treatment of black peppercorn, pecans, and almonds at 180°C for 13 s reduced the *Salmonella* spp. population below the detection limit (Ban et al., 2018).

2.2.2 Non-thermal processes

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

a) Chlorine Dioxide

Chlorine dioxide (ClO_2) is a strong oxidizing agent that has been used as a sanitizer both in the gaseous as well as in the aqueous form. The gaseous form has been widely used as an antimicrobial agent and is approved by Environmental Protection Agency (EPA) and the U.S. Food and Drug Administration (FDA) as a food additive for its antimicrobial action in food production (21 CFR 173.300) (Bhagat, Mahmoud, and Linton, 2010). The size of the ClO_2 gas molecule is relatively small (0.124 nm; Chai et al.,

2020); therefore, it can easily penetrate and disrupt the cell membrane and protein synthesis in microorganisms (Vandekinderen et al., 2009). Due to the strong penetration ability, ClO_2 gas is regarded as an effective and promising non-thermal technology for reducing the microbial load in various food products (Park and Kang, 2015).

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

Gaseous ClO_2 slowly dissociates into chlorine and oxygen and becomes explosive under pressure when stored for an extended period of time (Demirci and Ngadi, 2012). Due to its explosive and unstable nature, ClO_2 is usually produced on-site either by acidification of sodium chlorite ($NaClO_2$) or sodium chlorate ($NaClO_3$) or by oxidation of sodium chlorite with chlorine (Cl_2). Alternatively, ClO_2 can also be produced by the acidification of sodium chlorite and sodium hypochlorite (NaOCl) (White, 2010). The main reactions for the generation of ClO_2 is given below (Lee et al., 2015):

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

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

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

The literature survey revealed that the application of ClO_2 gas successfully eliminated foodborne pathogens in various high moisture foods (Table 2.5). It can be noted from the Table that gas concentration and treatment time were the primary factors whose effect on pathogen reduction was evaluated in most of the studies. Han et al. (2001) conducted systematic research and developed a response surface model to assess the effect of gas concentration, temperature, relative humidity, and treatment time on the inactivation of *E. coli* O157:H7 in green peppers. Results from this study indicated that the highest reduction of *E. coli* (5.5 log) was achieved when green pepper was treated with 0.4 mg/L of ClO_2 for 65 min at 20°C and 85% relative humidity. Han et al. (1999) reported that the concentration, exposure time, and relative humidity play an essential role in the inactivation of microorganisms during the ClO_2 gas treatment.

Rane et al. (2020) investigated the efficacy of chlorine dioxide gas to control the foodborne pathogens in whole black peppercorns and almonds. The moisture content of the almond kernels was increased by 4% to increase the efficiency of chlorine dioxide gas. At 0.40 mg ClO_2/g, 2.6 log reduction of *Salmonella* was achieved in almonds after 6 h of exposure time. The treated samples were then heat treated 65°C to bring the final moisture content of almonds below 5%. The results showed that an additional 1.7 log reduction was achieved post heat treatment. However, for whole black peppercorns, 3.7 log reduction of *Salmonella* was achieved after 4 h of treatment at 80% relative humidity and 0.40 mg ClO_2/g peppercorn. No heat treatment was performed for whole peppercorns post gas treatment.

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

b) Ozone

Ozone has been used for the purification of bottled water, swimming pools, and wastewater since the 19th century (Guzel-Seydim et al., 2004). It is regarded as a potent antimicrobial gas due to its high oxidizing capacity. In the food industry, ozone has been applied both in gaseous as well as aqueous forms for the decontamination of foods. The use of ozone for the treatment of raw commodities was approved by the U.S. Food and Drug Administration in 2001 and is also registered with the U.S. Environmental Protection Agency as a food contact sanitizer (Selma et al., 2008). Ozone is the strongest oxidant with an oxidation potential of 2.07 V, which is higher than that of hydrogen peroxide (1.80 V), chlorine (1.36 V) and chlorine dioxide (0.95 V) (Bialka and Demirci, 2007; Khadre and Yousef, 2001).

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

Ozone is regarded as a broad-spectrum antimicrobial agent due to its high reactivity and related oxidizing power of free radicals (Brodowska et al., 2017). Ozone is highly unstable both in the aqueous and gaseous forms, which decomposes to form highly reactive free radicals such as hydroperoxyl, hydroxyl, and superoxide radicals when stored for long period of time (Brodowska et al. 2017; Manousaridis et al., 2005; Pirani, 2010). The mechanism of action of ozone on microbial inactivation has been researched and studied on several occasions. For instance, Giese and Christenser (1954) indicated that the cell surface of the bacteria as the major site for ozone activity. In addition, ozone is reported to target the enzymes, proteins, cytoplasm, nucleic acids, spore coats and virus capsids of microorganisms for inactivation (Brodowska et al., 2018; Greene et al., 2012; Guzel-Seydim et al., 2004; Oizumi et al., 1998; Pirani, 2010). Furthermore, Prat et al. (1968) and Scott (1975) reported the modification of pyrimidine bases in bacterial DNA of *E. coli*, upon ozonation, while thymine being more sensitive than uracil

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

Ozone gas has been used to inactivate gram-positive and gram-negative bacteria in various food products. The inactivation of microorganisms post ozone treatment is due to the oxidation of vital cellular components and damage of nucleic acids (Das et al., 2006). Table 2.6 summarizes the literature available on the use of gaseous ozone for microbial inactivation in both high and low moisture foods. The efficacy of ozone in low moisture foods was achieved when the relative humidity of the treatment chamber was above 70% (Akbas and Ozdemir, 2006; Akbas and Ozdemir, 2008). Zhao and Cranston (1995) reported that the effectiveness of ozone on the microbial reduction was strongly influenced by the moisture content of black pepper, while higher moisture content led to a greater reduction of the microbial population. The ozone treatment (6.7 mg/L) of black pepper for 60 min resulted in >3.0 log reduction of *Salmonella* and *E. coli* O157:H7; whereas in the case of dried oregano, 3.7 log reduction of *Salmonella* was achieved at a gas concentration of 5.3 mg/L treated for 120 min (Torlak et al., 2013; Zhao and Cranston, 1995). The effect of temperature on the efficacy of ozone treatment in microbial reduction is currently unknown, as most of the studies have either not

studied the effect of temperature or the experiments were conducted only at room temperatures (20-22°C). With an exception, Perry and Yousef (2013) ramped up the temperature to 55-58°C and obtained >6.0 log reduction of *Salmonella* in shell eggs when treated at 160 mg/L for 60 min.

Overall, the results from various studies given in Table 2.6 revealed that ozone is an effective antimicrobial gas in low moisture foods such as dried oregano, flaked red pepper and black pepper. However, there is a need to evaluate the efficacy of ozone in different low moisture foods by conducting a systematic study investigating the effect of gas concentration and treatment time at different temperature and relative humidity on microbial reduction.

c) Hydrogen Peroxide

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

The mode of action of hydrogen peroxide on pathogenic microorganisms follows similar mechanisms to other oxidizing agents. The microbial inactivation of hydrogen

peroxide is mainly attributed by the oxidative mechanism of sulfhydryl groups and inhibition of osmosis within the cytoplasmic membrane owing to the changes in the cell wall (Kitis, 2004). Furthermore, hydrogen peroxide is also known to denature the protein structure by targeting their side chains (Finnegan et al., 2010). The use of hydrogen peroxide for sanitizing different food products is classified as GRAS (generally recognized as safe) in the United States (Sapers and Simmons, 1998). Table 2.7 summarizes the studies applying aqueous hydrogen peroxide for inactivation of microorganisms in various food products.

Aqueous hydrogen peroxide is usually used at a concentration between 1-5% for sanitizing the food contact surfaces (Parish et al., 2003; Sapers and Simmons, 1998). However, at higher concentrations (4-5%), the efficiency of hydrogen peroxide was reported to be similar to chlorine treatment (Olmez and Kretzschmar, 2009). Beuchat (1997) reported 3.5 log reduction of *Salmonella* when alfalfa seeds were treated with 6% hydrogen peroxide for 10 min. Treating organic leafy greens with 3% hydrogen peroxide for 2 min was less effective in reducing the levels of *Salmonella* Newport (0.2-2.6 log) (Moore et al., 2011). Similarly, only 0.8 log reduction of *E. coli* O157:H7 was obtained when button mushrooms were treated for 30 s at 3% hydrogen peroxide. These studies primarily discussed the effect of hydrogen peroxide concentration and exposure time on bacteria reduction.

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

d) Ethylene Oxide

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

Ethylene oxide treatment first involves generation of vacuum inside the chamber before release of EtO gas to enhance diffusion capacity through the packaged product

which is critical for the inactivation of microorganisms (Phillips and Miller, 1973). The alkylation reaction is considered to be the primary mechanism behind the inactivation of microorganisms (Mendes et al., 2007). The addition of alkyl groups to proteins, DNA, and RNA in microorganisms by binding to the sulfhydryl, hydroxyl, and carboxyl groups prevents the cellular metabolism and growth of microorganisms, thus makes the microbes nonviable (Mendes et al., 2007).

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

Because EtO is a toxic and carcinogenic gas, a vacuum cycle is usually integrated with the fumigation process, which helps in preventing the exposure of gas to the operator.

However, the use of a low-pressure cycle and high temperature has been reported as the reason behind the loss of volatile compounds in spices (Farkas and Andrassy, 1988; Vajdi, 1970). Most of the EtO fumigation studies on food commodities were conducted prior to the 1970's; since then, this technology has been commercialized for decontamination of spice products (Gilbert et al., 1964; Phillips and Kate, 1949; Wesley et al., 1965). The gas concentration, relative humidity, and temperature were reported to have a considerable impact on the microbial inactivation during EtO fumigation. To support, Wei et al. (2021) reported significant linear relationship of temperature, RH, and exposure time on microbial inactivation. They demonstrated that EtO gas treatment at 53°C and 50 % RH could achieve 4.92± 0.13 log CFU/g reductions of *Salmonella* within 20 min of exposure time. Further, Chen et al. (2021) stated that RH greater than 40% is required during EtO treatment to achieve efficient microbial inactivation in cumin seeds. However, still there are no up-to-date standard protocols available to guide the spice industry on how to conduct an effective fumigation process, which can ensure the microbial safety of spices.

Newkirk (2016) tested the efficacy of a two commercial ethylene oxide system on the inactivation of *Salmonella* in whole black peppercorn and cumin seeds. The results from the study revealed that the ethylene oxide fumigation significantly reduced the microbial load on both the food products; however, there was considerable variability associated with the commercial-scale ethylene oxide treatments. While on an average, EtO treatment achieved 6.62 ± 0.62 log CFU/g reduction in black peppercorn and 4.9 log

CFU/g in cumin seeds, there were replicates samples that received only 1 log reduction. Therefore, it is critical to systematically evaluate the effect of ethylene oxide fumigation parameters (gas concentration, relative humidity, temperature, exposure time) on microbial inactivation. The identification of the optimal conditions will serve as a guide for the spice industry to conduct in-plant validation studies.

2.3 Summary

Despite the common assumption that low moisture foods are microbiologically safe, *Salmonella* contamination in low moisture foods has occurred time and time again. This is particularly concerning considering the long shelf life and enhanced survival of *Salmonella* in low moisture foods, which allows the tainted food to sit on consumers' shelves for a long time and cause future infections that are difficult to trace. Therefore, it is imperative to evaluate and validate the novel technologies as kill steps to mitigate the pathogens in low moisture foods.

Thermal processing methods such as radio frequency treatment, extrusion, and steam pasteurization have been extensively studied for microbial inactivation. Steam treatment or controlled condensation is very effective and commercial systems are available. However, the treated product may require drying operation to bring the moisture content to safe level. Moreover, it has a negative impact on the quality of low moisture foods such as spices and herbs, especially the loss of volatiles may occur due to high temperature treatment. Traditional dry heat treatments have been found to be

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

Since most pathogenic bacteria exhibit increased heat tolerance at low water activity conditions, gaseous technologies have gained interest recently due to their high penetration and diffusion properties. Chlorine dioxide, hydrogen peroxide, and ozone

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

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Table 2. 1 Foodborne illness outbreaks reported in various low moisture foods.

Year	Product	Pathogen	Location	Comments	Reference
1973	Milk powder	*Salmonella* Derby	Trinidad	Approx. 3000 people were infected mostly infants and small children.	Weissman et al. (1977)
1993	Paprika	*S.* Saintpaul, *S.* Rubislaw, and *S.* Javiana	Germany	Children <14 years of age were mostly affected among 1000 total estimated cases. Main sources of illness were paprika powder, spice mixtures, and snacks.	Lehmacher et al. (1995)
1994	Infant formula powder	*S.* Virchow	Spain	48 cases were confirmed in 14 regions in Spain out of 17. Children under the age of 7 months were mostly affected.	Usera et al. (1996)
2001	Peanuts	*S.* Stanley	Australia	97 cases were estimated	Kirk et al. (2004)
2001	Peanuts	*S.* Newport	Australia	12 cases were estimated	Kirk et al. (2004)
2001	German chocolate	*S.* Oranienburg	Germany	439 notifications were registered within 6 months in Germany.	Werber et al. (2005)

2002 - 2003	Tahini	*S.* Montevideo	Australia and New Zealand	68 cases were reported, and sesame seed-based food was found to be the main source of illness.	Unicomb et al. (2005)
2005	Infant formula powder	*S.* Agona	France	Total of 141 confirmed cases were reported. Cross-contamination through the environment between two different production lines was reported to be the cause of the illness.	Brouard et al. (2007)
2005	Raw almonds	*S.* Enteritidis PT30	Canada	168 confirmed cases were identified, and raw whole almonds collected from home warehouses retail and distribution was detected to be the source.	Isaacs et al. (2005)
2007	Peanut butter	*S.* Tennessee	USA	715 cases from 48 states were reported.	Sheth et al. (2011)
2008	Infant formula powder	*S.* Kedougou	Spain	31 children under the age of one year were confirmed to be infected.	Rodríguez-Urrego et al. (2010)

2008 - 2009	Peanut butter	*S.* Typhimurium	USA	529 people from 43 states and 1 person from Canada were infected and 116 were hospitalized including 8 deaths.	Cavallaro et al. (2011)
2010	Black and red pepper	*S.* Montevideo	USA	272 cases from 44 states were confirmed and the strain was isolated from ready to eat salami and sealed containers of black and red pepper.	Gieraltowski et al. (2013)
2011	Rice cakes	*Shiga toxin Escherichia coli* (STEC) O157	Japan	142 confirmed cases were associated with the outbreak.	Nabae et al. (2013)
2011	In-shell hazelnuts	*Escherichia coli* O157:H7	USA	In-shell hazelnuts purchased from the retail food stores was found to be the main source of illness, infecting 8 people.	CDC (2011)
2012	Peanut butter	*S.* Bredeney	USA	442 people were reported to be infected in 20 states, majority of which were children under the age of 10 years.	CDC (2012)

2013	Tahini sesame paste	*S.* Montevideo and *S.* Mbandaka	USA	16 cases with 1 death were reported due to contamination in tahini sesame paste.	CDC (2013)
2014	Nut butter spread	*S.* Braenderup	USA	6 cases were reported and 1 hospitalization due to consumption of contaminated butter.	CDC (2014a)
2014	Chia powder	*S.* Newport, *S.* Hartford, and *S.* Oranienburg	USA	31 cases were confirmed in 16 states.	CDC (2014b)
2014	Cashew cheese	*S.* Stanley	USA	17 cases were reported in 3 states; a brand of cashew cheese was associated with the illness	CDC (2014c)
2015	Spice mix	*S.* Enteritidis PT13a	Sweden	174 cases were associated with the infection.	Jernberg et al. (2015)
2015	Sprouted Nut butter spread	*S.* Paratyphi B variant L (+) tartrate (+)	USA	13 people were reported to be infected. People of all age group were found to be infected with age less than 1 year old.	CDC (2015a)

2015	Soft cheese	*Listeria monocytogenes*	USA	30 people got infected out of which 28 people got hospitalized and 3 died from the illness.	CDC (2015b)
2016	Flour	*E. coli* O121 or O26	USA	63 cases were reported in 24 states over exposure to raw flour.	CDC (2016a)
2016	Pistachios	*S.* Montevideo *and S.* Senftenberg	USA	11 cases were confirmed in several states.	CDC (2016b)
2017	Soynut butter	*E. coli* O157:H7	USA	32 people mainly younger ones lesser than 18 years of age were reported to be infected.	CDC (2017)
2018	Dried coconut	*S.* Typhimurium	USA	14 cases were reported from 8 different states.	CDC (2018)
2019	Tahini	*S.* Concord	USA	6 people got infected from consuming tahini.	CDC (2019a)
2019	Cake mix	*S.* Agbeni	USA	7 cases were reported, and the main source of illness was found to be cake mixes.	Ladd-Wilson et al. (2019)
2019	Flour	*E. coli* O26	USA	21 people were reported to be infected, and source of infection is	CDC (2019b)

				linked to flour mixes.	

Table 2. 2 Summary of studies applying extrusion process for inactivation of pathogens in different foods.

Product	Pathogen	Moisture content (%)	Fat content (%)	Screw speed (rpm)	Temperature (˚C)	Reduction (log CFU/g)	Reference
Whey protein powder	*Streptococcus thermophilus*	4-5	NR	50	143	4.2	Queguiner et al. (1989)
Corn-soybean (70:30, w/w)	*Bacillus globigii*	18	NR	80-160	110-130	1.0-7.0	Likimani and Sofos (1990)
Mixture of deboned turkey and white corn flour	*Clostridium sporogenes*	NR	NR	NR	93-115	2.0-5.0	Li et al. (1993)
Animal feed mash	*Salmonella*	28.5	NR	NR	83	Below detection level	Okelo et al. (2006)
Carbohydrate-protein meal	*Enterococcus faecium* NRRL B-2354	25-31	NR	80-125	65-85	5.0	Bianchini et al. (2012)
Corn meal	*Escherichia coli*	35	NR	NR	>75	Below detection level	Ukuku et al. (2012)

Whey protein	*E. coli*	36	NR	NR	95	Below detection level	Ukuku et al. (2012)
Oat flour	*S.* Agona	14-28	8.5	500	70-100	5.0	Anderson et al. (2017)
Oat flour	*S.* cocktail	14-26	5-15	75-225	65-85	1.0-8.0	Verma et al. (2018a)
Oat flour	*E. faecium* NRRL B-2354	14-26	5-15	75-225	75-95	1.0-5.0	Verma et al. (2018b)
Oat flour (twin-screw extrusion)	*S.* cocktail	14-26	5-15	75-225	65	Below detection limit	Verma and Subbiah (2019)

* NR: Not reported

Table 2. 3 Summary of studies applying radiofrequency for inactivation of pathogens in various low moisture foods.

Product	Pathogen	Treatment conditions (CUT)	Reduction (log CFU/g)	Reference
Paprika	*Salmonella* cocktail and *Enterococcus faecium* NRRL B-2354	80°C (1.6 min)	4.2 (Sal.), 1.9 (E.F.)	Ozturk, Kong, and Singh (2020)
White pepper		80°C (4 min)	3.3 (Sal.), 1.4 (E.F.)	
Cumin powder		80°C (6 min)	2.8 (Sal.), 0.9 (E.F.)	
Cumin seeds	*S.* cocktail and *E. faecium*	100°C (1.3 min)	5.8 (Sal.), 3.3 (E.F.)	Chen et al. (2019)
Corn flour	*S.* Enteritidis PT30 and *E. faecium*	85°C, held for 10 min and stored at -20°C for 48 h	6.6 (Sal.), 4.8 (E.F.)	Ozturk et al. (2019)
Wheat flour	*S.* Enteritidis PT30 and *E. faecium*	85°C (15 min), held for 18 min	5.0 (Sal.), 3.7 (E.F.)	Liu et al. (2018)
In-shell almonds	*S.* Enteritidis PT30	75°C (3.6 min)	5.0	Gao et al. (2011)
Broccoli powder	Total bacteria	80°C (5 min)	4.2	Zhao et al. (2017)
Whole black peppercorn	*S.* cocktail and *E. faecium*	96.5°C (2.5 min)	5.3 (Sal.), 5.3 (E.F.)	Wei et al. (2018)
Red pepper powder	*S. typhimurium*	70°C (1 min)	>5.0	Hu et al. (2018)
Ground black pepper	*S.* cocktail and *E. faecium*	80°C (2 min)	6.0 (Sal.), 3.9 (E.F.)	Wei et al. (2019)
Powdered infant formula milk	*Cronobacter sakazakii*	65°C (10 min), held for 21 h in the oven at 65°C	5.1	Lin et al. (2020)

Egg white powder	*S.* cocktail and *E. faecium*	80°C (55 min), held for 1 h in the oven at 80°C	4.7 (Sal.), 3.7 (E.F.)	Wei et al. (2020)
Dried basil leaves	*Salmonella* and *E. faecium*	100°C (1 min)	Below detection limit (<10 CFU/g)	Verma et al. (2021)

* CUT: Come-up time; Sal.: *Salmonella*; E.F.: *Enterococcus faecium*

Table 2. 4 Summary of studies applying steam treatment for inactivation of various pathogens in low-moisture foods.

Product	Pathogen	Temperature (°C)	Exposure time (s)	Reduction (log CFU/g)	Reference
Raw shelled almonds (Nonpareil and Mission)	*Salmonella* Enteritidis	93	65	>5.0 (Nonpareil), >4.0 (Mission)	Lee et al. (2006)
Almonds	*E. coli* O157:H7, *S.* Typhimurium, *S.* Enteritidis, *L. monocytogenes*	200	15	>5.8	G.-H. Ban and Kang (2016)
Pistachio	*E. coli* O157:H7, *S.* Typhimurium, *S.* Enteritidis, *L. monocytogenes*	200	30	>5.5	
Whole flaxseed	*S.* PT 30, *E. coli* O157:H7	95	60	>6.0	Shah et al. (2017)
Quinoa	*S.* PT 30, *E. coli* O157:H7	95	60	Below detection limit	
Black peppercorn	*S.* PT 30, *E. coli* O157:H7	95	60	>6.4	
Milled flaxseed	*S.* PT 30, *E. coli* O157:H7	95	60	Below detection limit	

Sunflower kernels	*S.* PT 30, *E. coli* O157:H7	95	120	>6.2	
Black peppercorn	*S. enterica*	85	120	>5.0	Duncan et al. (2017)
Cumin seeds	*S. enterica*	85	60	>5.0	
Black peppercorn, pecans, and almonds	*S. enterica*	180	13	Below detection limit	C. Ban et al. (2018)

Table 2. 5 Summary of studies applying gaseous chlorine dioxide for inactivation of pathogenic bacteria in various foods.

Product	Pathogen	Gas conc. (mg/L)	Temp. (°C)	Relative humidity (%)	Exposure time (min)	Reduction (log CFU/g)	Reference
Green pepper	*Escherichia coli* O157:H7	0.60	20	90-95	30	7.3	Han et al. (2000)
Green pepper	*E. coli* O157:H7	0.10-0.50	5-25	55-95	7-135	1.0-5.5	Han et al. (2001)
Apple	*L. monocytogenes*	4.00	21	90	30	>4.0	Du et al. (2002)
Strawberries	*E. coli*, *L. monocytogenes*	4.00	22	90	30	>5.0	Han et al. (2004)
Cabbage, Carrot, Lettuce	*S. enterica*	4.10	23	NR	30.8	4.4, 5.2, 1.6	Sy et al. (2005)
Cabbage, Carrot, Lettuce	*E. coli* O157:H7	4.10	23	NR	20.5	3.1, 5.6, 1.6	
Cabbage, Carrot, Lettuce	*L. monocytogenes*	4.10	23	NR	29.3	3.6, 5.9, 1.5	
Lettuce	*E. coli* O157:H7	5.00	NR	NR	14.5	5.0	Mahmoud and Linton (2008)
Lettuce	*S. enterica*	5.00	NR	NR	19.0	5.0	

Whole cantaloupe	*E. coli* O157:H7	5.00	22	90-95	10, 6	4.6	Mahmoud et al. (2008)
Whole cantaloupe	*L. monocytogenes*	5.00	22	90-95	10	4.3	
Whole cantaloupe	*S.* Poona	5.00	22	90-95	6	>5.0	
Hydroponic tomatoes	*S. enterica, L monocytogenes*	0.50	22	90	12	>5.0	Bhagat et al. (2010)
Roma tomatoes	*S. enterica*	10.00	NR	NR	3	4.9	Trinetta et al. (2010)
Navel Oranges	*S. enterica*	0.50	NR	NR	14	>5.0	Bhagat et al. (2011)
Spinach leaves	*E. coli* O157:H7, *S.* Typhimurium, *L. monocytogenes*	0.14	NR	90	15	<1.0	Park and Kang (2015)
Whole blueberries	*S. enterica*	5.50	NR	NR	60	5.6	Annous et al. (2020)
Almonds and black peppercorn	*Salmonella*	0.40 mg ClO_2/g	NR	80	360	2.6 (almonds), 3.7 (black peppercorn)	Rane et al. (2020)

* NR: Not reported

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

Product	Pathogen	Gas conc. (mg/L)	Temp. (°C)	Relative humidity (%)	Exposure time (min)	Reduction (log CFU/g)	Reference
Black pepper	*E. coli* O157:H7, *Salmonella*	6.7	NR	NR	60	>3.0	Zhao and Cranston (1995)
Pistachios	*E. coli* O157:H7	0.0021	20	70	360	3.5	Akbas and Ozdemir (2006)
Cherry tomatoes	*S.* Enteritidis	30.0	NR	NR	15	Below detection limit	Das et al., (2006)
Flaked red pepper	*E. coli* O157:H7	0.0021	20	70	360	2.0	Akbas and Ozdemir (2008)
Baby spinach	*E. coli* O157:H7	4.29	NR	95-100	15	1.8	Vurma et al. (2009)
Tomato	*L. innocua*, *S.* Typhimurium, *E. coli*	2.11	22	NR	3	>3.0	Fan et al. (2012)
Shell egg	*S.* Enteritidis	160	55-58	NR	60	>6.0	Perry and Yousef (2013)
Dried oregano	*Salmonella*	2.8	NR	NR	120	2.8	Torlak et al. (2013)
Dried oregano	*Salmonella*	5.3	NR	NR	120	3.7	

Parsley	*L. innocua*	0.95	21	85	20	1.3	Karaca and Velioglu (2014)
Parsley	*E. coli* O157:H7	0.95	21	85	20	1.1	

*NR: Not reported

Table 2. 7 Summary of studies applying aqueous hydrogen peroxide for inactivation of pathogenic bacteria in various foods.

Product	Pathogen	H_2O_2 conc. (%)	Temp. (°C)	Relative humidity (%)	Exposure time (min)	Reduction (log CFU/g)	Reference
Alfalfa seeds	*Salmonella* spp.	6	NR	NR	10	3.5	Beuchat (1997)
Lettuce	*E. coli* O157:H7, *S.* Enteritidis, *Listeria monocytogenes*	2	50	NR	1	>3.0	Lin et al. (2002)
Cantaloupe	*Salmonella* spp.	5	NR	NR	5	2.3	Ukuku (2004)
Honeydew	*Salmonella* spp.	5	NR	NR	5	3.0	
Baby spinach	*E. coli* O157:H7	2	50	NR	2	2.2	Huang and Chen (2011)
Romaine lettuce	*S.* Newport	3	NR	NR	2	0.4	Moore et al. (2011)
Iceberg lettuce	*S.* Newport	3	NR	NR	2	2.6	
Adult spinach	*S.* Newport	3	NR	NR	2	0.2	
Baby spinach	*S.* Newport	3	NR	NR	2	0.9	
Red bell peppers	*L. innocua* NCTC 10528	5	15	NR	2	2.3	Alexandre et al. (2012)
Button mushroom	*E. coli* O157:H7	3	NR	NR	0.5	0.8	Guan et al. (2013)

*NR: Not reported

Chapter III: Conical twin-screw extrusion is an effective inactivation process for *Salmonella* in low-moisture foods at temperatures above 65˚C

3.1 Abstract

Single-screw extrusion is an effective tool in reducing microbial contamination, however, limited data exists on the efficacy of twin-screw extrusion for microbial inactivation. The purpose of this study was to evaluate the effect of fat (5 to 15%), moisture content (14 to 26%), temperature (55 to 85˚C), and screw speed (75 to 225 rpm) on *Salmonella* inactivation during the twin-screw extrusion of oat flour. Results indicated that *Salmonella* population was below the detection limit (<10 CFU/g) at temperature >65˚C. However, at 55˚C, *Salmonella* reduction ranged from 0.0 to 9.0 log CFU/g. Therefore, this data (55°C) was used to develop the response surface equation. Fat content and screw speed showed a significant negative linear effect on *Salmonella* reduction, whereas moisture content showed a significant linear and quadratic effect. As the industrial extrusion is commonly performed at temperatures >65°C, twin-screw extrusion can be used as an effective process intervention step, after validation. The developed model can serve as a baseline for the industry to validate their extrusion process for food safety.

Keywords: Thermal inactivation; Response surface; food safety; fat content; process validation

3.2 Introduction

Salmonella, a gram-negative bacteria, has been implicated in numerous recalls and outbreaks of low-moisture foods ($a_w < 0.85$) such as spices, peanut butter, flour, and chocolate (Enache et al., 2015; GMA, 2009). As most of the bacteria requires a_w of 0.90 or greater (Anthony and Fontana, 2007), low moisture foods were generally perceived as microbiologically safe. However, one of the biggest challenges working with *Salmonella* is that it can survive in low numbers for a prolonged period in low-moisture foods (Podolak et al., 2010). Very low infectious dose such as one cell has been shown to pose a significant risk to consumers as demonstrated in previous outbreaks in chocolate (ACMSF, 2006), paprika potato chips (Lehmacher et. al., 1995), and peanut butter (Zink, 2008).

The Food Safety Modernization Act (FSMA) of 2011 mandates the food processors to focus on implementing and validating the process interventions used to prevent and control identified hazards. The processing interventions are critical to reduce the risk of *Salmonella* or other food-borne pathogens in low-moisture foods and ensure that food products are safe to consume (Brackett et al., 2014). There are several legacy technologies such as baking, extrusion, etc. may achieve some levels of microbial log reduction, but they need to be validated. If the industry can validate the existing legacy technologies as a kill step, then there is no need to invest in new process technologies. There exists a need to validate extrusion as an intervention technology.

In the last two decades, extrusion processing has gained popularity in the food as well as in the feed industry due to its versatility, cost of production, productivity, and product quality (Chao-Chi Chuang and Yeh, 2004). Extrusion is a thermal process that combines various unit operations like cooking, kneading, shearing, shaping, and forming (Riaz, 2000). The most common types of extruder used in the food industry are single- and twin-screw extruders. A single-screw extruder consists of only one screw in the extruder barrel, whereas the twin-screw extruder consists of two screws which can either be co-rotating or counter-rotating (Harper 1994). The twin-screw extruder is more commonly used in the food industry than single-screw because it offers many advantages such as more consistency of the product quality, a wide range of raw materials with different moisture contents can be processed and is easy to maintain and clean (Riaz 2000). During the extrusion cooking, the raw materials are transformed into a variety of finished products by the application of heat through the jacketed barrels or the frictional heat (Kumar et al., 2008; Yu et al., 2014). In the industry, the twin screw extruders are used to manufacture a wide range of products such as breakfast cereals, co-extruded snacks, texturized vegetable proteins, and pet food (Riaz, 2000). The use of heat, shear, and pressure not only improves the quality of extruded product but also their safety (Harper 1994). However, extruded products such as snacks, cereals, and pet food remain as one of the highly recalled low-moisture food products (CDC, 1998; CDC, 2008b).

Several studies have reported the efficacy of extrusion technology to inactivate microbial population in both high-moisture and low-moisture foods (Anderson et al., 2017; Bianchini et al., 2012; Crane et al., 1973; Li et al., 1993; Likimani et al., 1990; Verma et al., 2018). Results from these studies showed that, the product temperature at the die above 85˚C was effective in reducing the microbial load from single-screw extrusion of oat flour in lab-scale extruder (Verma et al., 2018), and pilot scale extruder (Anderson et al., 2017), and carbohydrate-protein meal (Bianchini et al., 2012). There is currently a lack of studies that address the effect of twin-screw extrusion on inactivation of *Salmonella*. Therefore, it is imperative to understand how *Salmonella* behaves in a twin-screw extruder when extruded at different process parameters and product compositions. Overall, this study will generate knowledge regarding the potential intervention which in this case is twin-screw extrusion, to improve the safety of low-moisture food, which could then be communicated to the food processors to help them evaluate and validate their preventive control strategies.

The objective of this study was to validate the use of a conical twin-screw extruder to inactivate *Salmonella* in low-moisture food when extruded at a wide range of moisture content, fat content, temperature, and screw speed.

3.3 Materials and Methods

3.3.1 Bacterial strains and inoculum preparation

Five different strains of *Salmonella enterica* were chosen (Table 3.1) based on their frequency of occurrence and resistance in low-moisture foods. All isolates were stored at -80˚C as frozen stocks in 40% glycerol.

All media used in this study were purchased from Becton, Dickinson, and Company (Sparks, MD, USA). The method used to prepare the *Salmonella* cocktail is described in Verma et al. (2018). Briefly, the frozen stock of *Salmonella* strains was thawed at room temperature and was individually streaked for resuscitation on tryptic soy agar with 0.6% (w/v) supplemented with yeast extract (TSAYE) agar plates (100 mm diameter and 15 mm deep) and incubated for 24 h at 37˚C. After 24 h, one isolated colony was aseptically transferred to 10 mL tryptic soy broth (TSB) using a 10 µL loop and was incubated at 37˚C for 24 h. The overnight broth culture (100 µL) was then used to create bacterial lawns by spreading it onto TSAYE plates, and the plates were incubated at 37°C for 24 h. The bacterial lawns were then harvested by adding 3 mL of 0.1% (w/v) buffered peptone water (BPW) to each lawn plate, and the cells were agitated into solution using an L-shaped spreader. Finally, the S*almonella* cocktail was prepared by pipetting an equal volume of inoculum from each strain into a sterile conical tube. The prepared cocktail was then vortexed for 30 s to achieve uniform distribution of cells. The *Salmonella* population in the cocktail was at a level of 10^8-10^9 CFU/g.

3.3.2 Sample preparation and inoculation

Whole grain oat flour was used as a low-moisture food model in this study. It was purchased from Bob's Red Mill (Milwaukie, OR, USA) and was stored under refrigerated conditions (4˚C) until it was used. The preparation of inoculated samples is described in Verma et al. (2018). Briefly, oat flour (1 kg) was aseptically taken into the mixer bowl of a commercial mixer (Stand Mixer No. W53294842, KitchenAid, Benton Harbor, MI) along with the required amount of vegetable oil (Great Value, Wal-Mart Stores, Inc., Bentonville, AR), double deionized water, and inoculum. After adding the desired amount of ingredients, the mixer was set to run for 10 min at the lowest speed. After mixing, the inoculated sample was transferred to Ziploc bags (S.C. Johnson, Racine, WI) and stored at room temperature (in biosafety hood) for five days before extrusion to achieve homogeneity and stability of microorganism in the low-moisture environment. The moisture content and fat content of the sample was adjusted to five different levels ranging from 14 to 26% (w.b.) and 5 to 15% (w/w), respectively.

3.3.3 Extrusion

Post inoculation, the extrusion was performed in a lab-scale counter-rotating conical twin-screw extruder (Model CTSE-V; C.W. Brabender, Hackensack, NJ, USA). The diameter of the screw used in the extruder decreased from 43 to 28 mm along the barrel length of 365 mm from feed to die. The extruder was operated by an Intelli-Torque Plasti-Corder (Type FE 2000, C.W. Brabender) which was powered by a 7.5 hp

motor. Figure 3.1 shows the schematic diagram of conical twin-screw extruder used in this study.

The inoculated sample was fed into the extruder through an external volumetric feeder (screw size: 18 mm, pitch: 19 mm; Brabender Technologie, Ontario, CA) whose speed was controlled by a frequency controller. To simulate industrial extrusion process, the extrusion was carried out under starve-fed conditions (75% barrel fill) in this study. Therefore, a preliminary experiment was conducted to identify the feed rates to achieve 75% barrel fill for different screw speeds, fat contents, and moisture contents (Table 3.2).

The extruder barrel was segmented into four temperature-controlled zones which were heated by electronically controlled heaters. The temperature of the first three zones was maintained at 50˚C, while the temperature of the last zone was adjusted to achieve the desired product temperature (55, 65, 75 and 85˚C) at the die. All zones were equipped with Type J lead thermocouples (accuracy of ±2.2˚C) that continuously monitored the temperature at each zone along the barrel using the extruder program software, WINEXT. A temperature transducer (Model No. TPT412CAN-1/2-10M-6/18-MST001; C.W. Brabender Instruments, South Hackensack, NJ) was inserted into the die face to measure the product temperature. This study represented the worst-case scenario, which was achieved by using lower moisture levels, lower temperature, higher screw speed, higher fat levels, and lower pressure (due to the use of no die).

Verma et al. (2018) reported that feeding the uninoculated sample for 20 min after every inoculated sample was enough to provide a non-detectable limit of *Salmonella* (<10 CFU/g). A preliminary trial was conducted at multiple random conditions, which showed that *Salmonella* population was below the detection limit (<10 CFU/g) after feeding the uninoculated sample for 10 min (data not shown). To avoid bacterial carryover, uninoculated oat flour was fed for 20 min (for an additional factor of safety) after every trial to clean the barrel as push-through sanitation. When the extrusion trials were completed for the day, the extruder was sanitized with alcohol and the barrel was heated to 300°C for 30 minutes for sanitation of barrel, screw and die.

3.3.4 Experimental design

A split-plot second order central composite design was used to conduct the experiment. In this design, the temperature was used as a whole-plot factor and moisture content, fat content, and screw speed as the split-plot factors, following the experimental design described in elaborate details in Verma et al. (2018). The five levels of each variable (moisture content, fat content, and screw speed) were calculated based on the coded levels -α, -1, 0, 1, and α (α =1.633). The central points of the design (fat content = 10%, moisture content = 20% and screw speed = 150 rpm) were replicated six times. The temperature was applied to a set of various split-plot treatment combinations (blocks). The order of processing in each block was randomized for both extruder runs and for samples under fat content, moisture content, and screw speed combinations processed under a given temperature.

3.3.5 Moisture determination

A Halogen Moisture Analyzer HR73 (Mettler Toledo Laboratory and Weighing Technologies, Greifensee, Switzerland) was used to measure the moisture content of the samples before and after processing them in the twin-screw extruder.

3.3.6 Recovery of *Salmonella* from oat flour

Salmonella population in the extruded and non-extruded (control) samples were determined by a spread plating method. Sample (25 g) was homogenized in 225 mL of 0.1% BPW for 60 s using a Seward 400 Stomacher (Seward Laboratory Systems, London, England). This homogenized fluid was serially diluted, followed with spread plating on selective (TSAYE overlaid xylose lysine deoxycholate) and non-selective (TSAYE) media for enumeration with incubation at 37˚C for 24 h. The use of non-selective medium allowed the recovery of heat-injured cells or any other bacteria capable of growing under the same incubation conditions. However, the use of selective media as an overlay allowed to select and differentiate for *Salmonella* during the recovery of heat-injured cells.

3.3.7 Statistical analysis

A response surface model (2^{nd} order) was fit to evaluate the effect of different processing parameters on *Salmonella* reduction during twin-screw extrusion of oat flour. A final model was developed by eliminating the non-significant ($p > 0.05$) terms and keeping all the higher order terms that had significant ($p < 0.05$) effect along with their lower order components. SAS 9.4 (SAS Institute, Cary, NC) was used to fit the

response surface model, Microsoft Excel v.2013 was used to create the tables, and MATLAB R2016b (The MathWorks, Inc., Natick, MA) was used to generate contour plots.

3.4 Results and Discussions

3.4.1 Extrusion of inoculated flour

The initial population of *Salmonella* cocktail in oat flour was approximately 8.36 ± 0.38 log CFU/g. The standard deviation of population among three sub-samples over five days of storage was less than 0.3 log CFU/g, which demonstrated that the *Salmonella* cocktail inoculum was homogeneously distributed within the sample. In total, the *Salmonella* population reduced by 1.89 log CFU/g after storing the samples for five days at room temperature (25°C). Majority of the reduction (~1.50 log CFU/g) took place during the inoculation process, and the remaining reduction (~ 0.40 log CFU/g) was found during the rest of the days. After five days of storage, the *Salmonella* population was stable, and high starting initial microbial population was present in the sample to conduct the microbial challenge study. Similar homogeneity and stability results in oat flour were reported by Anderson et al. (2017) and Verma et al. (2018).

Samples inoculated with *Salmonella* cocktail were also analyzed for moisture content before and after processing them in the extruder. A moisture loss of 1.5 to 2.0% was observed after the extrusion process. According to Riaz (2000), moisture loss of 4.0 to 7.0% is typically seen during the industrial extrusion (performed at temperature >100˚C) due to flash-off as the product expands at the die. Bianchini et al. (2012) and Verma et al. (2018) reported a moisture loss of 1.0 to 2.5% at temperatures ranging

from 65 to 85˚C. The lower moisture loss in the extrudate could have been due to the use of lower temperature in our study which may have resulted in lower flash-off of product temperature at the die.

The extrusion of inoculated samples was performed at 65, 75, and 85˚C. The results showed that at a temperature above 65˚C, *Salmonella* survivors were below the detectable limit (<10 CFU/g) for all the conditions tested in this study. One of the major reaction which happens during the extrusion cooking is the starch gelatinization. It is imperative for the starch to gelatinize during the extrusion process as it affects product expansion (Ukuku et al., 2012). To cause the starch granules to melt and gelatinize, the extrusion at the industrial scale is performed at a much higher temperature than 65°C, which makes twin-screw extrusion an effective process for inactivation of pathogens.

Due to no recovery of *Salmonella* at temperature >65˚C, the study was repeated at a lower temperature of 55˚C. The results showed that *Salmonella* reduction ranged from 0.0 to 9.0 log CFU/g and therefore, was used to develop the response surface equation. During the enumeration of *Salmonella*, the samples were plated on both selective (TSAYE overlay XLD) and non-selective (TSAYE) media. The results showed higher bacterial counts (~1 log CFU/g) on non-selective media than on the selective media. This was expected because non-selective media allows the growth of all the bacteria capable of surviving the extrusion process. Therefore, the data from selective media was used for the statistical analysis and to develop the response surface equation.

3.4.2 Response surface model

A response surface methodology was employed to study the effect of fat content (5 to 15%), moisture content (14 to 26%), and screw speed (75 to 225 rpm) on inactivation of *Salmonella*. The second order polynomial equation developed at 55˚C (Eqn. 3.1) was used to predict the *Salmonella* reduction (log CFU/g) as a function of fat content, moisture content, and screw speed. Only coefficients that showed a significant effect were used in the final model. The model fitted well with the coefficient of determination (R^2) of 0.81 and had a non-significant ($p > 0.05$) lack of fit. According to Myers and Montgomery (2002), models with non-significant lack of fit makes them as predictive models, whereas a model with significant lack of fit is not a good indicator of the response and should not be used for prediction. The intercept in the response surface equation (Eqn. 3.1) was calculated by taking the average of estimate of all the blocks and was added to the intercept given in Table 3.3.

$$Log\ \frac{N_o}{N_t} = 59.181 - 1.958^*F - 3.515^*M - 0.115^*S + 0.072^*M^2 + 0.075^*M^*F + 0.0003^*S^2 \quad (3.1)$$

where N_o is the population of bacteria before extrusion (CFU/g), N_t is the population of bacteria after extrusion (CFU/g), M is the moisture content (% w.b.), F is the fat content (%), and S is the screw speed (rpm).

The developed response surface equation showed a significant adverse linear effect of fat content. Moisture content and screw speed had both significant linear and

quadratic effect, whereas moisture content also had a significant interaction effect with fat content (Table 3.3).

a) Effect of fat content

The negative linear effect of fat content on *Salmonella* reduction is shown in Figure 3.2. In general, within each contour plot, as the fat content increases from 5 to 15%, *Salmonella* reduction decreases. For example, at 150 rpm and 14% moisture, as the fat content increases from 5 to 15%, *Salmonella* reduction decreases from 8.0 log CFU/g to 0.8 log CFU/g. Trends in the contour plot (100 rpm) also show that >5 log CFU/g of *Salmonella* was achieved when the fat content was below 10% at all moisture contents. A higher moisture content (>24%) and a screw speed of 150 rpm were required to achieve >5 log CFU/g of *Salmonella* at 15% fat. However, when the inoculated samples were extruded at a temperature above 65˚C, the bacterial population was under the detectable limit (<10 CFU/g) at all the conditions tested in this study. A similar effect of fat content was also seen at other screw speeds (100 and 200 rpm). The survival of *Salmonella* with increasing fat content during the extrusion process was also tested by Verma et al. (2018). Their study reported that, at 65˚C and 150 rpm, oat flour formulated with lower fat content (5%) achieved a higher log reduction (4.5 log vs. 2.0 log), when compared to oat flour with higher fat content (15%). On examining the results from both the studies, the conical twin-screw extruder was more effective than single-screw extruder in reducing the bacterial population. The protective effect of fat content was also reported by several other studies. Gurman et al. (2016) reported that

fat content of 5 and 17% in pork patties affected *Salmonella* inactivation, with the higher fat level being more protective. Juneja et al. (2001) reported an increase in the D-values for *Salmonella* in ground beef and pork with increasing fat levels (4 to 28%). Smith et al. (2001) also reported that *Salmonella* in beef with 19% fat was more heat resistant than the one containing 4.8% fat.

b) Effect of Moisture Content

Moisture content had a significant linear and quadratic effect on *Salmonella* reduction. Figure 3.2 shows a decline in *Salmonella* population from 8.0 to 4.8 log CFU/g when the moisture content increases from 14 to 22% at 5% fat and 150 rpm. Due to the quadratic effect of moisture content, a further increase in moisture from 22 to 26% resulted in an increase in log reduction from 4.8 to 5.6 log CFU/g. Similar trends were also seen at other screw speeds. The quadratic effect of moisture content on inactivation of *Salmonella* Agona during extrusion (pilot-scale single-screw) of oat flour was also reported by Anderson et al. (2017). Their results showed that microbial reduction decreased for moisture content from 14 to 19% (a_w of 0.74 to 0.84) and then increased from 20 to 28% (a_w of 0.84 to 0.96). Verma et al. (2018) also reported the quadratic effect of moisture content on *Salmonella* inactivation during single-screw extrusion of oat flour. The results from their study showed a decrease in log reduction from 5.0 to 4.5 log (14 to 20% moisture) followed by an increase in log reduction from 4.5 to 6.0 log (20 to 26% moisture) at 65˚C and 5% fat content. The inflection point of moisture (where the trend in inactivation changes) was 21% in a twin-screw extruder,

whereas it was 20% in single-screw extrusion. Anderson et al. (2017) reported a similar result on pilot scale extruder with an inflection point of 19% for *Salmonella* Agona. It is interesting to note that the bacterial resistance was the highest at the moisture content ranging from 19-22%. However, further decrease in moisture content from 18 to 14% resulted in an increase in the bacterial reduction. This may be due to higher friction and shear in the material at lower moisture content. Higher product shear may be synergistic with temperature for microbial reduction during extrusion.

c) Effect of Screw Speed

The linear effect of screw speed on *Salmonella* reduction is shown in Figure 3.2. As the screw speed increases from 100 to 200 rpm, *Salmonella* reduction decreases. The higher screw speed results in lower residence time of material inside the extruder which affects the microbial reduction (Okelo et al., 2006). Figure 3.2 shows that as the screw speed increases from 100 to 200 rpm, *Salmonella* reduction declines from 9.0 log CFU/g to 7.5 log CFU/g when the extrusion was performed with oat flour formulated at 5% fat and 14% moisture content. In general, microbial reduction ranged from 1.6 to 10.4 log at 100 rpm, 0.8 to 8.0 log at 150 rpm, and 0.0 to 8.0 at 200 rpm depending on the moisture content and fat content of the oat flour. Reduction of the microbial population with an increase in screw speed was also reported by Likimani et al. (1990), who studied the inactivation of *Bacillus globigii* during the extrusion of a corn-soy blend. The results from their study showed that increasing the screw speed from 80 to 160 rpm decreased the residence time which resulted in lesser bacterial reduction at 18% moisture content.

Verma et al. (2018) also reported that increasing screw speed from 100 to 200 rpm decreased *Salmonella* reduction for any combination of moisture content, fat content, and temperature.

3.5 Conclusion

This study investigated the efficacy of twin-screw extrusion on the reduction of *Salmonella* over a range of fat content, moisture content, temperature, and screw speed. At a temperature above 65˚C, the bacterial population was below the detection limit (<10 CFU/g). Therefore, a response surface model was developed at 55˚C to evaluate the effect of fat content, moisture content, and screw speed on the reduction of *Salmonella*. Fat content showed a significant negative linear effect with lower fat content resulting in higher log reduction of *Salmonella*. A significant linear and quadratic effect of moisture content was observed on bacterial reduction. As the moisture content increased from 14 to 22%, *Salmonella* reduction decreased and then increased from 22 to 26%. Screw speed demonstrated a significant linear effect with higher screw speed resulting in greater *Salmonella* reduction.

In terms of reducing *Salmonella* population, conical twin-screw extrusion demonstrated to be an effective process compared to the single-screw extrusion. The developed response surface model may aid the food industry in planning an extrusion validation study in their plants. As industrial extrusion is often performed at temperatures much higher than 65°C, conical twin-screw extrusion is an effective inactivation process.

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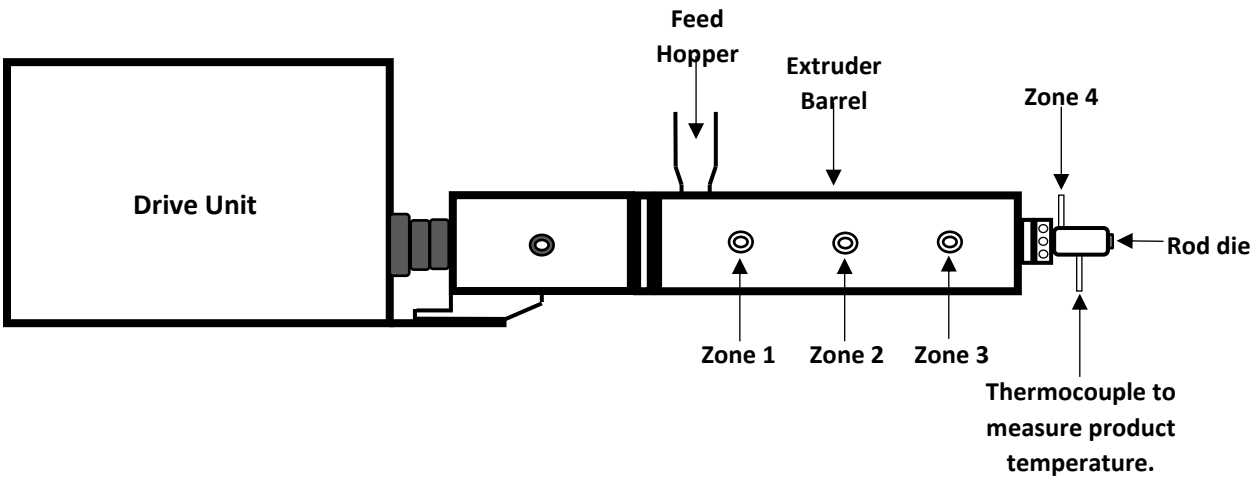

Figure 3. 1 Schematic diagram of the conical twin-screw extruder.

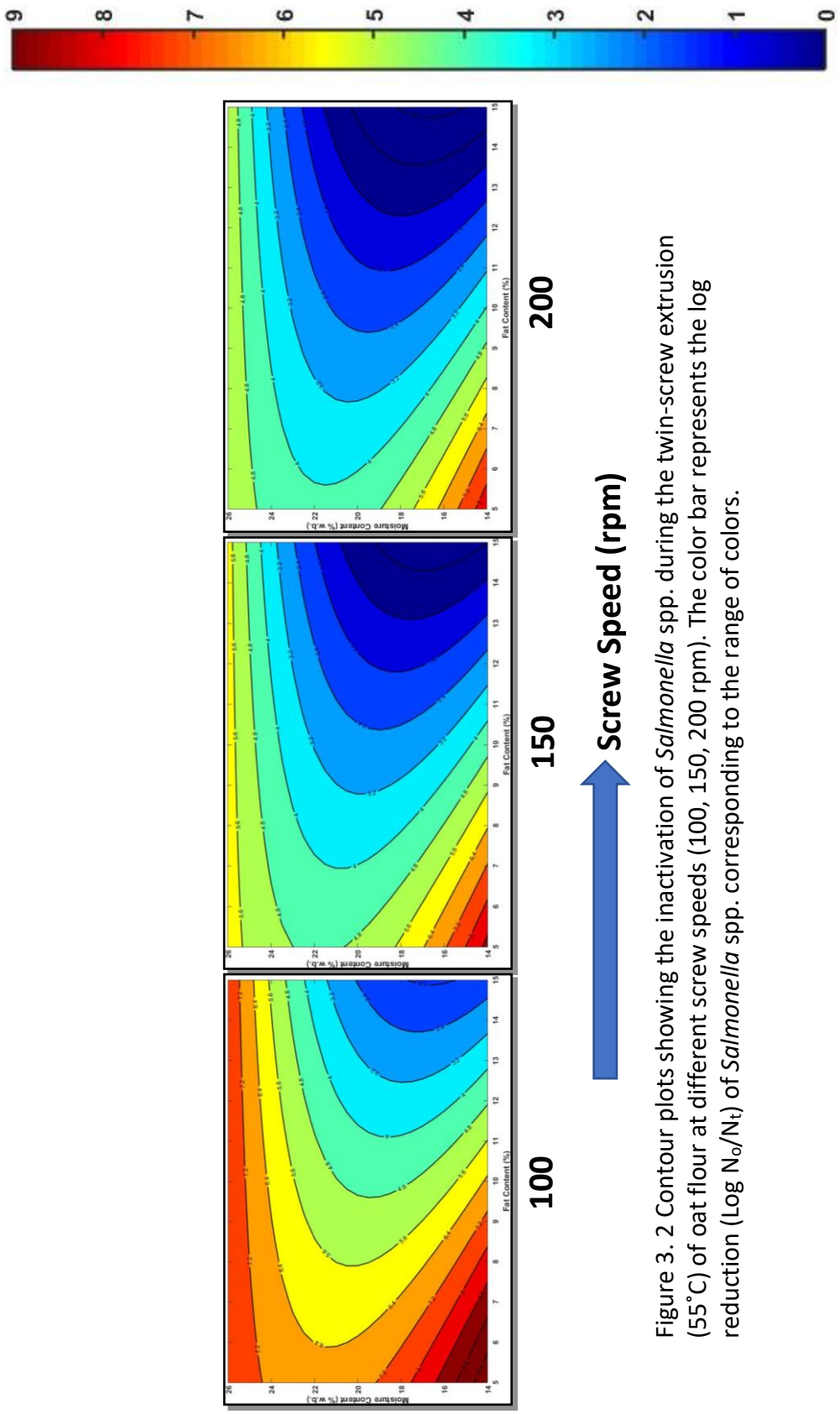

Figure 3. 2 Contour plots showing the inactivation of *Salmonella* spp. during the twin-screw extrusion (55˚C) of oat flour at different screw speeds (100, 150, 200 rpm). The color bar represents the log reduction (Log N_o/N_t) of *Salmonella* spp. corresponding to the range of colors.

Table 3. 1 *Salmonella* strains used in the study.

Microorganism	**Isolate no.**	**Source**	**Recall**
Salmonella Reading	*Moff 180418*	FDA Culture collection, Bedford Park, IL	Known desiccation and heat resistance in low moisture foods
Salmonella Agona	447967	FDA, ORA Arkansas Regional Lab (Jefferson, AR, USA)	Puffed rice cereal, Minnesota (CDC, 2008a)
Salmonella Tennessee	K4643	Dr. L. Beuchet, Universiy of Georgia (Griffin, GA, USA)	2006 Peanut butter outbreak (CDC, 2007)
Salmonella Mbandaka	698538	FDA, ORA Arkansas Regional Lab (Jefferson, AR, USA)	Sesame tahini from Turkey (CDC, 2013)
Salmonella Montevideo	488275	FDA, ORA Arkansas Regional Lab (Jefferson, AR, USA)	2009-2010 black pepper outbreak (CDC, 2010)

Table 3. 2 Feed rates and *Salmonella* reduction (log CFU/g) at different screw speeds, moisture content, and fat content under starve-fed condition (75%-barrel fill).

Run #	Fat content (%)	Moisture content (% w.b.)	Screw speed (rpm)	Feed rate (g/min)	Log reduction (log CFU/g)
1	5.00	20.00	150	68.9	5.65
2	15.00	20.00	150	93.8	2.42
3	10.00	14.00	150	66.9	5.68
4	10.00	26.00	150	100.1	5.95
5	10.00	20.00	75	53.9	3.31
6	10.00	20.00	225	98.2	3.37
7	10.00	20.00	150	88.1	8.28
8	10.00	20.00	150	90.1	3.31
9	6.96	16.35	196	61.6	6.04
10	13.04	16.35	104	43.8	4.22
11	6.96	23.65	104	45.2	3.39
12	13.04	23.65	196	84.1	3.42
13	10.00	20.00	150	87.2	7.41
14	10.00	20.00	150	87.8	1.94
15	6.96	16.35	104	38.3	5.49
16	13.04	16.35	196	67.1	7.59
17	6.96	23.65	196	71.4	8.13
18	13.04	23.65	104	50.4	3.65
19	10.00	20.00	150	88.9	3.48
20	10.00	20.00	150	87.5	3.48

Table 3. 3 SAS output for *Salmonella* inactivation in a twin-screw extruder.

Parameter	Coefficient estimate	Standard error	*t* Value	*P* value
Intercept	59.5497	12.26933	4.85	0.0005
Block (Temp) 1 55	-0.7426	0.608293	-1.22	0.2477
Block (Temp) 2 55	-0.3626	0.608293	-0.6	0.5632
Block (Temp) 3 55	0	0	0	0
F	-1.9581	0.724863	-2.7	0.0206
M	-3.5133	0.989741	-3.55	0.0046
S	-0.115	0.044455	-2.59	0.0253
M*M	0.07201	0.022963	3.14	0.0095
F*M	0.07491	0.035888	2.09	0.0609
S*S	0.00035	0.000146	2.36	0.0379

M = Moisture content (%, w.b.); F = Fat content (%); S = Screw speed (rpm)

Chapter IV: Use of residence time versus screw speed in the response surface model for microbial inactivation during single-screw extrusion of low-moisture food

4.1 Abstract

The implementation of the Food Safety Modernization Act requires the food industry to validate the processing interventions as a kill-step for foodborne pathogens. During extrusion process, the mean residence time (MRT) of the material plays a critical role in microbial inactivation. Therefore, the objective of this study was to develop a response surface model for the MRT as a function of moisture, fat content, screw speed, and temperature. This model was further incorporated into the previously developed models for microbial inactivation by replacing screw speed with MRT and evaluated for goodness-of-fit. The oat flour formulated to different moisture (14 to 26%) and fat (5 to 15%) contents was extruded in a single-screw extruder at different temperatures (65 and 95˚C) and screw speeds (75 to 225 rpm). The MRT was measured using 0.5 g of Congo red/kg of formulated sample. With this wide range of product and process variables tested, the range of MRT was minimal (111-144 s). The temperature had no significant effect on MRT in the range tested. Screw speed, moisture, and fat content showed a significant linear and quadratic effect on the MRT. The use of MRT instead of screw speed in the previously developed inactivation models slightly improved the R^2 value from 0.83 to 0.85 for *Salmonella* and 0.84 to 0.89 for *E. faecium*. As MRT is challenging to measure with high repeatability when compared to screw speed, the

slight increase in the goodness-of-fit measure does not warrant the use of MRT in inactivation models.

Keywords: response surface analysis; single screw extrusion; *Salmonella*; *E. faecium*; fat content

4.2 Introduction

Extrusion processing has gained popularity in the food and feed industry due to various reasons (Yu et al., 2014). These include the production of various food products at lower cost, higher productivity, and enhanced product quality by retaining heat-sensitive components of food. (Chao-Chi Chuang and Yeh, 2004). Food extrusion, as described by Riaz (2000), is a thermal process where the food material is exposed to a variety of conditions such as mixing, heating, and shear. The food material exits the extruder barrel under pressure through a die where the food material usually puffs due to the evaporation of moisture.

During the extrusion process, the amount of time that the feed material spent in the extruder barrel is expressed as the residence time (Ainsworth et al., 1997; Davidson et al., 1983). Knowledge of residence time during the extrusion process is necessary because the properties of the extrudate depend on various extruder conditions like temperature, screw speed, pressure, and shear (Yu et al., 2014). During the scaling-up of the process and determining the optimal conditions, residence time would serve as a useful tool for comparison (Ainsworth et al., 1997; Gogoi and Yam, 1994). The determination of residence time during the extrusion process involves the use of various

colored dyes such as erythrosine (Yeh et al., 1992), manganese dioxide (Fichtali et al., 1995), zinc oxide (Bounie, 1988), indigocarmine (blue dye; Ainsworth et al., 1997), congo red tracer (Iwe et al., 2001), lithium chloride (Jager et al., 1995), and sodium chloride (De Ruyck, 1997). The use of colored dyes in the raw material helps to track the movement of the feed material through the extruder barrel. The time it takes for the colored dye to first emerge at the extruder barrel is minimum residence time, and it relates to the fastest moving material.

Several researchers have studied the effect of various extrusion conditions and product compositions on the mean residence time. It was found that product compositions such as moisture content, and extrusion conditions such as screw speed, temperature, screw configuration, and die diameter are some of the variables that influence the mean residence time (Davidson et al., 1983; Harper, 1989; Kumar et al., 2006; Nwabueze and Iwe, 2010; Yu et al., 2014). However, there is limited literature on the comprehensive study investigating the interaction of product compositions and extrusion process variables on the mean residence time. Also, the effect of feed composition like fat content on the mean residence time has not been systematically evaluated.

The extrusion process has been validated to be a beneficial tool in reducing the pathogen load in various food products due to the application of heat, shear, and pressure. The studies conducted by different researchers showed that the extrusion conditions like temperature, screw speed, die type, screw type, and product

compositions like moisture and fat content has a significant effect on reducing the microbial load in various food products (Anderson et al., 2017; Bianchini et al., 2012; Likimani and Sofos, 1990; Likimani et al., 1990; Okelo et al., 2006). We previously developed a response surface model for *Salmonella* spp. reduction (Verma et al., 2018a) and its surrogate *Enterococcus faecium* NRRL B-2354 (Verma et al., 2018b) as a function of temperature, screw speed, moisture content, and fat content during the single screw extrusion of oat flour by following second order central composite design. However, these studies used screw speed instead of mean residence time as one of the factors affecting microbial inactivation. Peng et al. (1994) suggested that it is crucial to consider mean residence time rather than screw speed for considerations in nutrient degradation, improving food safety, and control of the product quality.

To fill the gaps, a detailed study is required to investigate the effect of different variables such as moisture content, fat content, screw speed, and temperature on the mean residence time. Therefore, the objective of this study was to develop a response surface model for the mean residence time as a function of moisture content, fat content, screw speed, and temperature. The developed model was then used to replace the screw speed in a previously developed response surface model for microbial inactivation (Verma et al., 2018a, 2018b). The goal is to weigh the benefits of improvement in accuracy of response surface model as determined by goodness-of-fit due to incorporation of mean residence time, as mean residence time is hard to measure in extrusion when compared to screw speed. This will aid the food industry to

use appropriate extrusion parameters for microbial inactivation when the validation study is conducted.

4.3 Materials and Methods

4.3.1 Preparation of sample

Based on the industry input, Anderson et al. (2017) identified whole grain oat flour as a model low-moisture and high-fat food for process validation of a pilot-scale extruder. The same model low-moisture food, whole grain oat flour, was used to perform validation in a lab-scale extruder with an extended range of process and product composition variables (Verma et al. 2018a, 2018b). The whole grain oat flour was acquired from Bob's Red Mill (Milwaukie, OR, USA) and was kept under refrigerated condition (4˚C). The initial moisture and fat content of the oat flour was 8.73% (w.b.) and 5%, respectively. The moisture content levels of the oat flour were chosen based on the preliminary trials. It was hard to operate the extruder when the moisture content was below 14%, however, above 26%, the oat flour looked like a dough and was no longer a low moisture food. The range of fat content in the oat flour (5 to 15%) and the temperature of the extruder (65-95°C) were selected based on the industry inputs as well as to represent the worst-case scenario. Therefore, the moisture and fat contents of the oat flour were adjusted from 14 to 26% (w.b.) and 5 to 15% (w/w), respectively, by adding a calculated amount of water and vegetable oil (Wal-Mart Stores, Inc., Bentonville, AR) based on their natural moisture and fat content. The ingredients (flour, oil, and water) were blended in a commercial kitchen aid mixer (Model C-100, Hobart

Corporation, Troy, OH) for 10 min at the lowest speed. The prepared samples were then packed in the whirl pack bags and were kept overnight under refrigerated conditions (4˚C) for moisture stabilization. The moisture content of the prepared samples was measured using a Halogen Moisture Analyzer HR73 (Mettler Toledo, Greifensee, Switzerland) at 105˚C.

4.3.2 Extrusion process

Extrusion cooking of the prepared sample was carried out in a single screw extruder (Brabender Intelli-Torque, South Hackensack, NJ). The extruder screw had a uniform flight with a pitch of 19 mm, a compression ratio of 3:1, and a length/diameter ratio of 20:1. The extruder was connected to an external feeder (Brabender Technologie, Ontario, CA) whose screw speed was controlled by a frequency controller. The extruder was starve-fed (75% barrel fill) in order to replicate the industrial conditions. Verma et al. (2018a) identified the feed rates at 75% barrel fill for different screw speeds, fat, and moisture contents.

The single screw extruder consisted of three zones where the temperature of zone 1 and zone 2 was maintained at 50˚C, and the temperature of zone 3 was adjusted to attain the desired temperature of the product when exiting the extruder (65 and 95˚C). The temperature-controlled heating system installed at each zone was used to heat the barrel. The temperature at each zone was monitored using a Type J lead thermocouple (accuracy = ±2.2˚C), and the temperature of the product was monitored by a temperature transducer (C.W. Brabender Instruments, South Hackensack, NJ) which was

installed in the die face. An extruder software, WINEXT, was used to monitor the steady-state condition as well as record the data. The product was allowed to free flow (no die) without any obstruction, which resulted in the creation of minimal back-pressure inside the barrel. Therefore, this study investigates conservative conditions due to the use of lower temperatures, lower moisture levels, higher fat levels, and no back-pressure.

4.3.3 Residence time measurement

The extrusion was performed at a steady-state condition for each extrusion run, as defined by the experimental design in Section 4.3.4. To achieve a steady-state condition, the oat flour was fed continuously. The stable values of product temperature and torque were used to determine the steady-state condition. The mean residence time was determined by introducing 0.5 g of Congo red tracer (Sigma-Aldrich Co., St. Louis, MO) at the feeding zone of the extruder, and the timer was started. Extruded samples were collected every 10 s interval for 5 min until the dye was no longer visible. A total of 30 samples were collected for each treatment. Each collected sample was ground, and the color (L^*= lightness (positive) and darkness (negative); a^*= redness (positive) and greenness (negative); b^*= yellowness (positive) and blueness (negative)) was measured using a colorimeter (Model BC-10, Minolta Co. Ltd., Osaka, Japan). The ground sample was placed in a container, the top surface was flattened with a spatula, and the color was measured at five random locations. A white tile was used to calibrate the colorimeter before the measurements were taken. The mean residence time; mean

time spent by material in the extruder, was calculated by using the redness color approach rather than the concentration (Peng et al., 1994)

$$\bar{t} = \frac{\sum_0^\infty ta(t)\Delta t}{\sum_0^\infty a(t)\Delta t}$$

where $\bar{t}$ is the mean residence time (s); $a(t)$ is the redness color value measured at the outlet at time, t; Δt is the time interval

4.3.4 Experimental design and statistical analysis

A split-plot central composite design is usually applied to the experiments that involve variables that are hard to change. Similarly, in this experiment, the temperature was one factor that is hard to modify, which makes it challenging to stabilize the extruder. Therefore, using a split-plot design was appropriate to conduct this study with the temperature being used as a whole-plot factor and other independent variables such as moisture content, fat content, and screw speed was employed as split-plot factors. The different levels of all the independent variables used in this study were calculated depending on the coded levels -α, -1, 0, 1, and α (α =1.633) which has been described in detail in Verma et al. (2018a).

A response surface model was developed to evaluate the effect of independent variables on mean residence time. The final model included the terms that have a significant impact ($p < 0.05$) along with their lower-order components. The response surface model was fit using SAS 9.4 (SAS Institute, Cary, NC), and contour plots were generated by MATLAB R2018b (The MathWorks, Inc., Natick, MA).

4.4 Results and Discussions

4.4.1 Response surface model

A second-order response model was generated for the mean residence time of oat flour in a single screw extruder at different temperatures, moisture contents, fat contents, and screw speeds evaluated in this study. The coefficient of determination was used to verify the model fit. The developed model fits well with $R^2 = 0.92$. The final intercept in the response surface equation was calculated by averaging the estimate of all the blocks and then adding it to the intercept presented in Table 4.1. The response equation for the mean residence time is shown in Equation 4.1.

$$\bar{t} = 225.927 + 4.269^{*}F - 7.261^{*}M - 0.542^{*}S + 0.194^{*}M^2 - 0.210^{*}F^2 + 0.001^{*}S^2 \quad (4.1)$$

$\bar{t}$ = Mean residence time (s)

F = Fat content (% w/w)

M = Moisture content of the sample before extrusion (%, wet basis)

S = Screw speed (rpm)

The response surface equation developed for the mean residence time is specific to an extruder and the product matrix. Therefore, this response surface model needs to be determined for each extruder and each type of product matrix, which makes it difficult for the industry to use. However, this study provides a baseline for the food industry to perform their validation study on the industrial-scale extruders.

Table 4.1 shows the significance of each coefficient. The developed response surface model showed a significant linear and quadratic effect of moisture content, fat content, and screw speed on the mean residence time. An increase in temperature from 65 to 95°C did not significantly ($p > 0.05$) affect the mean residence time of the oat flour. Therefore, the temperature was not considered while developing the final model. The non-significant effect of temperature on the mean residence time has also been reported by Altomare and Gossi (1986) and Gogoi and Yam (1994). The larger the magnitude of *t*-value, the more significant is the corresponding coefficient (Khuri and Cornell, 1987). The *t*-values given in Table 4.1 indicated that the screw speed has the highest significant effect on the mean residence time. Moreover, none of the interactions of temperature with the other parameters were significant and therefore were not included in the response surface model as well.

a) Screw speed

The effect on the mean residence time due to the screw speed is shown in Figure 4.1 at different levels of fat (5 to 15%) and moisture (14 to 26%) contents. Screw speed plays an important role during the extrusion process, which affects the mean residence time substantially (Ganjyal and Hanna, 2002). A significant ($p < 0.05$) linear as well as a quadratic effect of screw speed has been reported in the response surface model (Equation 4.1). In general, increasing the extruder screw speed from 100 to 200 rpm reduced the mean residence time of the oat flour in the extruder. At conditions such as 5% fat content and 14% moisture content, when the screw speed went from 100 to 200

rpm, the mean residence time dropped by approximately 22%. A significant ($p < 0.05$) drop in the mean residence time was seen from 140 to 108 s as the screw speed increased 100 to 200 rpm depending on the fat content and moisture content of the oat flour.

Our results remain consistent with several studies which have reported the similar negative effect of screw speed on the mean residence time (Ainsworth et al., 1997; Davidson et al., 1983; Iwe et al., 2001; Nwabueze and Iwe, 2010; van Zuilichem et al., 1988; Yu et al., 2014). Altomore and Ghossi (1986) mentioned in their study that screw speed had a strong impact on the mean residence time during the twin screw extrusion of rice flour. Ollett et al. (1989) published in their study that the residence time of wheat starch decreased by approximately 50% when the screw speed of twin screw extruder increased from 75 to 300 rpm. Singh and Rizvi (1998) reported that the residence time in the twin screw extruder dropped considerably from 119.2 to 80.8 s when the screw speed was changed from 150 to 200 rpm. However, a further increase in the extruder screw speed from 200 to 250 rpm, decreased the mean residence time moderately from 80.8 to 74.8 s.

b) Moisture content

Figure 4.1 shows the change in the mean residence time due to the moisture content of the oat flour. The mean residence time was significantly ($p<0.05$) affected by increasing the moisture content from 14 to 26%. The response surface equation (Equation 4.1) showed that moisture content had a significant linear and quadratic

effect. In Figure 4.1, at 100 rpm and 5% fat content, as the moisture content increased from 14 to 19%, the mean residence time decreased slightly from 132 to 128 s. However, the mean residence time increased as the moisture content was raised from 19 to 26%. For example, an increase in moisture content from 19 to 26% increased the mean residence time from 128 to 136 s. This is due to the quadratic effect of moisture content seen in the response surface equation.

The decrease in mean residence time with an increase in moisture content was also reported by Nwabueze and Iwe (2010). Their study showed that as the moisture content increased from 15 to 27%, the mean residence time decreased by half during the single screw extrusion of African breadfruit mixtures. However, they also reported a slight quadratic effect of moisture content (Nwabueze and Iwe, 2010). This may have occurred potentially due to the slipping of the product at higher moisture content. Yu et al. (2014) also found that increasing the moisture content from 25 to 35% resulted in a decrease in the mean residence time from 87 to 62 s. It was reported that an increase in the moisture content of the sample results in the higher steam pressure which accelerates the movement of the food product, contributing to the lower residence time (Yu et al., 2014).

Conversely, Gomez and Aguilera (1984) reported that an increase in the moisture content from 14 to 32% did not affect the mean residence time significantly during the extrusion of corn starch. van Zuilichem et al. (1988) also reported that moisture content up to 22% did not have an effect on the mean residence time during the single screw

extrusion of maize and soya. Also, a marginal effect of moisture content on the mean residence time was reported by Altomare and Ghossi (1986) and Gogoi and Yam (1994).

c) Fat content

The fat content had a significant ($p < 0.05$) negative linear as well as a quadratic effect on the mean residence time (Equation 4.1). As illustrated in Figure 4.1, an increase in fat content from 5 to 10%, the mean residence time increased from 132 to 136 s at 100 rpm and 14% moisture content. However, under the same conditions, the mean residence time went back to 132 s when the fat content increased from 10 to 15%. This may be due to the quadratic effect of fat content, as seen in the response surface model. While the quadratic effect is statistically significant ($p < 0.05$), the actual change in the mean residence time is very minimal. As the coefficient for the quadratic term is significant (Table 4.1), the term was left in the response surface model to provide a good fit. Similar trends were observed at different moisture contents and screw speeds.

4.4.2 Comparison of response surface models

Table 4.2 shows the comparison of the R^2 values from response surface models (screw speed vs. mean residence time) for the inactivation of *Salmonella* spp. and *E. faecium* NRRL B-2354. Verma et al. (2018a, 2018b) developed a model for *Salmonella* spp. reduction and its surrogate *E. faecium* NRRL B-2354 as a function of various independent variables such as moisture content, fat content, screw speed, and temperature with the R^2 value of 0.83 and 0.84, respectively. The response surface

model developed for the mean residence time in this study (Equation 4.1) was used to model the inactivation of *Salmonella* and *E. faecium* as a function of residence time instead of screw speed along with other parameters. The purpose of this comparison was to evaluate the accuracy of the models by using mean residence time instead of screw speed for the microbial inactivation. The results presented in Table 4.2 showed that the use of mean residence time instead of screw speed slightly improved the R^2 value for the response surface model from 0.83 to 0.85 for *Salmonella* spp. and 0.84 to 0.89 for *E. faecium* NRRL B-2354 inactivation. Nevertheless, a marginal improvement in the accuracy may not call for the use of mean residence time instead of screw speed because it is much easier and convenient for the food industry to use screw speed rather than the mean residence time.

The response surface model developed for the mean residence time in this study and microbial inactivation models developed by Verma et al. (2018a, 2018b) selected all product and process parameters according to the second order central composite design. When screw speed values were replaced with the mean residence time as determined by the response surface model, the mean residence time do not follow the central composite design. Therefore, the verification of this model should ideally be performed. However, the slight improvement in goodness-of-fit does not warrant the use of these newly developed models and therefore, verification becomes irrelevant.

4.5 Conclusions

This research investigated the effect of various extruder parameters (temperature, screw speed) and product composition (moisture content, fat content) on the mean residence time of oat flour in a single screw extruder. Temperature showed a non-significant ($p > 0.05$) effect on the mean residence time, in the range of 65-95°C. Moisture content, fat content, and screw speed showed a significant linear and a slight quadratic effect on the mean residence time. Although, a wide range of process variables and product compositions were tested, the mean residence time changed by only 30% for both 65 and 95°C.

On comparing the R^2 values from response surface models (screw speed vs. residence time) for inactivation of *Salmonella* spp. and *E. faecium* NRRL B-2354, the results showed a higher goodness-of-fit measures when mean residence time was used instead of screw speed. However, the slight improvement in the accuracy of the model may not warrant the use of mean residence time due to the complexity in the determination of mean residence time with high repeatability when compared to screw speed.

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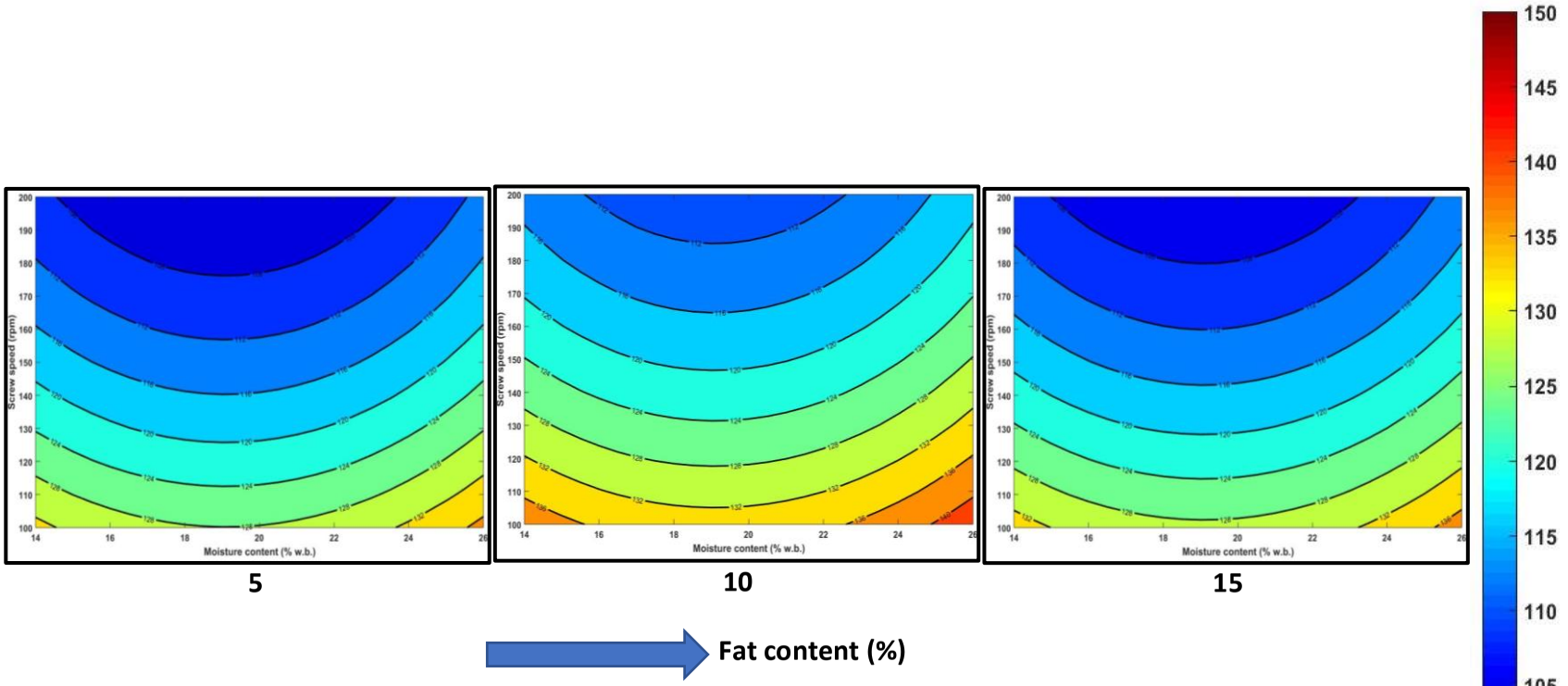

Figure 4. 1 Contour plots showing the effect of moisture content, fat content, and screw speed on the mean residence time during the single-screw extrusion of oat flour. The color bar represents the mean residence time corresponding to the range of colors.

Table 4. 1 Estimates of response surface model parameters for the mean residence time during the single-screw extrusion of oat flour (R^2 = 0.92).

Parameter	Estimate	SE	t-value	P-value
Intercept	228.9425172	20.3887	11.23	<.0001
block(temp) 1 65	-2.0491667	1.7434	-1.18	0.2497
block(temp) 2 65	-7.2949783	1.63082	-4.47	0.0001
block(temp) 3 65	-0.1861667	1.7434	-0.11	0.9157
block(temp) 4 95	-1.6736667	1.7434	-0.96	0.3453
block(temp) 5 95	-6.8911033	1.63082	-4.23	0.0002
block(temp) 6 95	0	.	.	.
F	4.2686956	1.27234	3.35	0.0023
M	-7.2610926	1.75384	-4.14	0.0003
S	-0.5417816	0.0845	-6.41	<.0001
M*M	0.1944359	0.04366	4.45	0.0001
F*F	-0.2100159	0.06289	-3.34	0.0024
S*S	0.0011647	0.00028	4.18	0.0003

F = Fat content (%); M = Moisture content (% wb); S = Screw speed (rpm)

SE = Standard error

Table 4. 2 Comparison of R^2 values from response surface models (screw speed vs. mean residence time) for *Salmonella* spp. and *E. faecium* NRRL B-2354.

Microorganism	With screw speed	With mean residence time
***Salmonella* spp.**	-7.9212 + 0.342*T – 0.268*F – 1.007*M + 0.0762*S – 0.0011*S*T + 0.0277*M^2 (R^2 = 0.83)	41.073 – 0.404*T – 0.270*F – 0.783*M – 0.327*$\bar{t}$ + 0.022*M^2+ 0.005*$\bar{t}$*T (R^2 = 0.85)
***Enterococcus faecium* NRRL B-2354**	4.458 + 0.243*T – 1.06*F – 1.099*M – 0.008*S + 0.047*F*M + 0.0349*F^2 + 0.014*M^2 – 0.009*F*T (R^2 = 0.84)	-5.695 + 0.243*T – 1.212*F – 0.546*M + 0.035*$\bar{t}$ + 0.047*F*M + 0.042*F^2 – 0.009*T*F (R^2 = 0.89)

T = Temperature (°C); M = Moisture content (%, wb); F = Fat content (%); S = Screw speed (rpm); $\bar{t}$ = Mean residence time (s)

Chapter V: Thermal inactivation kinetics of *Salmonella* and *Enterococcus faecium* NRRL B-2354 on dried basil leaves

5.1 Abstract

The enhanced heat resistance of *Salmonella* developed at low water activity makes it a serious challenge to eliminate them during thermal processing. The objectives of this research are to (i) investigate the effect of water activity on thermal inactivation of *Salmonella* cocktail (Agona, Tennessee, Mbandaka, Montevideo, and Reading) in dried basil leaves, and (ii) evaluate *Enterococcus faecium* NRRL B-2354 as an appropriate surrogate for *Salmonella* in dried basil leaves. Dried basil leaves, inoculated with a *Salmonella* cocktail and *E. faecium* separately, were equilibrated to different water activities (a_w: 0.40, 0.55, and 0.70) in a humidity-controlled chamber. The basil samples were packed (1.6±0.1 g) in aluminum pouches and thermally treated at 70, 75, and 80°C using a dry heating method for 0 to 180 minutes to obtain the thermal death curve. The microbial survival data was fit using two primary models (Log-linear and Weibull model). Results from AIC_c showed that the log-linear model fits well for thermal inactivation of both microorganisms. As the a_w decreases from 0.70 to 0.40 at 75°C, the *D*-value increases from 3.30 to 9.14 min for *Salmonella* and 6.53 to 14.07 min for *E*. *faecium*. Based on the AIC_c values, the modified Bigelow model fits the *D*-values better than the response surface model for both the microorganisms. The kill ratio of surrogate to pathogen ranged from 1.4 to 2.8, indicating that it is a conservative surrogate for *Salmonella* for performing validation of the thermal pasteurization process. The

identification of suitable surrogate and development of modified Bigelow model will help the spice industry in developing the thermal processes for improving the safety of basil leaves.

Keywords: Validation; spices; surrogate; thermal lethality; low moisture food; *Salmonella*

5.2 Introduction

Foodborne pathogens are a serious public health concern worldwide. According to an Annual Report by U.S. Centers for Disease Control and Prevention (CDC), in 2017, a total of 841 foodborne outbreaks were reported, which resulted in 14,481 illnesses, 827 hospitalizations, and 20 deaths (CDC, 2019). In the same report, *Salmonella* was listed as the second most common microorganism responsible for 29% outbreaks and 34% illnesses.

Throughout history, low moisture food products like flour, spices, nuts, herbs have been considered as low-risk commodities for microbial contamination. This is due to the low water activity ($a_w < 0.70$; Blessington et al., 2013) of these foods, which acts as a hurdle for the growth of pathogenic bacteria. However, low moisture foods have been repeatedly involved in numerous product recalls and outbreaks. Between 2006 to 2019, 26 salmonellosis outbreaks were reported to be associated with the consumption of contaminated low moisture foods like nuts (CDC, 2009a; 2014; 2016a), spices (CDC, 2010), peanut butter (CDC, 2009b), pet food (CDC, 2008a), and puffed cereals (CDC,

2008b). Therefore, *Salmonella* is an emerging issue in low moisture foods, as reflected by different recalls and outbreaks.

Sweet Basil (*Ocimum basilicum*) is reported to be an essential culinary herb that has antimicrobial and antifungal properties (Meyers, 2003). In 2007, fresh basil was found to be associated with a *Salmonella* Senftenberg outbreak. The outbreak started in the United Kingdom with 55 human cases, and eventually spread to the United States in 2008 and caused 1,019 illnesses (Pezzoli et al., 2007). Pathogen such as *Salmonella* is present in the environment, and therefore it can penetrate the internal plant tissues via the root and survive inside plants in the field (Gorbatsevich et al., 2013). High moisture foods such as fresh basil leaves (>80% wet basis) are known to have a short shelf life and can shelter pathogenic bacteria like *Salmonella,* where they can survive and multiply faster (Olaimat and Holley, 2012; Ziuzina et al., 2014). A forced-air drier (≤120°F/50°C) is generally used to dry the fresh basil leaves slowly. The slow drying process maintains the color of dried basil, aroma, and high oil content (Bączek et al., 2019). Even though the quality of dried basil is maintained, its microbial safety is compromised because the drying method used is not a pasteurization process. Dried basil leaves combined with other spices and herbs may be added to soups, stews, vegetables, chicken, and fish after the cooking step (Meyers, 2003). Because there may be no additional cooking step involved, the presence of any pathogenic bacteria in dried basil leaves may pose a severe public health risk.

The U.S. Food and Drug Administration (FDA) Food Safety Modernization Act passed in 2011 requires the food industry to validate their process control and document all the data in the validation report (Brackett et al., 2014). Usually, thermal methods are used to mitigate the bacterial population in low moisture foods (Anderson, 2018; Duncan et al., 2017; Lathrop et al., 2014; Verma et al., 2018b; Wei et al., 2019; Zhang et al., 2020). However, at desiccated conditions, pathogenic bacteria like *Salmonella* are known to develop enhanced heat resistance, which compromises the effectiveness of the thermal process (Podolak et al., 2010). Therefore, it is essential to determine the thermal inactivation kinetics of pathogenic bacteria in a specific food matrix before conducting the process validation study.

To conduct an in-plant validation study, it is often recommended to identify and use a suitable non-pathogenic surrogate, considering that the introduction of a pathogen into the facility would be undesirable. *Enterococcus faecium* NRRL B-2354 has been identified as a valid surrogate for various low moisture foods such as oat flour (Verma et al., 2018a), black peppercorns (Wei et al., 2019), cumin seeds (Chen et al., 2019), white pepper (Ozturk et al., 2020), cocoa powder (Tsai et al., 2019), and wheat flour (Liu et al., 2018). However, the surrogates are specific to product matrix and process and currently, there is no information available in the literature on the acceptability of *E. faecium* NRRL B-2354 as an ideal surrogate for *Salmonella* in dried basil leaves.

Therefore, the objectives of this research were to (i) investigate the effect of water activity and temperature on thermal inactivation of *Salmonella* spp. in dried basil leaves,

and (ii) evaluate the suitability of *Enterococcus faecium* NRRL B-2354 as an appropriate surrogate for *Salmonella* spp. in dried basil leaves.

5.3 Materials and Methods

5.3.1 Dried basil leaves

Three production batches of steam-sterilized dried sweet basil leaves (Hsieh et al., 1989) were supplied by McCormick & Company, Inc. (Hunt Valley, MD) and stored at ambient conditions due to extended shelf life. The dried basil leaves were in the form of the crushed sample instead of whole leaves. To improve the repeatability, the steam-sterilized samples with negligible background microflora were used in this study. Upon receiving, the samples were tested for water activity, moisture content, and background microflora (aerobic plate counts). The water activity and moisture content of dried basil were determined using a dew point water activity meter (Model: 4TE, Meter Group, Pullman, WA) and halogen moisture analyzer (Model: HR73, Mettler Toledo, Greifensee, Switzerland), respectively. Five 25-g samples were taken from random locations to test the background microflora present in the dried basil leaves. Each sample was diluted with 225 mL of 0.1% buffered peptone water (BPW; Difco, Sparks, MD) and plated onto tryptic soy agar supplemented with 0.6% (w/w) yeast extract (TSAYE, Difco, Sparks, MD) with incubation at 37°C for 24±2 h. This procedure was repeated for the other two batches of dried basil leaves.

5.3.2 Bacterial strains

A cocktail of five serotypes of *Salmonella enterica* (NACMCF, 2010) previously associated with outbreaks and recalls in low moisture foods was used in this study. A non-pathogenic bacterium, *Enterococcus faecium* NRRL B-2354, was used to conduct the surrogate study. Table 5.1 presents detailed information about these strains. For long-term preservation, all the isolates were stored as a frozen stock at -80°C in tryptic soy broth (TSB; Difco, Sparks, MD) supplemented with 40% (v/v) glycerol.

5.3.3 Inoculum preparation

A frozen stock of each strain (*Salmonella* spp. and *E. faecium*) was taken out of the ultrafreezer (-80°C) and thawed at room temperature for 10 min. This frozen stock was used to prepare the working stock plates that were eventually used to prepare the inoculum. The working stock plates for each strain was prepared by adding 1 mL of frozen culture to 10 mL TSB and incubated for 24±2 h at 37°C. To obtain the isolated colonies, a loopful (10 µL) of the overnight culture was streaked onto TSAYE agar plates with incubation at 37°C for 24±2 h. The parafilm wrapping film (PM-999; Bemis) was used to cover the prepared working stock plates and stored in a refrigerator at 4°C. These plates were used within 30 days to prepare the inoculum.

One isolated colony (each *Salmonella* strain or *E. faecium*) from the working stock plate was transferred to tryptic soy broth supplemented with 0.6% yeast extract (TSBYE; Difco, Sparks, MD) and incubated at 37°C for 24±2 h. The bacterial lawn was created for each strain by spread plating 0.1 mL of overnight broth culture onto TSAYE agar plates

and incubated for 24±2 h at 37°C. Lastly, the lawns were harvested by adding 3 mL of BPW to each agar plate and agitating the bacterial cells using an L-shaped spreader. The *Salmonella* cocktail was prepared by adding each strain in equal amount into a 15 mL sterile conical tube and vortexed for 30 s to homogenize the mixture.

5.3.4 Basil inoculation with *Salmonella* and *E. faecium*

The inoculation activities were carried out in the biosafety cabinet. Dried basil leaves (100±0.1 g) were aseptically transferred to a sterile whirl pak bag to which 2 mL of either *Salmonella* cocktail or *E. faecium* inoculum was sprayed. The whirl pak bag was carefully closed, and the sample was hand-massaged for 10 min. The inoculated sample was then placed on a sterile aluminum tray (230 x 300 x 15 mm) and transferred to a custom-designed relative humidity chamber. The humidity of the chamber was set to different levels in order to condition the samples to three water activity levels: 0.40, 0.55, and 0.70. The water activity of the sample was measured before the inactivation study was conducted.

5.3.5 Homogeneity and viability of inoculum

It is essential that the bacteria are homogeneously distributed within the sample and physiologically adapted to the low water activity environment before subjecting the samples for thermal treatment. Therefore, the homogeneity and viability of both microorganisms were evaluated for each batch for 15 days. This was done by taking three 3±0.1-g samples from the humidity chamber on 0, 1, 2, 3, 6, 9, 12, and 15 days. Each sample was diluted with 27 mL of BPW in a whirl pak bag and was stomached for

60 s. The homogenized fluid was serially diluted, and appropriate dilutions were spread plated in duplicate onto differential media. For *Salmonella*, TSAYE supplemented with 0.03 (w/v) sodium thiosulfate (Fisher Scientific, Fair Lawn, NJ), and 0.05% (w/v) ammonium iron citrate (Sigma Alrich, St. Louis, MO) (m-TSA) was used, whereas, for *E. faecium*, TSAYE supplemented with 0.05% (w/v) ammonium iron citrate and 0.025% (w/v) esculin hydrate (Acros, NJ) (e-TSA) was used. The black colonies observed on m-TSA and e-TSA were considered presumptive *Salmonella and E. faecium*, respectively. The standard deviation of the bacterial population among the three subsamples of <0.3 log CFU/g indicated a homogeneous distribution of inoculum (Hildebrandt et al., 2020).

5.3.6 Thermal treatment and bacterial enumeration

A thermal death time sandwich system, designed and developed by Lau and Subbiah (2020), was used to conduct the thermal inactivation process. The thermal treatments were performed at 70, 75, and 80°C for *Salmonella* and *E. faecium*. The inoculated and pre-conditioned dried basil samples (1.6±0.1 g) were packed in a sterile aluminum pouch (75 x 75 x 1 mm) and hermetically sealed to prevent any moisture loss during the thermal treatment. The come-up time (CUT; time required for the center of the sample to reach 0.5°C below its target temperature) was measured by inserting a T-type thermocouple into the center of an aluminum pouch filled with uninoculated basil sample (1.6±0.1 g). CUT was measured at each temperature and was referred to as time zero. A total of six pre-determined time points (including time zero) were used,

depending on the temperature, to achieve a 3-5 log reduction of *Salmonella* and *E. faecium*.

At a given temperature, for every time point, one pouch was removed from the sandwich system and immediately immersed in an ice water bath for 2 min to stop the thermal inactivation. The sample (1.6±0.1 g) was diluted with 14.4 mL of BPW and homogenized in a stomacher for 60 s. The fluid was serially diluted using 9 mL dilution tubes, and appropriate dilutions were spread plated in duplicate onto m-TSA for *Salmonella* and e-TSA for *E. faecium*. The entire experiment was replicated on the two more production batches of dried basil leaves.

5.3.7 Comparison of inactivation models

a) Primary models

Two different inactivation models, Log-linear (Eqn. 5.1) and Weibull (Eqn. 5.2) (Peleg, 2006), were used to fit the inactivation data of *Salmonella* and *E. faecium* in dried basil leaves:

$$\log_{10}\left(\frac{N}{N_0}\right) = -\frac{t}{D} \tag{5.1}$$

$$\log_{10}\left(\frac{N}{N_0}\right) = -\left(\frac{t}{\delta}\right)^{\alpha} \tag{5.2}$$

where N_0 is the initial microbial count (CFU/g); N is the number of microbial survivors (CFU/g) at time t; t is the treatment time (min); D is the decimal reduction time (min; time required to reduce the bacteria by 1-log at a given temperature, °C); δ is

the time taken for 1st log reduction (min); α is the shape parameter and describes the linear (α =1) or concavity/convexity (α<1 or α>1) of the curve.

After fitting the survivor data, the following statistical indices were calculated to evaluate the goodness-of-fit of the log-linear and Weibull model (Motulsky & Cristopoulus, 2004).

Root Mean Square Error: $$RMSE = \sqrt{\frac{\sum_{i=1}^{n}\left[\log_{10}\left(\frac{N}{N_{o\,(data,i)}}\right)-\log_{10}\left(\frac{N}{N_{o\,(model,i)}}\right)\right]^2}{n}} \quad (5.3)$$

where $\log_{10}\left(\frac{N}{N_{o\,(data,i)}}\right)$ is the measured bacterial log reduction (CFU/g); $\log_{10}\left(\frac{N}{N_{o\,(model,i)}}\right)$ is the predicted bacterial log reduction (CFU/g); n is the number of observations.

Akaike Information Criterion: $$AIC_c = n\ln\left(\frac{SS}{n}\right) + 2K + \frac{2K(K+1)}{n-K-1} \quad (5.4)$$

where n is the number of observations; SS is the error sum of squares; K is the number of parameters plus 1. AIC_c serves as a tool to justify if the decrease in residual sum of squares is related to the addition of parameters in a model (Smith et al., 2016). A lower value of AIC_c indicates that the chosen model is correct for the data. Therefore, AIC_c was selected as an indicator for the evaluation of primary models. A freeware tool, GInaFiT (Geeraerd and Van Impe Inactivation Model Fitting Tool), was used to fit both the primary models (Geeraerd et al., 2005).

b) Secondary models

Two different secondary models, response surface model (Eqn. 5.5) and modified Bigelow model (Eqn. 5.6; Gaillard et al., 1998), were developed for the selected primary model to evaluate the effect of temperature and water activity on the *D*-values.

$$D_{(T,a_w)} = \beta_o + \beta_1 * T + \beta_2 * a_w + \beta_3 * T^2 + \beta_4 * a_w^2 + \beta_5 * T * a_w \quad (5.5)$$

where β_o is the overall intercept; β_1 is the linear regression coefficient for temperature, T ; β_2 is the linear regression coefficient for water activity, a_w; β_3 is the quadratic regression coefficient for temperature; β_4 is the quadratic regression coefficient for water activity; β_5 is the linear regression coefficient for the interaction of temperature and water activity; a_w is the water activity; T is the temperature (°C). The significance of each variable and its interactions was tested at $p < 0.05$. The terms that had a significant effect on the *D*-value were used to develop the final response surface model. A statistical package, SAS version 9.4 (SAS Institute, Cary, NC), was used to test the significance of the terms and fit the final response surface model.

$$D_{(T,a_w)} = D_{ref}.10^{\frac{T_{ref}-T}{z_T}}.10^{\frac{a_{w\,ref}-a_w}{z_{a_w}}} \quad (5.6)$$

where T_{ref} and $a_{w\,ref}$ are the optimized reference temperature and water activity; D_{ref} is the time required to achieve 1-log reduction of bacteria at T_{ref} and $a_{w\,ref}$; T is the temperature (°C); a_w is the water activity; z_T is the increase in temperature required to decrease the *D*-value by 1-log; z_{a_w} is the increase in water activity required to decrease the *D*-value by 1-log. The analysis for Bigelow model was performed in

MATLAB version 2016b (The Mathworks, Inc., Natick, MA) using the ordinary least square (OLS) minimization with *nlnfit*. AIC_c and RMSE were used to evaluate the performance of both the secondary models.

5.4 Results and Discussions

5.4.1 Basil inoculation

The initial moisture content and water activity of dried basil leaves were 10.18±0.39% wet basis (w.b.) and 0.549±0.002, respectively. Therefore, a_w = 0.55 was used as the natural water activity of the dried basil leaves. The effect of water activity on the thermal resistance of *Salmonella* and *E. faecium* was determined by adjusting the water activity of dried basil leaves to two additional levels (low and high): 0.40 and 0.70. Based on our preliminary studies (data not shown), the water activity of the thermally treated sample was around 0.42 due to the moisture loss. Therefore, a_w = 0.40 was selected as a lower level and higher level of water activity was also selected to develop a model. The specific values of water activity were chosen after discussing it with the industry partner. The moisture content of dried basil leaves at a_w = 0.40 and 0.70 was measured as 9.26±0.23% and 12.44±0.09% respectively. Before the inoculation process, the background microflora in basil was also tested. The results indicated that the aerobic plate count in the dried basil leaves was <10 CFU/g.

The bacterial counts (day 0) of *Salmonella* and *E. faecium* in basil were at a level of 8.38±0.02 and 7.91±0.06 log CFU/g, respectively (Figure 5.1). The inoculated samples were then placed in the relative humidity chamber for 15 days to achieve the desired

water activity and allow the bacteria to acclimatize to the new environment. Pathogenic bacteria like *Salmonella* require $a_w > 0.92$ to multiply and grow in the food product. However, the introduction of bacteria in low water activity foods ($a_w < 0.70$; Blessington et al., 2013; Esbelin et al., 2018) creates a stressful environment for which a higher bacterial reduction is usually achieved when a microbial challenge study is conducted immediately after inoculation (Jeong and Kang, 2014). Therefore, it is imperative to expose the bacteria to the desiccated environment for a few days, allowing them to stabilize before the microbial challenge study is performed. This may lead to the higher heat resistance of bacteria and simulates more realistic industrial conditions (Wei et al., 2019).

The inoculation method also plays a critical role in the stability and thermal resistance of bacteria in low moisture foods. Hildebrandt et al. (2016) studied the influence of different inoculation methods on the stability and thermal resistance of bacteria in wheat flour. The study concluded that the lawn based method (wet inoculation) could provide a stable population of bacteria with repeatable *D*-values. Therefore, the wet inoculation method was preferred over dry inoculation to conduct this study. The wet inoculation method has been used to inoculate various low moisture foods such as oat flour (Anderson et al., 2017; Verma et al., 2018a; 2018b), carbohydrate-protein meal (Bianchini et al., 2012), cumin seeds (Chen et al., 2019), and black peppercorn (Wei et al., 2019).

Figure 5.1 shows the results from the viability test of *Salmonella* and *E. faecium* in dried basil leaves monitored for 15 days. It can be noted that on day 1, the *Salmonella* population in basil dropped considerably (ca. 0.5 log CFU/g), whereas *E. faecium* population dropped only by 0.2 log CFU/g. However, from day 5 to 15, *Salmonella* and *E. faecium* population did not change significantly, and a higher bacterial count (*Salmonella*: 7.61±0.08 log CFU/g; *E. faecium*: 7.73±0.13 log CFU/g) was achieved prior to the thermal inactivation study. Therefore, the inoculated samples were allowed to stabilize for at least five days before the thermal inactivation study was conducted. Wei et al. (2019) and Chen et al. (2019) also reported in their studies that storing the inoculated samples for five days in the relative humidity chamber (for the desired a_w) resulted in a stable population of bacteria in black peppercorn and cumin seeds, respectively. The homogeneous distribution of inoculum in the dried basil leaves was also tested. The error bars in Figure 5.1 represent one standard deviation among the three subsamples tested on each day. A standard deviation of <0.3 log CFU/g among the subsamples confirmed that the inoculation method used resulted in a homogeneous distribution of inoculum. However, a standard deviation of ≥0.3 log CFU/g would mean a heterogeneous or non-uniform distribution of inoculum in the sample.

5.4.2 Thermal resistance of *Salmonella* and *E. faecium*

a) Primary models

The CUTs in dried basil leaves were found to be 98, 100, and 94 s at 70, 75, and 80°C, respectively. Table 5.2 presents the calculated *D*-value (Log-linear), δ- and α-value

(Weibull) for *Salmonella* and *E. faecium* NRRL B-2354 at different temperatures and water activities. For the log-linear model, it can be noted that as the temperature and water activity increases, the *D*-value of *Salmonella* and *E. faecium* decreases. For example, at 75°C, the *D*-value of *Salmonella* and *E. faecium* at a_w = 0.40, 0.55, and 0.70 were 9.14, 6.64, and 3.30 min, and 14.07, 9.57, and 6.53 min, respectively. The enhanced thermal resistance of *Salmonella* spp. developed at lower water activity in dried basil leaves is in agreement with the literature published on low moisture foods. Vasquez (2018) reported that the $D_{75°C}$ of *Salmonella* spp. decreased from 42.86 to 1.51 min when the water activity of ground black pepper increased from 0.25 to 0.65. Similarly, Liu et al. (2018) reported that the *D*-value of *Salmonella* Enteritidis at 75°C ranged from 24.5 to 12.0 min as the water activity of wheat flour increased from 0.30 to 0.60.

An inverse relationship of water activity and the δ-value was also observed in the Weibull model as well. For example, at 70°C, as the water activity of the basil increased from 0.40 to 0.70, the δ-value for *Salmonella* and *E. faecium* decreased from 9.78 to 2.35 min and 14.48 to 5.78 min, respectively. Figure 5.2 shows the survival curves for *Salmonella* using log-linear and Weibull model at different temperatures and water activities. Weibull model has been commonly used to describe the non-linear inactivation of microorganisms in low moisture foods such as red pepper powder (Zhang et al., 2020), paprika and cumin powder (Ozturk et al., 2020), wheat flour (Smith et al., 2016), peanut butter (Ma et al., 2009), and almonds (Villa-Rojas et al., 2013).

Villa-Rojas et al. (2013) reported that the thermal inactivation of *Salmonella* Enteritidis PT 30 was explained better by the Weibull model than the log-linear model. However, this conclusion was completely based on the coefficient of determination (R^2). Similarly, other studies have reported the use of RMSE values to explain the efficacy of the primary models (Ozturk et al., 2020; Farakos et al., 2013; Liu et al., 2018). However, Dolan et al. (2013) and Smith et al. (2016) stated that AIC_c should be used as a parameter for evaluating the fit of the primary models as it describes that whether a decrease in residual sum of squares is justified by the addition of parameters to the model. Therefore, in this study, AIC_c was used as an indicator for evaluation of the primary models. Lower AIC_c values indicates a better model to explain the thermal inactivation. According to the AIC_c values reported in Table 5.2, the log-linear model fits well for thermal inactivation of both *Salmonella* and *E. faecium* in dried basil leaves. Thus, the data from the log-linear model was used to generate the secondary models, response surface and modified Bigelow model, to evaluate the effect of temperature and water activity on the *D*-values. The z_T-value of *Salmonella* and *E. faecium* in dried basil leaves at a_w = 0.40, 0.55, and 0.70 were determined to be 11.34, 11.04, 10.68°C and 13.51, 13.88, 13.75°C for log-linear model, respectively (Figure 5.3).

b) Secondary models

A split-plot design analysis with water activity as the whole-plot factor and temperature as the split-plot factor was used to determine the effect of temperature and water activity on the *D*-values of *Salmonella* and *E. faecium*. The first secondary

model, response surface model, was developed for *Salmonella* spp. (Eqn. 5.7) and *E. faecium* (Eqn. 5.8) to evaluate the effect of temperature and water activity on the *D*-values. Only the terms that showed a significant ($p < 0.05$) effect were considered in the final response surface model.

<u>*Salmonella*</u>: $D = 686.4670 - 14.7080^{*}T - 270.5266^{*}a_w + 0.0773^{*}T^2 + 3.3236^{*}a_w{}^{*}T \ \ (AIC_c = 76.15;\ RMSE = 0.60\ min)$ (5.7)

<u>*E. faecium*</u>: $D = 1955.6983 - 44.9972^{*}T - 586.9727a_w + 0.2583^{*}T^2 + 7.2524^{*}a_w{}^{*}T\ (AIC_c = 128.94;\ RMSE = 1.80\ min)$ (5.8)

where D is the D-value (min); T is the temperature (°C); a_w is the water activity of the sample.

The final response surface model showed a significant ($p < 0.0001$) linear and quadratic effect of temperature on the *D*-value of *Salmonella* and *E. faecium*. The water activity of the sample showed a significant ($p < 0.0001$) negative linear as well as its positive interaction effect with temperature on the *D*-values for both the microorganisms. These response surface models can be used by the spice industry to predict the time required to achieve the desired microbial lethality. Figure 5.4 shows the contour plots for *Salmonella* and *E. faecium* using the response surface model.

The following is the second secondary model, modified Bigelow model, developed for *Salmonella* spp. (Eqn. 5.9) and *E. faecium* (Eqn. 5.10):

<u>*Salmonella*</u>: $D = 5.69 * 10^{\frac{75-T}{12.73}} * 10^{\frac{0.55-a_w}{0.86}}$ $(AIC_c = 0.91;\ RMSE = 0.90\ min)$ (5.9)

<u>*E. faecium*</u>: $D = 9.88 * 10^{\frac{75-T}{11.97}} * 10^{\frac{0.55-a_w}{0.73}}$ $(AIC_c = 23.36;\ RMSE = 1.36\ min)$ (5.10)

where D is the D-value (min); T is the temperature (°C); a_w is the water activity of the sample. Figure 5.5 shows the contour plots for *Salmonella* and *E. faecium* using the modified Bigelow model.

AIC_c and RMSE values were used to determine the performance of the secondary models for *Salmonella* and *E. faecium*. A lower value of AIC_c and RMSE indicated that the modified Bigelow model had a relatively better fit compared to the response surface model for both the microorganisms. The results remain consistent with Smith et al. (2016) and Valdramidis et al., (2006) which also reported that Bigelow model fits the inactivation data better compared to the other models tested in their study. Smith et al., (2016) mentioned that the Bigelow model comprises of parameters in the model that have phenomenological meaning unlike the response surface model which contains numerous parameters and is hard to extrapolate outside the ranges tested. The contour line for the value at 4 min for *E. faecium* response surface model (Fig. 5.4b) shows a curvature, which cannot be explained with phenomenological meaning and therefore indicates an overfitting. While the predicted values at measured points are accurate, the interpolated values in the upper (top-right) quadrant would have errors for the *E. faecium* response surface model. Thus, the Bigelow model is more suitable for interpolating the *D*-value of *Salmonella* and *E. faecium*.

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

It is imperative to identify an appropriate surrogate for *Salmonella* to help the food industry with in-plant validation studies. The second part of this research proposed to evaluate *E. faecium* as an appropriate surrogate for *Salmonella* in dried basil leaves. Table 5.2 also shows the comparison of *D*-values of *Salmonella* and *E. faecium* at all the conditions in dried basil leaves using the log-linear model. It can be noted from the graph that *E. faecium* exhibited a higher thermal resistance than *Salmonella* at all three temperatures tested in this study. For example, at a_w = 0.55, the *D*-value of *Salmonella* and *E. faecium* at 70, 75, and 80°C was 15.02, 6.64, and 1.86 min, and 26.84, 9.57, and 5.09 min, respectively.

The applicability of *E. faecium* NRRL B-2354 as a surrogate for *Salmonella* has been well-established for different low moisture foods such as extrusion of oat flour (Verma et al., 2018a) and carbohydrate-protein meal (Bianchini et al., 2014), radiofrequency heating of ground black pepper (Wei et al., 2019), cumin seeds (Chen et al., 2019), white pepper (Ozturk et al., 2020). Because the surrogates are product and process specific, it is necessary to evaluate its suitability in dried basil leaves as well. The *D*-values of *E. faecium* in dried basil leaves were approx. 1.4 to 2.8 times greater than *D*-values of *Salmonella* at all the conditions tested in this study. Therefore, the consistently high thermal resistance indicates that *E. faecium* may be used as a conservative surrogate for *Salmonella* in dried basil leaves as well.

5.5 Conclusion

A log-linear model was used to describe the thermal inactivation of *Salmonella* and *E. faecium* in dried basil leaves. The results from this study showed that water activity plays an essential role during microbial inactivation. As the water activity of the sample decreases, the time required to achieve the desired bacterial reduction increases significantly. Based on AIC_c and RMSE, the Bigelow model performed better compared to the response surface model for the inactivation data of *Salmonella* and *E. faecium*. The output of this study may be used by the spice industry in developing the thermal processes for improving the safety of dried basil leaves. Additionally, industry may use *E. faecium* as a conservative surrogate for *Salmonella* for performing validation of the thermal pasteurization process due to its higher thermal resistance.

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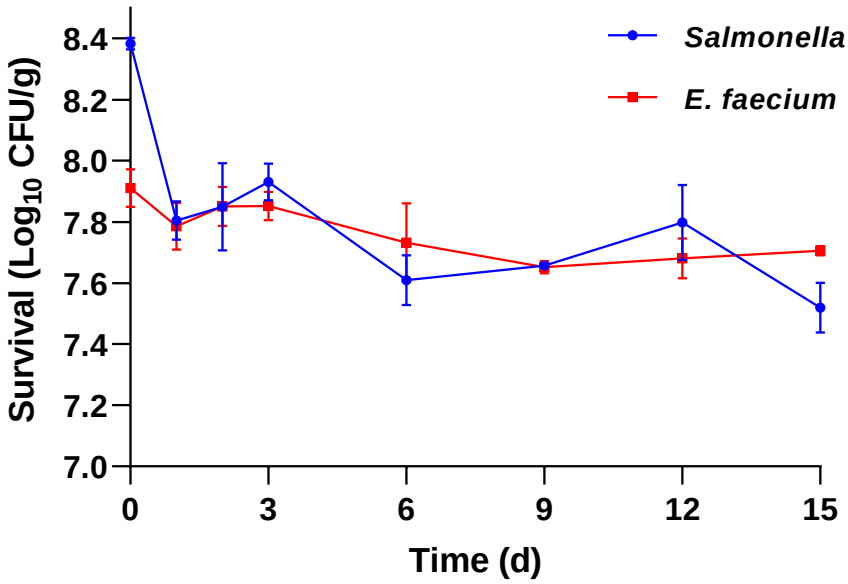


Figure 5. 1 Viability and homogeneity (± one standard deviation as error bars) test of *Salmonella* and *E. faecium* NRRL B-2354 in dried basil leaves for 15 days at a_w=0.55 (n=3).

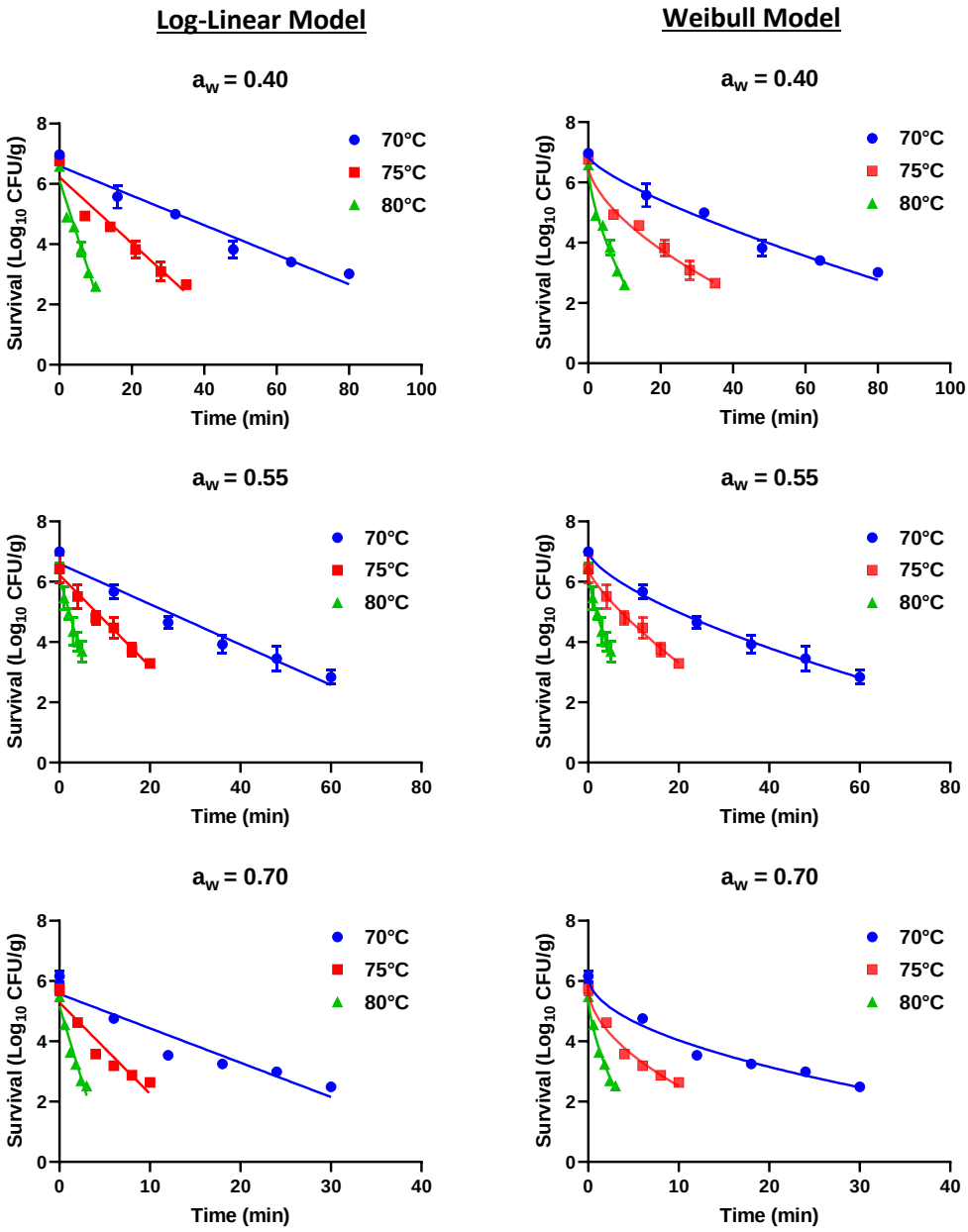


Figure 5. 2 Survival curves for *Salmonella* using log-linear and Weibull model at different temperatures and water activities (n=3). Error bars represent mean ± standard deviation.

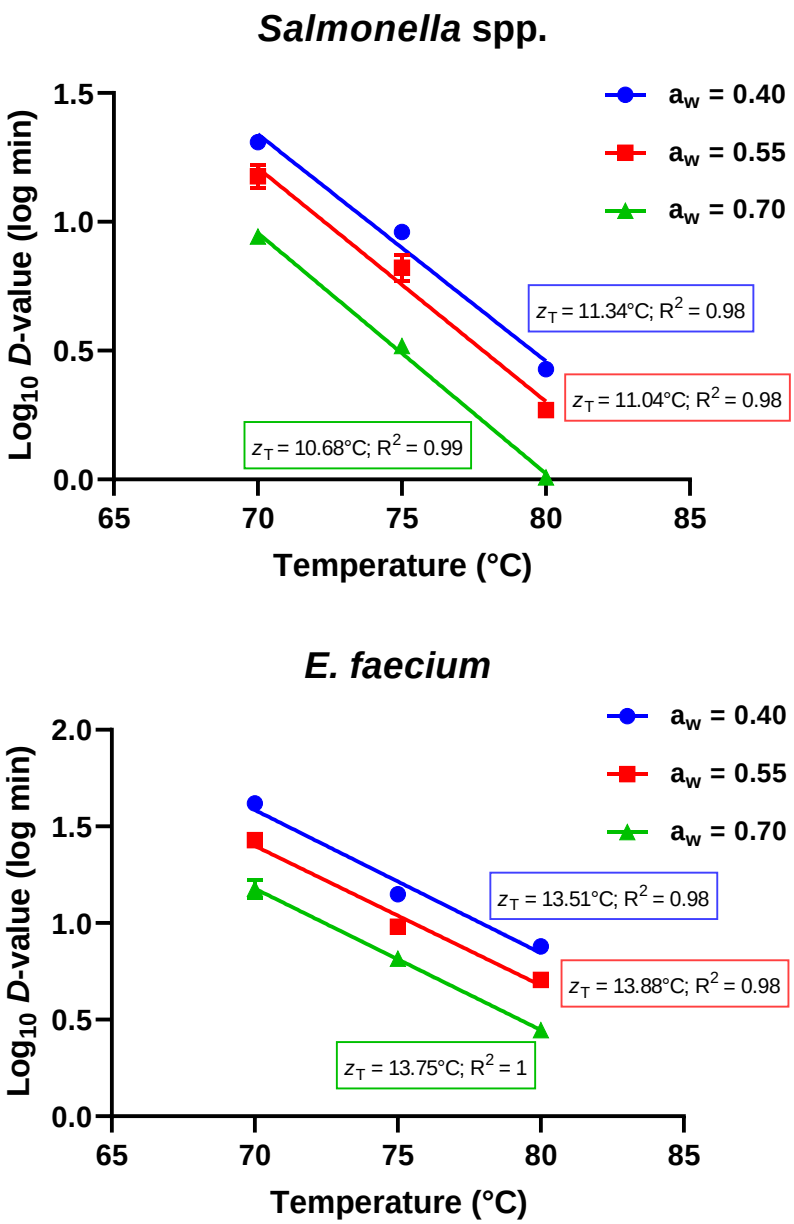
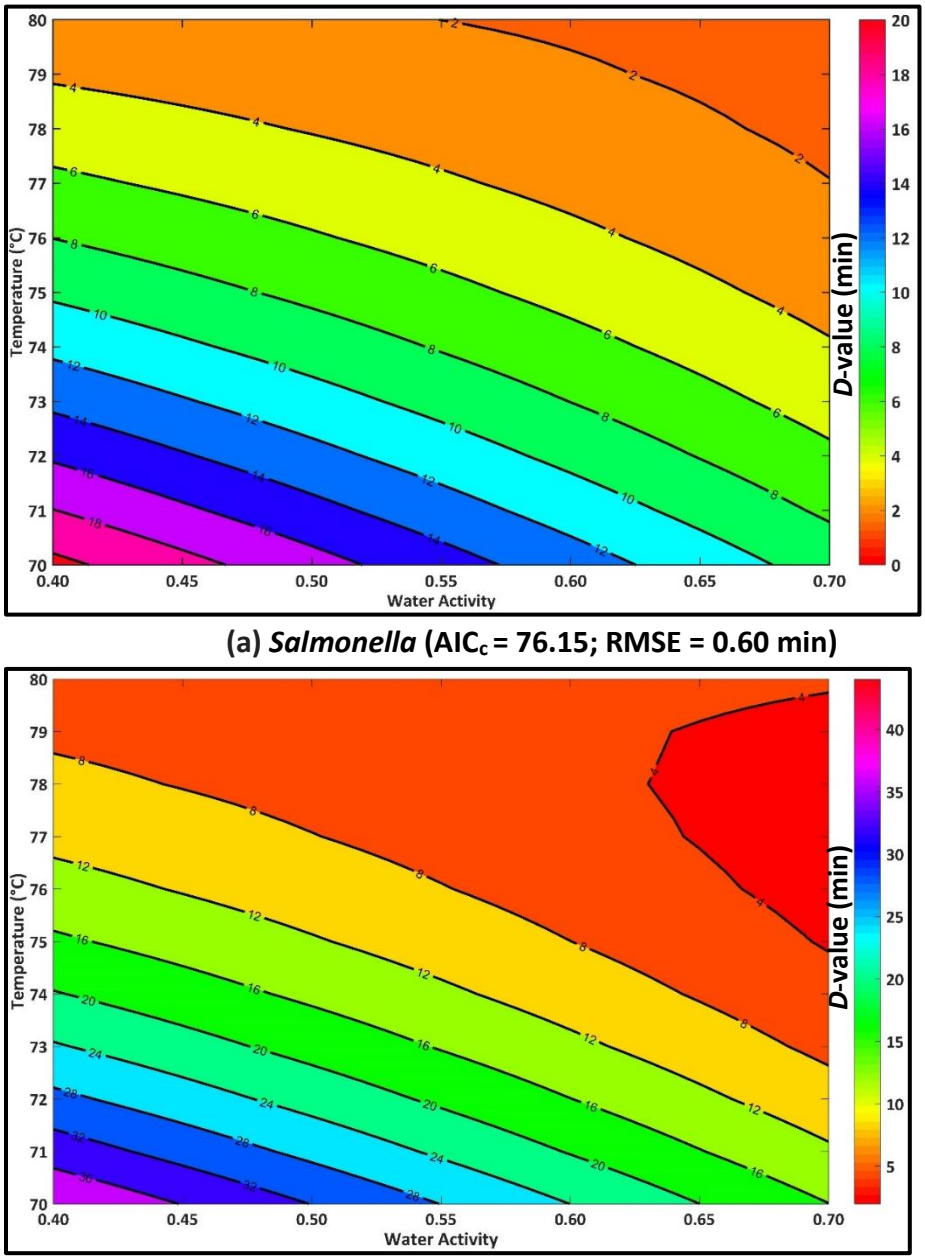


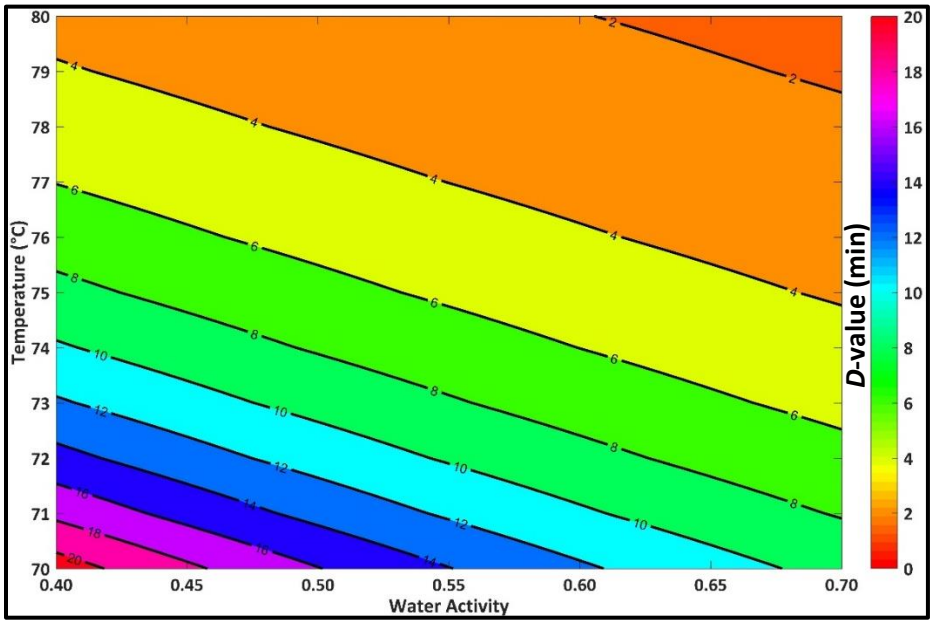
Figure 5. 3 z_T-values of *Salmonella* and *E. faecium* NRRL B-2354 in dried basil leaves at a_w=0.40, 0.55, and 0.70.



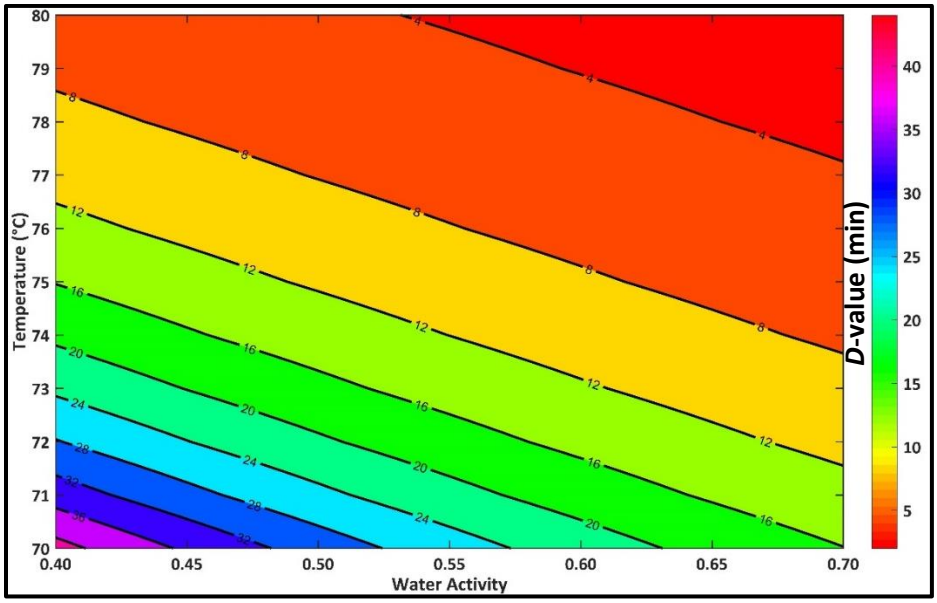
(a) *Salmonella* (AIC_c = 76.15; RMSE = 0.60 min)

(b) *E. faecium* (AIC_c = 128.94; RMSE = 1.80 min)

Figure 5. 4 Contour plots showing the *D*-values for (a) *Salmonella* and (b) *E. faecium* using the response surface model. The color bar represents the *D*-values (min).



(a) *Salmonella* (AIC_c = 0.91; RMSE = 0.90 min)



(b) *E. faecium* (AIC_c = 23.36; RMSE = 1.36 min)

Figure 5. 5 Contour plots showing the *D*-values for (a) *Salmonella* and (b) *E. faecium* using the modified Bigelow model. The color bar represents the *D*-values (min).

Table 5. 1 Bacterial strains used in this study.

Microorganism	**Strain**	**Source**	**Original Isolation Source**
Salmonella Agona	447967	FDA, ORA, Arkansas Reg. Lab, Jefferson, AR (USA)	Oat cereal (CDC, 1998)
Salmonella Mbandaka	698538	FDA, ORA, Arkansas Reg. Lab, Jefferson, AR (USA)	Sprouts (Jackson et al., (2013))
Salmonella Montevideo	488275	FDA, ORA, Arkansas Reg. Lab, Jefferson, AR (USA)	Black and red pepper (CDC, 2010)
Salmonella Reading	*Moff* 180418	FDA culture collection, Bedford Park, IL (USA)	Alfalfa sprouts (CDC, 2016b)
Salmonella Tennessee	K4643	Dr. L. Beuchet, University of Georgia, Griffin, GA (USA)	Peanut butter (CDC, 2007)
Enterococcus faecium	NRRL B-2354	USDA, ARS, Peoria, IL (USA)	-

Table 5. 2 Parameter estimates for log-linear and Weibull model for inactivation of *Salmonella* and *E. faecium* NRRL B-2354 in dried basil leaves.

Bacteria	Temperature (°C)	Water activity	Log-linear Model			Weibull Model			
			D-value (min)	RMSE (log CFU/g)	AIC_c	δ (min)	α	RMSE (log CFU/g)	AIC_c
Salmonella	70	0.40	20.41 ± 0.15^{c}	0.35	3.02	9.78 ± 2.40^{b}	0.66 ± 0.12^{bcd}	0.24	25.25
	75		9.14 ± 0.29^{e}	0.41	4.76	3.16 ± 1.10^{defg}	0.58 ± 0.08^{bcde}	0.19	24.08
	80		2.68 ± 0.07^{ij}	0.35	3.06	1.15 ± 0.06^{hi}	0.63 ± 0.09^{bcde}	0.21	25.02
	70	0.55	15.02 ± 1.57^{d}	0.33	2.17	6.83 ± 1.04^{bc}	0.66 ± 0.04^{abc}	0.10	16.07
	75		6.64 ± 0.78^{g}	0.17	-6.03	4.71 ± 1.00^{def}	0.77 ± 0.07^{a}	0.10	16.32
	80		1.86 ± 0.11^{jk}	0.24	-1.41	0.96 ± 0.24^{hi}	0.63 ± 0.04^{bcde}	0.06	9.45
	70	0.70	8.77 ± 0.13^{ef}	0.51	7.41	2.35 ± 0.59^{fghi}	0.50 ± 0.09^{fg}	0.23	26.44
	75		3.30 ± 0.13^{i}	0.41	4.97	1.11 ± 0.28^{hi}	0.53 ± 0.10^{efg}	0.20	24.45
	80		1.02 ± 0.07^{k}	0.30	1.04	0.51 ± 0.02^{i}	0.63 ± 0.09^{bcde}	0.15	21.44
E. faecium	70	0.40	41.57±1.13^{a}	0.50	7.34	14.48 ± 5.57^{a}	0.60 ± 0.04^{cdef}	0.11	17.43
	75		14.07±0.66^{d}	0.50	7.34	3.65 ± 0.73^{defgh}	0.54 ± 0.03^{efg}	0.10	15.75
	80		7.56±0.20^{fg}	0.64	10.14	1.17 ± 0.43^{hi}	0.43 ± 0.02^{g}	0.06	9.66
	70	0.55	26.84±1.49^{b}	0.49	6.95	7.61 ± 0.52^{bc}	0.57 ± 0.03^{def}	0.10	15.59
	75		9.57±0.35^{e}	0.29	0.80	5.44 ± 0.30^{cde}	0.73 ± 0.03^{ab}	0.11	12.58
	80		5.09±0.26^{h}	0.51	7.44	1.50 ± 0.30^{ghi}	0.53 ± 0.03^{efg}	0.08	7.27
	70	0.70	15.04±1.67^{d}	0.40	4.47	5.78 ± 2.18^{cd}	0.60 ± 0.04^{cdef}	0.10	16.22
	75		6.53±0.43^{g}	0.35	2.79	2.80 ± 1.01^{efghi}	0.63 ± 0.03^{bcde}	0.07	12.04
	80		2.79±0.07^{ij}	0.48	6.73	0.73 ± 0.50^{i}	0.51 ± 0.05^{fg}	0.13	12.81

*Values presented in the table are mean ± standard deviation (n=3).

[a-k] Within a column, values with different letter are significant different (P<0.05).

RMSE = Root mean square error

Chapter VI: Pasteurization of dried basil leaves using radio frequency heating: A microbial challenge study and quality analysis

6.1 Abstract

The current microbial reduction techniques have low consumer acceptance or may have an adverse effect on the quality of spices and herbs. This requires a need for an alternative decontamination method that can effectively reduce the microbial load and minimize the food quality losses. Radio frequency (RF) heating is a dielectric heating method that has been proven effective to pasteurize various low-moisture foods. The present research was designed to evaluate the efficacy of RF heating for the reduction of *Salmonella* in dried basil leaves, evaluate the suitability of *Enterococcus faecium* NRRL B-2354 as a surrogate for *Salmonella,* and assess the quality of dried basil leaves post RF treatment. Dried basil leaves inoculated with a *Salmonella* cocktail or *E. faecium* were conditioned to a higher moisture content such that the moisture content of the treated sample post RF treatment fall within the typical range. The inoculated samples were packaged in the teabag and placed in the identified cold spot (top center) of a laminated paper tray filled with uninoculated sample. Samples were subjected to RF heating for 45, 55, and 65 s during which the cold-spot temperature reached 65, 80, and 100°C, respectively. The results showed that at 55 s, *Salmonella* and *E. faecium* population decreased by 4.8 and 2.7 log CFU/g, respectively. Both microorganisms reached below the detection limit (>6.5 log CFU/g reduction) at 65 s of RF heating. Therefore, the quality analysis of the dried basil leaves was performed with the samples treated with

RF for 65 s. The results showed that the RF heating had no significant effect on the quality parameters (color, total volatiles, total phenolics, and antioxidant activity) of the dried basil leaves. Therefore, RF processing results in rapid heating of the dried basil leaves enhancing food safety with insignificant impact on quality. Furthermore, the food processing facility may use *E. faecium* as an appropriate surrogate for *Salmonella* when conducting an in-plant validation study.

Keywords: RF heating, process validation, thermal lethality, surrogate, *Salmonella*

6.2 Introduction

Although low-moisture foods are generally considered microbiologically safe, the association of *Salmonella* with various low-moisture food outbreaks and recalls has raised food safety concerns among industry, regulators, and consumers. Due to the low water activity ($a_w < 0.70$; Blessington et al., 2013), the growth of microorganisms in low-moisture foods is highly restricted (Beuchat, 1981); however, certain pathogens may likely to survive in a dry environment for a prolonged period of time. Multiple low-moisture foods have been directly associated with *Salmonella* and salmonellosis cases, including peanut butter (Medus et al., 2009), toasted oat cereals (CDC, 1998), powdered infant formula (Angulo et al., 2008), raw almonds (CDC, 2004), pet foods (Imanishi et al., 2014), paprika seasoned potato chips (Lehmacher et al., 1995), among others.

Spices and herbs have been historically used as ready-to-eat seasonings due to their flavor properties. Usually, spices and herbs are imported into the United States from

various developing countries where they are sometimes grown under inadequate sanitation conditions. During this time, pathogens such as *Salmonella* can easily contaminate spices and herbs even before they are harvested (Zweifel and Stephan, 2012). The other stages where further microbial contamination can potentially occur include harvesting, processing, storage, transportation, and packaging (McKee, 1995). Due to the desiccated environment, pathogens may survive for a long time in spices and herbs. Because the spices and herbs may be added to the cooked food with no additional cooking step, the presence of pathogens can pose a severe public health threat. The outbreaks associated with spices and herbs such as black and red pepper (CDC, 2010), paprika (Lehmacher et al., 1995), basil leaves (Pezzoli et al., 2008), white pepper (Kennelly, 2010), cilantro (Campbell et al., 2001), and fennel seeds (Ilic et al., 2010) indicate that the current processing methods may be inadequate to ensure the microbial safety of these products.

Ethylene oxide (EtO), steam, and irradiation are some of the microbial reduction techniques that have been identified by the American Spice Trade Association to enhance the microbial safety of spices and herbs (ASTA, 2017). However, poor consumer acceptance of EtO-treated and irradiated food products, as well as an adverse effect on the food quality due to steam treatment, have limited the use of these techniques in the spice industry (Lee et al., 2006; Schneider, 1993; Schweiggert et al., 2007; Waje et al., 2008). Basil leaves contain natural chlorides, which leads to the formation of toxic ethylene chlorohydrin when fumigated with ethylene oxide.

Therefore, the use of ethylene oxide with basil leaves is prohibited under U.S. regulation 40 CFR 180.151 (EPA, 2009).

Radio frequency (RF) heating is a dielectric heating method that operates in the frequency range of 3 kHz to 300 MHz. During RF heating, the heat is generated inside the food product due to the molecular friction caused by ionic conduction and dipole rotation of water molecules. The microbial efficacy of RF heating has been evaluated in various spices such as whole black peppercorns (Wei et al., 2018), ground black pepper (Wei et al., 2019), cumin seeds (Chen et al., 2019), black and red pepper spice (Kim et al., 2012), and paprika, white pepper, and cumin powder (Ozturk et al., 2020). These studies demonstrated that radio frequency heating is an effective technique in reducing the microbial load while minimizing the quality loss of the spices.

With the implementation of the Food and Safety Modernization Act, food processors in the U.S. are required to validate their process preventive controls to ensure that their operations are effectively controlling and minimizing the identified hazard. Usually, the use of surrogate in the food processing facility is recommended for conducting an in-plant microbial challenge study (NACMCF, 2010). A surrogate is a non-pathogenic microorganism that behaves similar to the target pathogen when the same treatment is applied (Anderson and Lucore, 2012). Several surrogates for *Salmonella* have been investigated in various foods/processes including *Bacillus stearothermophilus* (Animal feed; Okelo et al., 2006), *Pediococcus acidilactici* (Ground and formed beef jerky; Borowski et al., 2009), and *Pantoea agglomerans* SPS2F1 (Dry roasted almonds; ABC,

2007b). For low-moisture foods, *Enterococcus faecium* NRRL B-2354 has been identified as the most suitable surrogate for *Salmonella* for conducting the validation studies (ABC, 2007a; Bingol et al., 2011; Chen et al., 2019; Liu et al., 2018; Ozturk et al., 2020; Verma et al., 2019; Wei et al., 2019). However, there is no information available on the suitability of *E. faecium* as a surrogate for *Salmonella* during RF heating of dried basil leaves.

Therefore, the objectives of this research were to (i) investigate the efficacy of RF heating on the inactivation of *Salmonella* in dried basil leaves, (ii) evaluate the suitability of *E. faecium* NRRL B-2354 as a surrogate for *Salmonella* in dried basil leaves, and (iii) evaluate the effect of RF heating on the quality parameters of dried basil leaves.

6.3 Materials and Methods

6.3.1 Dried basil leaves

Three different production batches of steam-sterilized dried sweet basil leaves were provided by McCormick & Co., Inc. (Hunt Valley, MD) and were stored at room temperature (ca. 25°C). The moisture content and water activity were determined upon receiving the dried basil leaves using a halogen moisture analyzer (Model: HR73, Mettler Toledo) and a dew point water activity meter (Model: 4TE, Meter Group; 25°C), respectively. The moisture content and water activity of the samples were also measured before and after RF treatment.

6.3.2 Background microorganisms

The aerobic plate count was used to estimate the background microflora in the dried basil leaves for each production batch. Five 25-g samples were taken from random locations in each batch and diluted with 225 mL of 0.1% buffered peptone water (BPW; BD Difco, Sparks, MD). The diluted sample was homogenized using stomacher for 1 min, plated on trypticase soy agar (TSA; BD Difco, Sparks, MD) supplemented with 0.6% (w/w) yeast extract (YE; BD Difco, Sparks, MD), and incubated at 37°C for 24±2 h.

6.3.3 Bacterial cultures

Five serotypes of *Salmonella enterica,* namely *Salmonella* Agona (447967), *Salmonella* Mbandaka (698538), *Salmonella* Montevideo (488275), *Salmonella* Tennessee (K4643), and *Salmonella* Reading (Moff 180418) were selected based on their implication in outbreaks and recalls related to low-moisture foods. *Salmonella* Agona (447967), *Salmonella* Mbandaka (698538), and *Salmonella* Montevideo (488275) were obtained from the Food and Drug Administration, Office of Regulatory Affairs, Regional Laboratory in Jefferson, AR and were associated with foodborne outbreaks in oat cereals (CDC, 1998), sprouts (Jackson et al., 2013), and black and red pepper (CDC, 2010), respectively. *Salmonella* Reading (Moff 180418) associated with a foodborne disease outbreak in alfalfa sprouts (CDC, 2016) was obtained from the FDA culture collection in Bedford Park, IL. *Salmonella* Tennessee (K4643) that was implicated in the PCA peanut butter outbreak (CDC, 2007), was obtained from the University of Georgia in Griffin, GA.

To conduct the surrogate study, *Enterococcus faecium* (NRRL B-2354) was used as a potential surrogate and was obtained from the United States Department of Agriculture, Agriculture Research Services (USDA, ARS) in Peoria, IL. All the bacterial cultures were stored as a frozen stock at -80°C in trypticase soy broth (TSB; BD Difco) supplemented with 40% (v/v) glycerol (G31-1, Fisher Chemicals).

6.3.4 Radio Frequency (RF) processing of dried basil leaves

a) Inoculum preparation and inoculation

The working stock plates of *Salmonella* serotypes and *E. faecium* were used to prepare the inoculum. To prepare the working stock plate for each strain, 1 mL of thawed frozen stock from each serotype (*Salmonella* and *E. faecium*) was added to the 10 mL TSBYE, and the tubes were incubated for 24±2 h at 37°C. A loopful (10 µL) of the TSBYE broth was used to streak the TSAYE plates, followed with incubation at 37°C for 24±2 h. The prepared working stock plates were wrapped with the parafilm (PM-999, Bemis) and stored at 4°C. These plates were used within 30 days for inoculum preparation.

The inoculum was prepared by transferring one isolated colony from each working stock plate (*Salmonella* serotype or *E. faecium*) to 10 mL TSBYE with incubation at 37°C for 24±2 h. The overnight TSBYE broth (100 µL) was spread plated onto TSAYE agar plates (100 mm diameter x 15 mm height) to create bacterial lawns and incubated at 37°C for 24±2 h. Finally, 3 mL of BPW was added to each agar plate, and an L-shaped spreader was used to harvest the lawn. An equal amount of inoculum (2 mL) from each

Salmonella serotype was transferred to a 15 mL sterile conical tube (339650, Thermo Scientific) and vortexed for 30 s to prepare the *Salmonella* cocktail. The working inoculum (*Salmonella* cocktail or *E. faecium*) was used within 2 h for dried basil leaves inoculation.

All the inoculation activities were performed in the biological safety cabinet. Dried basil leaves (100±0.1 g) were aseptically placed into a sterile whirl bag to which 2 mL of either *Salmonella* cocktail or *E. faecium* was atomized. The bag was sealed, and the sample was manually mixed for 10 min to ensure the uniform distribution of inoculum onto the product surface.

b) Adjustment of water activity

The inoculated dried basil leaves were placed on a sterile aluminum tray (23 x 30 x 1.5 cm), and the tray was then moved to the relative humidity chamber. Radio frequency heating is a thermal process where a moisture loss from the food product is expected during the treatment. Therefore, the initial water activity of the dried basil leaves was adjusted to a higher level in order to maintain the final water activity of the product close to its natural level.

c) Homogeneity and stability of inoculum

The homogeneity and stability of inoculum were tested for 15 days before conducting the RF treatment of inoculated dried basil leaves. Three 3±0.1 -g samples were taken out of the relative humidity chamber on 0, 1, 2, 3, 6, 9, 12, and 15 days. Each

subsample was diluted with 27 mL of BPW and stomached for 1 min. The mixture was serially diluted using 9 mL BPW tubes, and suitable dilutions were spread plated onto TSAYE supplemented with 0.03% (w/v) sodium thiosulfate (Fisher Scientific), and 0.05% (w/v) ammonium iron citrate (Sigma Aldrich) (m-TSA) for *Salmonella* or TSAYE supplemented with 0.05% (w/v) ammonium iron citrate and 0.025% (w/v) esculin hydrate (Acros Organics) (e-TSA) for *E. faecium*. Black colonies observed on m-TSA and e-TSA were counted as *Salmonella* and *E. faecium*, respectively.

d) RF treatment

A pilot-scale parallel-plate RF heating system (6 kW, 27.12 MHz; Model: SO-6B, Monga Strayfield) was used to conduct this study. A laminated paper tray (ConAgra Brands, Omaha, NE) was used to pack 125±0.1 g of dried basil leaves for the RF treatment. The electrode gap in the RF system was adjusted to 10.5 cm, which provided the fastest heating rate without affecting the final food quality (Jiao et al., 2014). A few studies have shown that the hot spots are typically located at the edges and cold spots are located at the center (Boreddy et al., 2014; Jiao et al., 2014; Liu et al., 2018). Therefore, the hot spot and cold spot was determined by inserting six fiber optic sensors (accuracy of 0.6°C; Neoptix, Quebec City, Quebec) through the pre-drilled holes in the rectangular paper tray at different locations: T1: Top center; T2: Middle center; T3: Bottom center; T4: Top edge; T5: Middle edge; T6: Bottom edge (Figure 6.1). Instead of inoculating the whole sample (125 g), the inoculated pouch method was followed (Chen et al., 2019; Liu et al., 2018; Wei et al., 2019). Any effect of the pouch on the heating

profile was evaluated by placing a heat-sealable tea bag (7 x 6 mm) filled with 4.0±0.1 g of sample in the identified cold spot along with the remaining 121±0.1 g of sample. For direct comparison of *Salmonella* and *E. faecium* inactivation, the tea bag was split into halves, and each pouch (7 x 3 mm) filled with 2.0±0.1 g of *Salmonella* and the *E. faecium* inoculated sample was placed next to each other in the cold spot along with the 121±0.1 g of uninoculated dried basil leaves (Figure 6.2). The tray was covered with plastic wrap (Press'n Seal, The Glad Products Co.) to prevent excessive moisture loss and improve the heating uniformity. A venting nut was fixed at the center of the plastic wrap to allow the release of steam vapor generated during the RF heating. The prepared laminated paper tray was placed inside the RF system at the center of the bottom electrode and treated for 45, 55, and 65 s.

After the RF treatment, the wrap was removed, the inoculated packs were transferred to two separate sterile Whirl-Pak bags and immersed in the ice-water bath to stop the further thermal inactivation. The treated sample (2.0±0.1 g) was diluted with 18 mL of BPW and homogenized for 1 min in a stomacher. The dispersion was serially diluted using 9 mL BPW tubes, and suitable dilutions were spread plated in duplicate onto m-TSA for enumerating *Salmonella* and e-TSA for enumerating *E. faecium*. The RF treatment of dried basil leaves was replicated on two more production batches with each batch inoculated using a new frozen stock representing three biological replicates.

6.3.5 Quality analysis

The quality analysis of the dried basil leaves was performed by treating the uninoculated sample (125±0.1 g) using the conditions that achieved more than a 5-log reduction of *Salmonella*. Post RF treatment, the sample was transferred to a Ziploc bag and allowed to cool down to room temperature instead of an ice-water bath representing the worst-case scenario for quality loss.

The RF treated sample was ground in a Kitchen Aid spice blender (Model: BCG211OB) for 30 s. The ground sample was sieved using a U.S. Sieve No. 20 to achieve a uniform particle size to conduct the quality analysis. An extract from basil samples was prepared by diluting 1.0±0.1 g ground sample in 100 mL of 200 proof ethanol (Decon Labs Inc., PA). The mixture was magnetically stirred at 300 rpm for 18±2 h. The solution was then filtered through filter paper (Fisherbrand 09795G), and the basil extract was transferred to a 50 mL conical tube (Fisherbrand 055398). The tube was wrapped with aluminum foil and stored under refrigerated conditions (4°C) until used for quality analysis. The prepared basil extract was used to determine the total phenolic content and antioxidant activity of the dried basil leaves.

a) Color measurement

A colorimeter (Model: BC-10, Konica Minolta Co., Osaka, Japan) was used to measure the color of the ground dried basil samples. The samples were placed on a petri dish with the top surface flattened, and the color values of L^* (+: lighter; −: darker), a^* (+: redder; −: greener), and b^* (+: yellower; −: bluer) were measured at five

random locations. The colorimeter was always calibrated using a white tile before the measurements were taken. The effect of RF heating on the sample color was assessed by calculating the total color difference (ΔE) of the pre- and post RF treated sample using the following equation (Robertson, 1977):

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

b) Total phenolic content

The total phenolic content in dried basil leaves was determined using a modified Folin-Ciocalteu method (Singleton and Rossi, 1965). A calibration curve was prepared using gallic acid (Acros Organics) standard solutions at 2, 3, 4, 5, 6, and 7 µg/mL. To each standard solution and 0.2 mL basil extract, 2 mL of diluted Folin-Ciocalteu reagent (20% v/v; Sigma-Aldrich) were added. The solutions were vortexed for 5 s and stored in the dark. After 10 min, 2 mL of 7.5% (w/v) Na_2CO_3 (Acros Organics) was added to each solution and vortexed for 5 s. The total volume of each solution was brought to 5 mL with distilled water. The tubes were stored for 2 h at room temperature under dark conditions. Ethanol (0.2 mL) was treated the same as samples and standards and was used as blank.

The absorbances of standards, samples, and blank tubes were measured at 765 nm using a UV-1800 spectrophotometer (Shimadzu Corp., Kyoto, Japan). The total phenolic content in the basil extract was expressed as gallic acid equivalent (GAE). The values are reported as an average of triplicate analysis.

c) Antioxidant activity

The antioxidant activity of the basil extract (described at beginning of Section 6.3.5) was determined on the basis of the scavenging activity of the 2,2 diphenyl-1-picrylhydrazyl (DPPH; Bersuder et al., 1998). The DPPH solution (Alfa Aesar) was prepared by diluting 4 mg of DPPH with 100 mL of 200 proof ethanol. The prepared DPPH solution was covered with aluminum foil and stored under refrigerated conditions (4°C) until use. A volume of 2 mL of DPPH solution was added to 0.0, 0.2, 0.4, 0.6, 0.8, and 1.0 mL of basil extract, and ethanol was added to each tube to bring the final volume to 5 mL. Each tube was vortexed for 5 s and stored in the dark for 30 min at room temperature. Ethanol was used as blank. After 30 min, the solutions' absorbances were measured at 517 nm using a UV-1800 spectrophotometer (Shimadzu Corp., Kyoto, Japan). The following equation was used to calculate the scavenging activity of the DPPH radical in each sample (Bersuder et al., 1998):

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

where $A_{control}$ is the absorbance of a solution containing 0.0 mL of basil extract and A_{sample} is the absorbance of solutions containing 0.2-1.0 mL of basil extract. The values are reported as an average of triplicate analysis.

d) Total volatile analysis

The volatiles compositions of ground basil samples were determined by a Thermo Scientific headspace gas chromatography (Trace 1300) equipped with ISQ Mass

Selective Detector (MS). The 0.5 g of ground basil was placed in a 20 mL headspace vial with a magnetic screw cap, which enables magnetic pick up in the autosampler. The sample was incubated for 15 min at 72°C agitator temperature. The gas (0.7 mL) released from the sample during incubation was injected into GC-MS with a 1:5 split ratio. The dimension of the capillary column used for the analysis was 30 m x 0.25 mm ID x 0.25 dF. The oven temperature ranged from 40°C to 235°C with 30°C/min heating rate and maintained at this temperature for 4.5 min. The ionization energy was 70 eV, and the mass range was 10-650 amu. Mass transfer line and ion source temperatures for MS detector were 250 °C and 200 °C, respectively. The compositions of the samples were identified by NIST 11 mass spectral library.

6.4 Results and Discussion

6.4.1 Basil inoculation

The native moisture content and water activity of the dried basil leaves was recorded as 9.87±0.08% wet basis (w.b.) and 0.538±0.003, respectively. The results from the background microflora showed that the aerobic plate counts were below the detection limit (<10 CFU/g). Therefore, the background microflora would not affect our results due to the comparatively high inoculation level of *Salmonella* and *E. faecium* in dried basil leaves.

Because the food product loses moisture during RF heating, the dried basil leaves' initial water activity was adjusted to a higher level such that the final water activity of the sample was close to its natural level. From trial-and-error experiments, it was found

that upon adjusting the initial water activity to 0.62 (10.61% w.b.), the final water activity of the RF treated dried basil leaves would be 0.53 (9.20% w.b.) after 65 s of RF heating. Therefore, the water activity of the inoculated and uninoculated dried basil leaves was adjusted to 0.62 before the RF inactivation study was conducted. A similar approach was used during the RF treatment of whole black peppercorns and cumin seeds, where the initial water activity of the food product was adjusted to achieve the desired water activity of the RF treated samples (Chen et al., 2019; Wei et al., 2018). While the initial higher water activity may allow for rapid bacterial inactivation, the final appropriate water activity of product is critical for longer shelf life and preventing any form of chemical and enzymatic quality deterioration.

Post inoculation, the stability of *Salmonella* and *E. faecium* population in dried basil leaves was tested for 15 days at a_w=0.62 (Figure 6.3). A higher bacterial reduction is usually achieved when the low-moisture food undergoes a thermal treatment immediately after inoculation (Jeong and Kang, 2014). This is because the desiccated environment (a_w < 0.70; Blessington et al., 2013) creates stress for the pathogenic bacteria, which eventually results in rapid reduction during thermal treatment. Therefore, the inoculated food product should be equilibrated for a few days such that the bacteria physiologically adapt to the new environment. This may result in higher thermal resistance of bacteria and will simulate more realistic industrial conditions (Wei et al., 2019). From day 0 to day 2, a considerable drop (0.5 log CFU/g) in *Salmonella* population was seen, whereas *E. faecium* population dropped by only 0.2 log CFU/g.

However, both microorganisms exhibited a stable population from day 5 to 15 and a higher bacterial count was achieved (7.6±0.1 log CFU/g for *Salmonella*; 7.9±0.1 log CFU/g for *E. faecium*) to conduct the RF inactivation study. Therefore, the inoculated dried basil leaves (either with *Salmonella* or *E. faecium*) were allowed to equilibrate for a minimum of 5 days before the inactivation study was conducted. The error bars in Figure 6.3 represent one standard deviation among the subsamples tested for homogeneous distribution of inoculum on dried basil leaves. A standard deviation below 0.3 log CFU/g among the sub-samples was used as a threshold for using the samples for further experiments (Hildebrandt et al., 2020). Any samples with than 0.3 log CFU/g were considered non-homogeneous and were not used for the study.

6.4.2 Cold spot determination

Figure 6.4a shows the time-temperature profile of dried basil leaves (a_w = 0.622±0.001; MC = 10.61±0.13%) during RF heating for 65 s. It can be noted that the top center (T1) location was the coldest spot, which took 65 s to reach 100°C. However, the bottom edge (T6) location took only 58 s to reach 100°C. Jiao et al. (2015) also showed that the edges heat faster than the other locations in the container. This is because the radio frequency waves from multiple directions heat the food product on the edges more, resulting in the hot spots. Rapid heating results in steam generation, which enhances the heat distribution within the food product. The excessive steam generation which occurs inside the container diffuses through the sample (bottom to the top layer), resulting in a uniform distribution of temperature. Due to the steam movement in the

container, the bottom center (T3) and the middle center (T2) were heated up quickly to 100°C in 60 s. Even though the steam vent helps to release excess steam, the plastic film ballooned during heating. Liu et al. (2018) reported a good temperature uniformity in all the layers during the RF heating of wheat flour when the standard deviation was ≤4.4°C. In our study, the standard deviation of temperatures of 3 layers along the edge and center was 3.1°C and 2.8°C, respectively. Overall, a good temperature uniformity was obtained during RF heating of dried basil leaves. The container was covered with the plastic film, which helped reduce the moisture loss and improve the heating uniformity in the dried basil leaves. Several studies have reported the top center as the coldest spot during RF heating (Chen et al., 2019; Kim et al., 2012; Lin et al., 2020; Liu et al., 2018; Villa-Rojas et al., 2017; Wang et al., 2015; Wei et al., 2018; Wei et al., 2019). Different approaches such as forced hot air (Wang et al., 2010) and infrared heating (Fasina et al., 2001) have been used in combination with RF heating to improve the heating uniformity of low-moisture foods. However, this was not necessary for this study as the dried basil sample was uniformly heated.

The inoculated pack (*Salmonella* and *E. faecium*) was placed in the cold spot to evaluate the antimicrobial efficacy of RF heating. Similar RF inoculated pack studies have been conducted with other low-moisture foods including wheat flour (Liu et al., 2018), cumin seeds (Chen et al., 2019), and ground black pepper (Wei et al., 2019). Figure 6.4b shows the time-temperature profile of the inoculated pack placed in the top center (T1) of the container. It can be noted from the Figure that the inoculated pack also took

approx. 65 s to reach a temperature of 100°C. Therefore, it was concluded that the introduction of a teabag for holding the inoculated sample did not impact the heating profile of the dried basil leaves during RF treatment.

6.4.3 RF microbial inactivation and surrogate evaluation

The teabags containing *Salmonella* and *E. faecium* inoculated samples were placed in the cold spot. The tea bags were covered with a layer of uninoculated dried basil leaves (40±0.1 g) and was subjected to RF heating for 45, 55, and 65 s. Figure 6.5 shows the comparison of *Salmonella* and *E. faecium* reduction at 45 and 55 s of RF heating. At 45 s, only 1.0 and 0.7 log reductions of *Salmonella* and *E. faecium* were achieved in dried basil leaves. A significant ($p<0.05$) greater reduction was achieved (4.8 log CFU/g for *Salmonella*; 2.7 log CFU/g for *E. faecium*) when the dried basil leaves were heated for 55 s. Because the *Salmonella* reduction was below 5-log, the sample was further heated for 65 s which reduced the bacterial survivors below the detection limit (<10 CFU/g) resulting in greater than 5 log reduction (FDA, 2015).

RF heating has been demonstrated to be an effective process for reducing the microbial load from the food product. Ozturk et al. (2020) reported that the *Salmonella* population decreased by 4.2 log when paprika was RF heated to 80°C in 100 s of heating. Additionally, *Salmonella* population decreased by 5.3 log when the whole black peppercorns were heated in RF for 2.5 min (Wei et al., 2018). Similarly, other studies have reported the effectiveness of RF heating in inactivating pathogenic bacteria in various low moisture foods such as cumin seeds (Chen et al., 2019), wheat flour (Liu et

al., 2018), in-shell almonds (Gao et al., 2011), and corn flour (Ozturk et al., 2019). An additional holding time in the hot air oven was required post RF treatment of egg white powder and powdered infant formula in order to maintain the temperature to achieve a desired bacterial kill (Lin et al., 2020; Wei et al., 2020). However, in case of spices and herbs, there is no need for holding the product at high temperatures, as the antimicrobial compounds present assist in inactivating the pathogens (Chen et al., 2019; Wei et al., 2018, 2019). The come-up time recorded during RF heating of dried basil leaves was the lowest (65 s to reach 100°C) when compared with other RF microbial challenge studies for cumin seeds (Chen et al., 2019), powdered infant milk formula (Lin et al., 2020), black pepper (Wei et al., 2018, 2019). Thus, RF is a suitable rapid heating process for effectively inactivating microorganisms in dried basil leaves.

The identification of an appropriate surrogate is critical to help the spice and herb industry perform an industrial validation study on dried basil leaves. *Enterococcus faecium* NRRL B-2354 is a suitable surrogate for *Salmonella* which has been validated for various low moisture foods such as RF heating of cumin seeds (Chen et al., 2019), black pepper (Wei et al., 2019), wheat flour (Liu et al., 2018), extrusion of oat flour (Verma et al., 2018), cocoa powder (Tsai et al., 2019) and almond kernels (ABC, 2007a). Although *E. faecium* is an appropriate surrogate for *Salmonella*, it is imperative to evaluate its suitability and conduct the validation for a specific process and product matrix. It can be noted from Figure 6.5, the log reduction of *E. faecium* was significantly ($p<0.05$) lower than that of *Salmonella* at both heating times (45 and 55 s). Due to its higher thermal

resistance, *E. faecium* can be used as a conservative surrogate for *Salmonella* during RF heating of dried basil leaves.

6.4.4 Quality analysis

Because the bacterial population was below the detection quantification (< 10 CFU/g) at 65 s, the quality analysis of dried basil leaves was performed with the uninoculated samples treated by RF for 65 s. The RF treated samples were cooled down at room temperature to represent the worst-case scenario for quality analysis. Table 6.1 summarizes the quality analysis data for both untreated and treated samples. The moisture content and water activity of the untreated dried basil leaves were significantly ($p<0.05$) different from the RF treated samples. Post RF treatment, the moisture content of the dried basil leaves dropped by 1% and was recorded as 9.20±0.06%, whereas the final water activity of the dried basil leaves was close to its natural level (0.529±0.001). Moisture content and water activity affects not only the quality of the dried basil leaves but also its microbial safety (Wei et al., 2019). Since RF heating reduces a significant amount of moisture from the sample, the initial moisture content was increased to meet the final moisture content and water activity requirements. This approach has also been used for various low-moisture foods such as black peppercorns (Wei et al., 2018) and cumin seeds (Chen et al., 2019), where the initial moisture content of the food product was raised in order to comply with the safe storage requirements given by American Spice Trade Association.

The color values (L^*, a^*, and b^*) did not show any significant ($p<0.05$) difference between untreated and RF treated samples. The color difference (ΔE) value for the RF treated sample was 0.51, which means that the human eye cannot notice any difference (Mokrzycki and Tatol, 2011). Although the dried basil leaves were heated up to a very high temperature (100°C), the color of the food product was not significantly affected due to the short processing time.

The total phenolic content in the dried basil leaves was also determined and is presented in Table 6.1. The results indicated that there was a slight decrease in the total phenolic content in RF treated dried basil leaves; however, the decrease was not significantly ($p>0.05$) different from the untreated samples. Wei et al. (2019) and Chen et al. (2019) also measured the total phenolic content in the RF treated black pepper and cumin seeds, respectively. The results from their studies showed no significant difference between treated and untreated samples. Phenolic components such as flavonoids, phenolic acids, and anthocyanins play an essential role in the antioxidative effect of herbs and spices (Javanmardi et al., 2003). Wangcharoen and Morasuk (2007) reported that the total phenolic contents of white and red holy basils were approximately 9.0 and 12.5 mg GAE/g, respectively at 95% ethanol extraction solvent. This was significantly greater than the total phenolic content measured in dried sweet basil (7.80±0.43 mg GAE/g). The difference in phenolic content in various basil types could be due to solvent (ethanol) concentration used in the study as well as genetic factors and environmental conditions of the basil (Bravo, 1998).

The DPPH (2,2 diphenyl-1-picrylhydrazyl) assay was used to assess the antioxidant activity of the dried basil leaves. DPPH is a free radical that measures the disappearance of DPPH, which is a useful index to estimate the total free scavenging capacity of the food product (Li et al., 2007). Upon reacting with the hydrogen donating antioxidant compound, DPPH changes color from purple to yellow, which can easily be measured with a UV-spectrophotometer at 517 nm (Zhang et al., 2009). Figure 6.6 shows the antioxidant activity of untreated, and RF treated dried basil leaves. It can be seen that there was no significant difference between the untreated and RF treated samples. Therefore, RF heating effectively improved the microbial safety of dried basil leaves while maintaining the overall food quality.

A total of 25 volatile compounds were identified in the untreated and RF treated dried basil leaves. Among 25 compounds, 1,8-cineole, linalool, methyl chavicol, E-methyl cinnamate, and trans-α-bergamotene accounted for 69% of the total volatile compounds in the dried basil leaves. These major compounds are responsible for the typical basil aroma (Lee et al., 2005). Table 6.2 summarizes and compares the total volatile compounds between untreated and RF treated samples. None of the five identified major compounds experienced any significant drop. Only one minor volatile compound, camphor, experienced a significant *($p<0.05$)* drop post RF treatment. The drop in the volatile compounds ranged from 5-10% which could have been due to the high temperature experienced by the dried basil leaves during treatment. A study by Calin-Sanchez et al. (2012) reported a significant loss (55% decrease) in the

concentration of total volatile concentration during the drying of fresh sweet basil using the hot air method at 40°C. Diaz-Maroto et al. (2004) reported that the cuticle layer of the basil leaves collapses during the drying process. This leads to the expansion of the cell structure and causes the volatile compounds to release into the atmosphere. Any loss in the major volatile compounds can adversely affect the flavor and aroma of the dried basil leaves. In the RF treated dried basil leaves, a nominal drop in the volatile compounds was observed, and thus the overall effect on the flavor and aroma is minimal. However, the RF treated dried basil leaves were cooled at room temperature. If rapid cooling was implemented, the volatile loss caused by RF heating may be lower. Thus, RF can be considered as a high-temperature short-time process due to rapid heating while minimizing quality deterioration.

6.5 Conclusion

Steam generation in the food product during RF heating enhanced the heating uniformity. *Salmonella* population decreased by 1.0 and 4.8 log CFU/g at 45 and 55 s of RF heating. The corresponding reductions for *E. faecium* were 0.7 and 2.7 log CFU/g. At 65 s, both microorganisms were below the quantification limit (<10 CFU/g), resulting in greater than 6.5 log CFU/g reduction, without adversely affecting the quality of dried basil leaves. A significant drop seen in one minor volatile compound (camphor) post RF treatment. However, this volatile loss can be further reduced by cooling the food product immediately after the treatment. Furthermore, *E. faecium* proved to be an appropriate surrogate for *Salmonella* due to its increased thermal resistance at the

experimental conditions. The results from this study can help the spice industry adopt RF as effective pasteurization and a drying method for the basil leaves.

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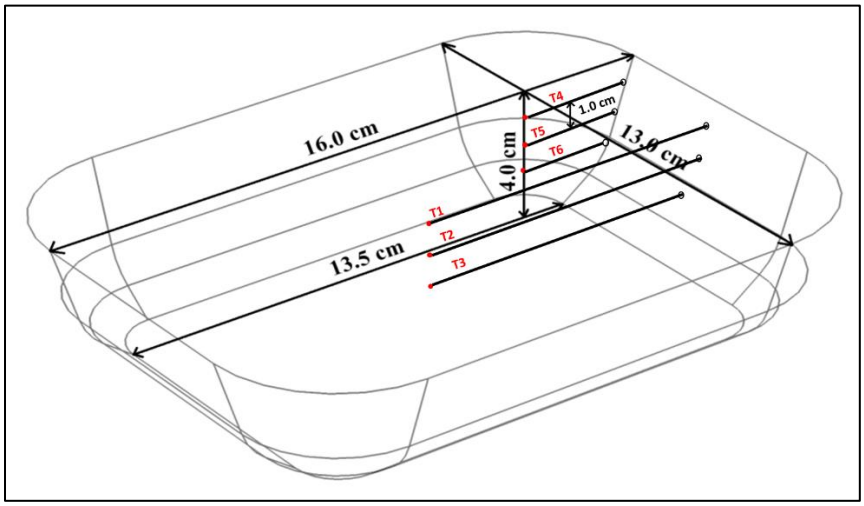


Figure 6. 1 Location of six fiber optic sensors in the rectangular laminated tray (T1: Top Center; T2: Middle Center; T3: Bottom Center; T4: Top Edge; T5: Middle Edge; T6: Bottom Edge).

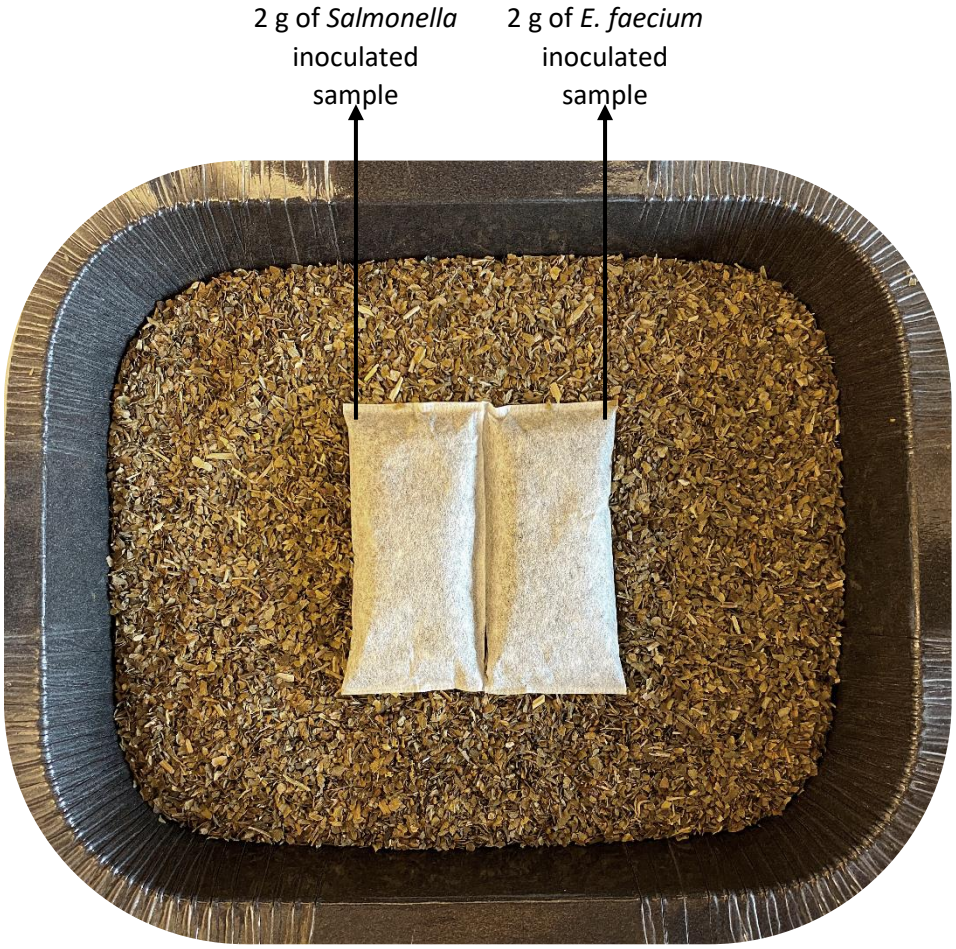


Figure 6. 2 Teabags filled with *Salmonella* (2.0±0.1 g) and *E. faecium* (2.0±0.1 g) inoculated dried basil leaves were placed in the cold spot. The teabags were covered with a layer (40±0.1 g) of dried basil leaves prior to RF treatment.

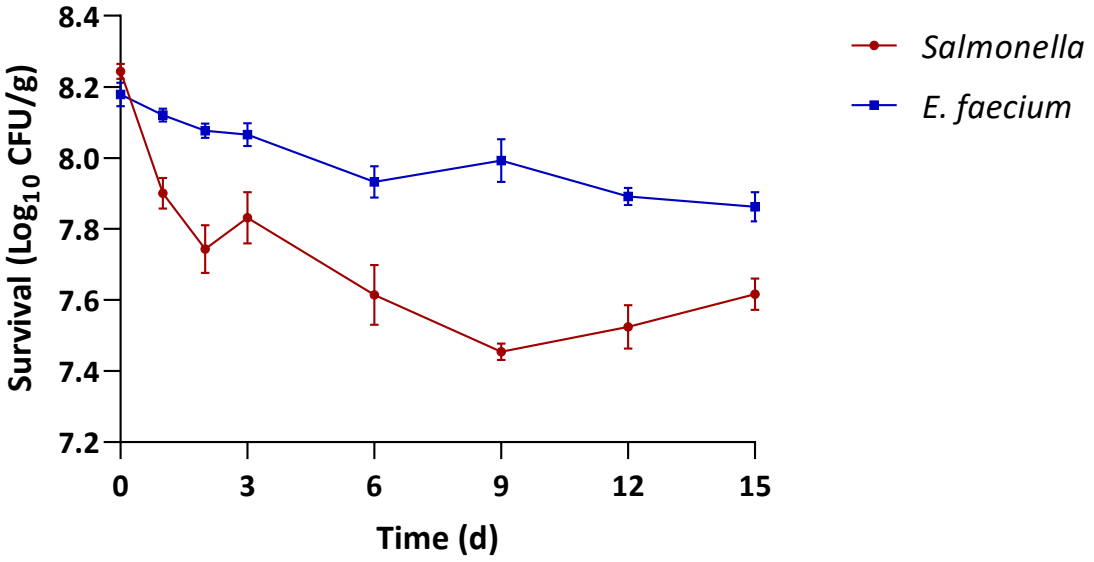
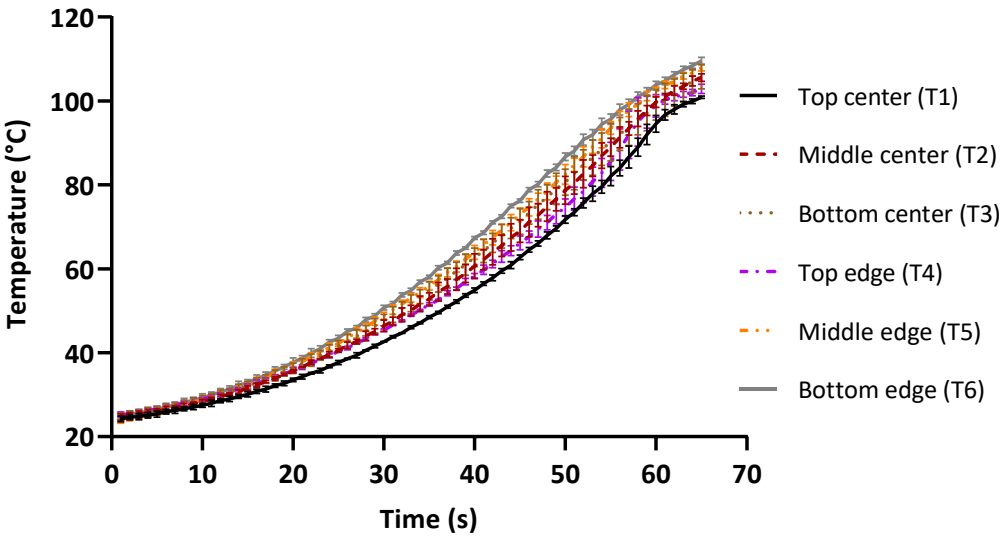
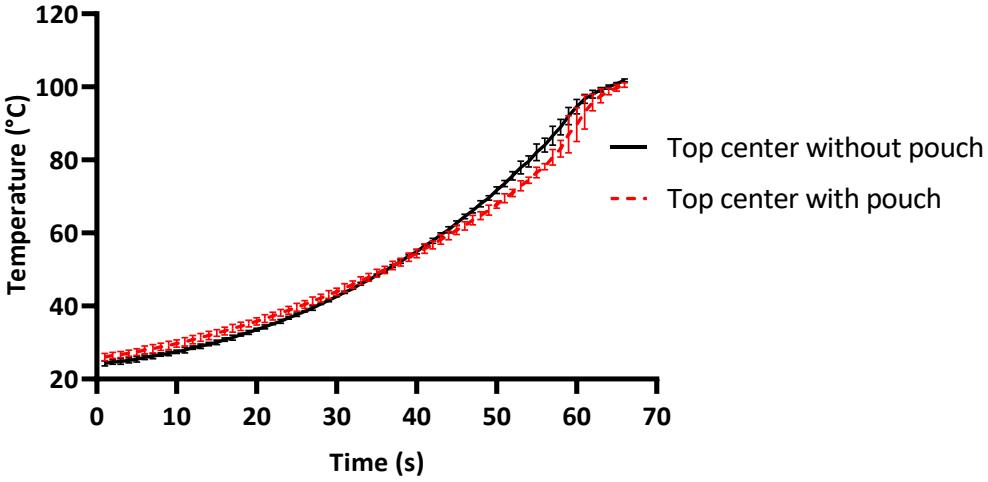


Figure 6. 3 Stability and homogeneity of *Salmonella* and *E. faecium* NRRL B-2354 population in dried basil leaves for 15 days at a_w = 0.62 (n=3).



(a)



(b)

Figure 6. 4 Time-temperature profile of dried basil leaves during RF heating (a) Without pouch (b) Comparison of top center (T1) with and without pouch. The locations are identified in Figure 6.1. Error bars indicate standard deviation of temperatures from the same location for three replications.

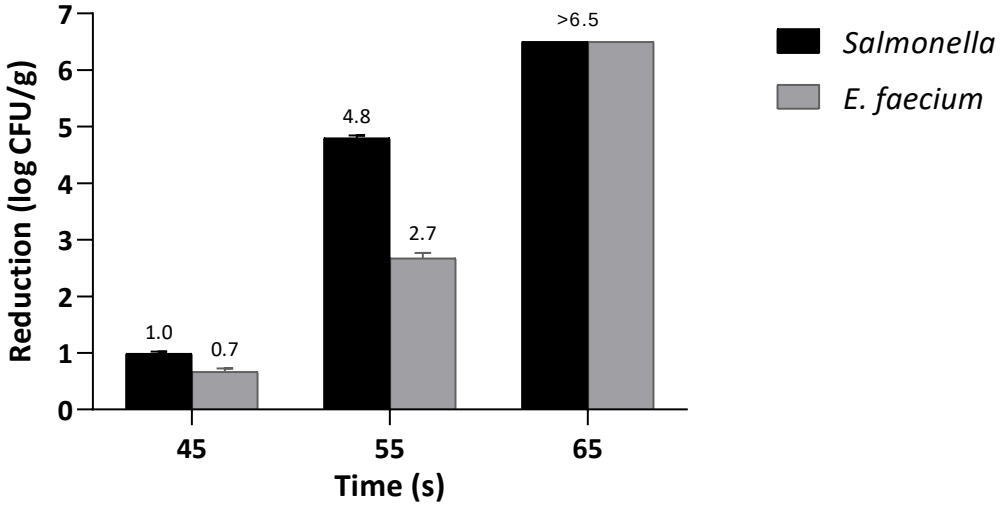


Figure 6. 5 Inactivation of *Salmonella* and *E. faecium* NRRL B-2354 during RF heating for 45, 55, and 65 s. Error bars indicate the standard deviation of microbial log reductions from three replicates. The population of both microorganisms was below the detection limit (<10 CFU/g) at 65 s of RF treatment, resulting in >6.5 log (CFU/g) reduction.

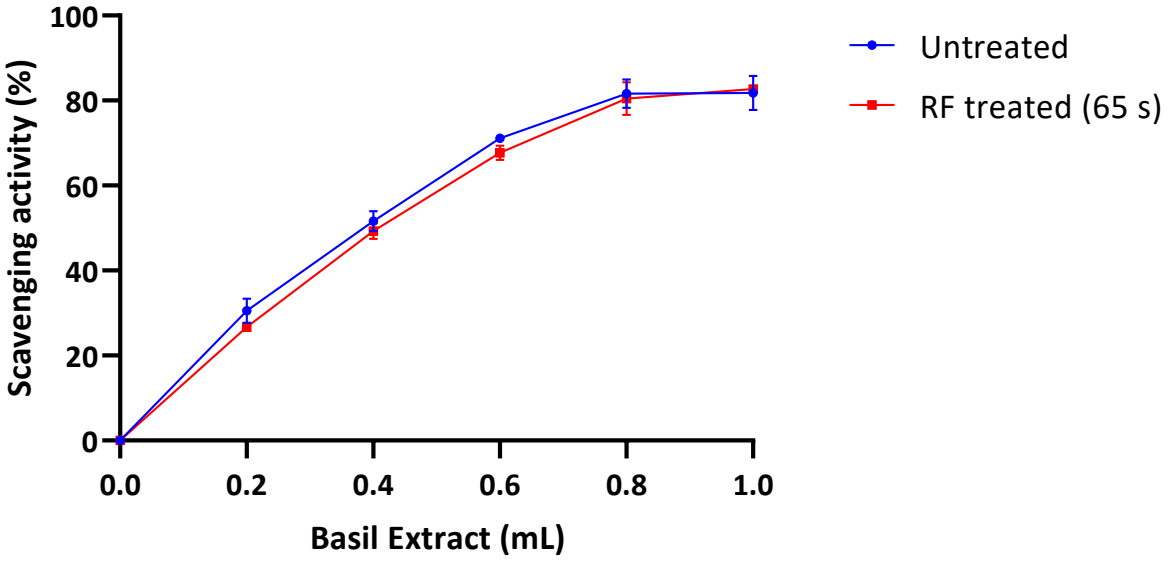


Figure 6. 6 Antioxidant activity of untreated and RF treated (65 s) dried basil leaves. Error bars indicate the standard deviation of antioxidant scavenging activity from three replicates.

Table 6. 1 Comparison of quality parameters of untreated and RF treated (65 s) dried basil leaves.

Parameter	Untreated	RF treated (65 s)
Water activity	0.622±0.001[a]	0.528±0.001[b]
Moisture content (%)	10.66±0.13[a]	9.20±0.05[b]
Color		
L^*	52.15±0.38[a]	52.55±0.47[a]
a^*	3.73±0.43[a]	3.65±0.35[a]
b^*	12.05±0.24[a]	12.08±0.53[a]
ΔE	0	0.51±0.37
Total phenolic (mg GAE/g)	8.25±0.64[a]	7.80±0.46[a]

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

Table 6. 2 Comparison of total volatile compounds in untreated and RF treated (65 s) dried basil leaves.

Compound	% Area	
	Untreated	**RF Treated (65 s)**
β-Myrcene	1.26±0.35[a]	1.29±0.41[a]
α-Terpinen-7-al	0.75±0.59[a]	0.77±0.61[a]
1,8-Cineole	6.38±1.19[a]	6.38±1.50[a]
γ-Terpinene	0.17±0.15[a]	0.18±0.15[a]
α-Terpineolene	0.91±0.09[a]	0.90±0.09[a]
6-Methyl-2-(2-oxiranyl)-5-hepten-2-ol	0.17±0.07[a]	0.14±0.11[a]
Linalool	27.72±1.96[a]	26.78±2.24[a]
Camphor	1.63±0.52[a]	1.44±0.41[b]
Borneol	0.46±0.10[a]	0.45±0.09[a]
Terpinen-4-ol	1.51±0.08[a]	1.56±0.15[a]
Methylchavicol	23.99±3.08[a]	23.33±3.62[a]
Borneol acetate	0.74±0.05[a]	0.75±0.04[a]
α-Copaene	0.49±0.15[a]	0.50±0.13[a]
Eugenol	0.71±0.15[a]	0.69±0.08[a]
E-Methyl cinnamate	3.15±1.46[a]	3.25±1.63[a]
trans-α-Bergamotene	7.65±1.88[a]	8.09±2.16[b]
α-Caryophyllene	0.58±0.29[a]	0.63±0.22[a]
4-Methoxycinnamaldehyde	0.12±0.01[a]	0.12±0.01[a]
α-Copaene	0.22±0.10[a]	0.23±0.11[a]
Cedrene	0.13±0.04[a]	0.12±0.04[a]
Guaiene	0.16±0.12[a]	0.17±0.11[a]
γ-Cadinene	1.74±0.49[a]	1.77±0.51[a]
Cis-α-Bisabolene	0.12±0.06[a]	0.13±0.07[a]
Cis-Muurola-3,5-diene	0.26±0.25[a]	0.25±0.24[a]
Cadina-1,4-diene	0.48±0.38[a]	0.44±0.29[a]

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

Chapter VII: Antimicrobial efficacy of gaseous chlorine dioxide for inactivation of *Salmonella* on dried basil leaves

7.1 Abstract

The repeated association of low moisture foods with foodborne outbreaks requires the application of novel antimicrobial processing technologies to ensure food safety. Chlorine dioxide gas is a promising non-thermal treatment that has been employed to reduce the microbial load in various food products and is approved under U.S. regulation 21 CFR 173.300. This research aims to (i) investigate the efficacy of chlorine dioxide gas on the inactivation of *Salmonella* on dried basil leaves, and (ii) evaluate the suitability of *E. faecium* as a surrogate for *Salmonella*. Dried basil leaves (2.0±0.1 g) inoculated with a 5-strain *Salmonella* cocktail or *E. faecium* were packed in a mesh tea bag, heat-sealed, and placed inside a chamber designed for the chlorine dioxide treatment. The samples were exposed to different levels of gas concentration (5, 10, and 15 mg/L) and relative humidity (RH; 60, 70, 80%) for various exposure times (0-5 h) to develop a microbial inactivation model. The treated samples were diluted with 18 mL of neutralizing buffer (NB) and homogenized for 1 min. The dispersions were 10-fold serially diluted with NB and plated onto tryptic soy agar modified to enumerate *Salmonella* and *E. faecium.* The log-linear model was used to fit the inactivation data for both microorganisms. As the relative humidity increases from 60 to 80% at 10 mg/L, the *D*-value decreases from 70.5 to 41.8 min for *Salmonella* and 121.7 to 52.6 min for *E.*

faecium. Based on the AIC_c values, the modified Bigelow model performed better than the response surface model for estimation of the *D*-values. A greater resistance exhibited by *E. faecium* indicated that it may be used as an ideal surrogate for *Salmonella* during chlorine dioxide treatment of dried basil leaves. This research will provide food processors with a starting point for implementing gaseous technologies to improving the microbial safety of low moisture foods.

Keywords: Inactivation kinetics; *E. faecium*; non-thermal treatment; low moisture food

7.2 Introduction

The American Spice Trade Association (ASTA) defines spices as any commodity which is derived from plants and is further dried for seasoning purposes (ASTA, 1990). Historically, spices and herbs have been consumed as ready-to-eat seasonings due to their aroma and flavor properties. In the present time, the demand for spices and herbs has significantly increased not only to enhance the flavor of the food but also for their potential health benefits (Tapsell et al., 2006). The spice market in the United States is estimated to reach 7.3 billion dollars by 2025, growing at a compound annual growth rate of 4.07% from 2020-2025 (Wunsch, 2020).

Spices in the United States are usually imported from various developing countries where they are sometimes grown and harvested under inadequate sanitary conditions (ASTA, 2017). After harvesting, spices and herbs are spread on the ground to sundry with no or minimal protection from domestic animals, pests, or wildlife. Therefore, it is

not surprising that the spices can easily get contaminated with various microorganisms during this time. Spices and herbs fall under the category of low moisture foods (a_w < 0.70). The inherent low water activity of spices/herbs prevents the microorganisms from growing, thereby improving their shelf life. However, pathogenic bacteria such as *Salmonella* can survive for a long time in desiccated environments, leading to multiple recalls of various low moisture foods (Podolak et al., 2010). A study by Keller et al. (2013) reported that *Salmonella* survived in black pepper for over one year when stored at ambient conditions (25°C, 40% relative humidity). According to the Food and Drug Administration (FDA) Reportable Food Registry, from 2009-2014, 74 recalls were associated with spices and seasonings, out of which 65 were due to contamination with *Salmonella.* Therefore, *Salmonella* is a pertinent microorganism in spices and herbs that must be controlled effectively to avoid any potential threat to human health.

With the enactment of the Food Safety Modernization Act in 2011, the food processors must validate their process controls as interventions to prevent and control food safety hazards identified in their processes. Because spices are considered ready-to-eat products, microbial reduction techniques need to be employed and validated to assure the safety of spices and herbs. Currently, the U.S. spice industry uses ethylene oxide fumigation as an intervention to control various pathogens such as *Salmonella* and *E. coli*. Microbial validation studies conducted by Wei et al. (2021) and Chen et al. (2021) showed that ethylene oxide fumigation was effective in reducing the *Salmonella* population in whole black peppercorns and cumin seeds, respectively.

According to ASTA, ethylene oxide is used to fumigate 40-85% of spices in the U.S each year (ASTA, 2017). The U.S. Environmental Protection Agency (EPA) regulates the ethylene oxide treatment under 40 CFR 180.151, and strict tolerances for ethylene oxide and ethylene chlorohydrin residues in spices have been set at 7 and 940 ppm, respectively. However, herbs such as dried basil have been listed as an exception that cannot be treated with ethylene oxide because the treatment may result in the formation of high levels of ethylene chlorohydrin due to the presence of naturally occurring chlorides (Gurtler et al., 2014). Therefore, it is essential to explore other technologies for effectively control the microbial load in dried basil leaves.

Another treatment method, steam has been commonly used in reducing the microbial load in spices (ASTA, 2017). However, the major disadvantage of using steam is that the moisture content of the treated product increases significantly, which requires an additional drying step for spices. Additionally, there is a possibility that the heat-sensitive components such as volatiles in spices and herbs might decrease due to the involvement of high temperatures during steam treatment. Therefore, investigating non-thermal technologies would be beneficial for controlling the microbial load in dried basil as well as maintaining the final product quality.

Gaseous chlorine dioxide is a non-thermal method that has been used to reduce the microbial load in various high moisture foods such as green peppers (Han et al., 2000), apples (Du et al., 2002), strawberries (Han et al., 2004), and tomatoes (Trinetta et al., 2010). The use of gaseous chlorine dioxide has also been approved by EPA and FDA as a

food additive for antimicrobial purposes under 21 CFR 173.300 (FDA, 1998). However, there is a lack of studies that evaluates the efficacy of gaseous chlorine dioxide on microbial inactivation in different low moisture foods. A recent study by Rane et al. (2020) evaluated the antimicrobial effect of chlorine dioxide gas in almonds and black peppercorn for microbial inactivation. The study reported that at 0.40 mg ClO_2/g product, 2.6 and 1.7 log reduction of *Salmonella* was achieved in almonds (6 h exposure) and whole black peppercorns (4 h exposure), respectively. However, the study did not evaluate the effect of different levels of gas concentrations and relative humidity on microbial inactivation. During the chlorine dioxide treatment, gas concentration, relative humidity, and exposure time play an important role in microbial inactivation (Han et al., 1999). Therefore, it is critical to conduct a comprehensive study to help the food processors for effective implementation of chlorine dioxide gas treatment for improving the microbial safety of spices and herbs.

The objectives of this research were to (i) investigate the efficacy of chlorine dioxide gas treatment on the inactivation of *Salmonella* on dried basil leaves at different levels of gas concentration, relative humidity, and exposure time, and (ii) evaluate the suitability of *Enterococcus faecium* NRRL B-2354 as an appropriate surrogate for *Salmonella* during chlorine dioxide treatment of dried basil leaves.

7.3 Materials and Methods

7.3.1 Bacterial strains and inoculum preparation

Five serotypes of *Salmonella enterica* were selected based on their occurrence in different low moisture food outbreaks and recalls and were used in this study as a cocktail. For the surrogate study, *Enterococcus faecium* was used. The detailed information about the microorganisms is presented in Table 7.1.

The inoculum preparation was divided into two steps: preparing working stock plates and using the working stock plates to prepare inoculum. The frozen culture for each serotype and surrogate was removed from the ultra-freezer (-80°C) and thawed at room temperature for 10 min. The thawed culture (1 mL) was transferred to 10 mL tryptic soy broth supplemented with 0.6% yeast extract (TSBYE; BD Difco) and incubated at 37°C for 24±2 h. The overnight TSBYE broth (10 µL) was used to streak tryptic soy agar supplemented with 0.6% yeast extract (TSAYE; BD Difco) plates followed with incubation for 24±2 h at 37°C. These plates were wrapped with parafilm (PM-999, Bemis), stored at refrigerated conditions (4°C), and were called working stock plates. The working stock plates were used within 30 days, and after 30 days, a new set of plates were prepared.

The working stock plates were used to prepare the *Salmonella* cocktail and *E. faecium* inoculum. One isolated colony from each working stock plate (*Salmonella* serotype or *E. faecium*) was transferred to 10 mL TSBYE and incubated for 24±2 h at 37°C. The TSBYE broth (0.1 mL) was spread plated onto TSAYE plates with incubation for 24±2 h at 37°C. The following day, the bacterial lawn was harvested by adding 3 mL of

0.1% (w/v) buffered peptone water (BPW; BD Difco) to each plate, and an L-shaped spreader (14665230; Fisherbrand) was used to scrape the lawn. The *Salmonella* cocktail (10 mL) was prepared by adding an equal volume of each serotype (2 mL) to a sterile conical tube. The conical tube was vortexed for ca. 30 s for the uniform distribution of cells. For *E. faecium*, the harvested lawn was also aseptically transferred to a conical tube and vortexed for ca. 30 s. The prepared inoculum (*Salmonella* cocktail and *E. faecium*) was used within 2 h for the inoculation of dried basil leaves. A new set of working stock plates was prepared using a new frozen stock for inoculation of the other two batches, representing the three biological replicates.

7.3.2 Preparation and inoculation of dried basil leaves

Three batches (replicates) of steam-sterilized dried basil leaves were procured from McCormick & Co., Inc. (Hunt Valley, MD). Each batch of dried basil leaves came from a different production lot. The water activity and moisture content of the samples were measured using a dew point water activity meter (Model: 4TE, Meter Group; 25°C) and a halogen moisture analyzer (Model: HR73, Mettler Toledo), respectively. Before conducting the experiment, the background microflora of dried basil leaves was estimated using the aerobic plate count method. For each batch, five 25-g samples were removed from different locations, diluted with 225 mL of BPW, and homogenized for 1 min in a stomacher. The homogenized fluid was spread plated onto TSAYE plates in duplicates with incubation at 37°C for 24±2 h.

The inoculation activities were carried out in the biosafety cabinet. Dried basil leaves (100±0.1 g) were aseptically weighed in a sterile whirl bag to which 2 mL of inoculum (*Salmonella* cocktail or *E. faecium*) was sprayed. The bag was then sealed, and the inoculated sample was manually mixed for 10 min to ensure that the inoculum was homogeneously mixed onto the product surface. Post inoculation, the sample was placed on a sanitized aluminum tray (23 x 30 x 1.5 cm) and transferred to the custom-designed relative humidity chamber (Kiat and Subbiah, 2020). The relative humidity of the chamber was set to 55% to bring back the samples to their natural water activity level.

7.3.3 Gaseous chlorine dioxide

The gaseous chlorine dioxide treatment was carried out in a polypropylene closed chamber (28.9 in. (length) x 17.4 in. (width) x 26.9 in. (height)) designed and prepared by ClorDiSys Solutions, Inc. (Branchburg, NJ). A temperature and humidity transducer (Testo 6621; accuracy of ±2.0% relative humidity, ±0.5°C) was mounted on top of the chamber to continuously monitor the temperature and relative humidity during the experiment. An ultrasonic humidifier (EE-5301, Crane) was connected to the gas chamber with a tubing (01000774, Fisherbrand) and was used to inject the humid air into the gas chamber. Two USB-powered fans (38HX82; Grainger) were placed inside the chamber to allow the circulation of chlorine dioxide gas. The schematic of the gas chamber is presented in Figure 7.1.

The ClorDiSys Minidox-M portable gas generator was used to conduct the experiments. The gas generator consists of three cartridges, which contain sodium chlorite as an active ingredient. An external reagent gas cylinder composed of 2% chlorine/98% nitrogen (SG G2676973; Matheson) was connected to the generator, which reacts with the sodium chlorite to produce chlorine dioxide gas. The chlorine dioxide gas generated was transferred to the chamber through polytetrafluoroethylene (PTFE) tubing. The Minidox-M gas generator was equipped with a photometric sensor which measured the chlorine dioxide concentration in real time. The decontamination cycle consisted of five phases: pre-condition, condition, charge, exposure, and aeration. The detailed information about each phase is given below:

Pre-conditioning: During pre-condition, a chamber leak test was performed. After leak testing, moisture was introduced into the chamber to raise the relative humidity to a specified set point.

Conditioning: Relative humidity was maintained during this time in the chamber for the selected amount of time.

Charge: Chlorine dioxide gas was generated and introduced into the chamber to achieve the target gas concentration.

Exposure: During this time, the target gas concentration is continuously monitored and maintained. If the gas concentration falls below the setpoint, gas makeup was activated, and additional gas was supplied to the chamber.

Aeration: This allows the gas to be removed from the chamber. The gas generator is connected to a scrubber (BSC-001; ClorDiSys Solutions, Inc., Branchburg, NJ). The scrubber is a carbon-based scrubbing system used to remove chlorine dioxide gas from the chamber after the desired exposure time was completed. The air leaving the scrubber was monitored using a handheld gas detector (Accuracy: ±5%; D16 Portasens III (Sensor: 00-1005); Analytical Technology, Inc., Collegeville, PA) for chlorine dioxide gas. If any gas was detected, the protocol is to replace the activated carbon pellets inside the scrubbing system.

7.3.4 Treatment of inoculated samples

The Minidox-M has its own built-in control system which was used to control relative humidity, gas concentration, and exposure time. The inoculated dried basil leaves (2.0±0.1 g) were packed in a heat-sealable tea bag (2.5 x 2.75 in.) and placed inside the gas chamber on a plastic petri dish (100 mm diameter x 15 mm height). The treatments were performed at different levels of chlorine dioxide gas concentration (5, 10, and 15 mg/L) and relative humidity (60, 70, and 80%). A total of five pre-determined exposure times were used, depending on the gas concentration and relative humidity, to achieve a 3 to 5 log reduction of *Salmonella*. The same treatment conditions were performed for *E. faecium* to evaluate its suitability as a surrogate for *Salmonella*. For all the treatments, the pre-conditioning and conditioning cycle was set for 10 min. Post-treatment, the gas chamber was allowed to aerate for 5 min. The ATi Portasens III gas

detector was used to check the presence of any chlorine dioxide gas inside the chamber before the treated samples were removed.

7.3.5 Microbial enumeration

Each treated sample (2.0±0.1 g) was removed from the chamber and diluted with 18 mL of neutralizing buffer containing 0.016% (w/v) of sodium thiosulfate (Fisher Scientific, Fair Lawn, NJ) and 0.00425% (w/v) of monopotassium phosphate (Fisher Scientific, Fair Lawn, NJ). The neutralizing buffer was used to inactivate the bactericidal effect of chlorine compounds. The diluted sample was homogenized in a stomacher for 1 min, and appropriate serial dilutions were prepared using tubes containing 9 mL neutralizing buffer. The diluent was spread plated in duplicate onto m-TSAYE and e-TSAYE agar plates for *Salmonella* and *E. faecium*, respectively. The m-TSAYE media was composed of TSAYE supplemented with 0.03 (w/v) sodium thiosulfate (Fisher Scientific, Fair Lawn, NJ), and 0.05% (w/v) ammonium iron citrate (Sigma Alrich, St. Louis, MO), and e-TSAYE was composed of TSAYE supplemented with 0.05% (w/v) ammonium iron citrate and 0.025% (w/v) esculin hydrate (Acros, NJ).

7.4 Inactivation models

7.4.1 Primary model

The log-linear model (Eqn. 7.1) was used to fit the inactivation data of *Salmonella* and *E. faecium* in dried basil leaves treated with chlorine dioxide gas.

$$\log_{10}\left(\frac{N}{N_0}\right) = -\frac{t}{D} \tag{7.1}$$

where N_0 is the microbial counts before the gas treatment (CFU/g); N is the microbial survivor count after the gas treatment (CFU/g); D is the decimal reduction time (min; time required to decrease bacterial population by 1-log at a given gas concentration and relative humidity); and t is the exposure time (min).

The adjusted coefficient of determination (R^2) and root mean square error (RMSE) were used to evaluate the goodness-of-fit of the log-linear model. A scientific software, GraphPad Prism version 8.0.2 (GraphPad Software, San Diego, CA) was used to fit the primary model and generate the inactivation curves for *Salmonella* and *E. faecium*.

7.4.2 Secondary models

Two secondary models, a second-order response surface model (Eqn. 7.2) and the modified Bigelow model (Eqn. 7.3), were developed to evaluate the effect of gas concentration and relative humidity on the *D*-values of *Salmonella* and *E. faecium*.

$$D_{(C,RH)} = \beta_o + \beta_1 * C + \beta_2 * RH + \beta_3 * C^2 + \beta_4 * RH^2 + \beta_5 * C * RH \quad (7.2)$$

where β_o is the intercept; β_1 is the linear regression coefficient for gas concentration, C; β_2 is the linear regression coefficient for relative humidity; β_3 and β_4 are the quadratic regression coefficients for gas concentration and relative humidity, respectively; β_5 is the linear regression coefficient for the interaction of gas concentration and relative humidity; C is the gas concentration (mg/L); RH is the relative humidity (%).

After fitting the response surface model, the effect of each variable was tested at *p<0.05*, and only the significant terms were considered while developing the final response surface model. An open-source statistical tool, RStudio version 1.3.1073 (Integrated Development for RStudio, Inc., Boston, USA) was used to fit the response surface model and estimate its parameters.

The following is the equation for the modified Bigelow model:

$$D_{(C,RH)} = D_{ref}.10^{\frac{C_{ref}-C}{z_C}}.10^{\frac{RH_{ref}-RH}{z_{RH}}} \quad (7.3)$$

Where GC is the gas concentration (mg/L); RH is the relative humidity (%); C_{ref} and RH_{ref} are the optimized reference gas concentration and relative humidity; D_{ref} is the reference decimal reduction time (min); z_C is the increase in gas concentration required to decrease the *D*-value by 1-log; z_{RH} is the increase in relative humidity required to decrease the *D*-value by 1-log. The modified Bigelow model was developed with MATLAB version 2020b (The Mathworks, Inc., Natick, MA) using the ordinary least square minimization with *nlnfit*. Akaike Information Criterion (AIC_c; Eqn. 7.4) was used to evaluate the goodness-of-fit of both secondary models.

$$AIC_c = n\ln\left(\frac{SS}{n}\right) + 2K + \frac{2K(K+1)}{n-K-1} \quad (7.4)$$

where n is the number of observations; K is the number of parameters plus 1; SS is the error sum of squares.

7.5 Results and Discussions

7.5.1 Inoculation of dried basil leaves

The results from the background microflora test showed that the aerobic plate count was below the detection limit (<10 CFU/g), which means that the background microflora will not interfere during the bacterial enumeration. The water activity and moisture content of the dried basil leaves were recorded as 0.54±0.01 and 9.85±0.15% wet basis, respectively. The dried basil leaves were inoculated with *Salmonella* and *E. faecium* at a level of 7.52±0.08 and 7.73±0.13 log CFU/g, respectively. Post inoculation, the samples were conditioned to their native water activity of 0.54.

Usually, a higher water activity ($a_w > 0.92$) is required by pathogenic bacteria such as *Salmonella* to grow and multiply in a food product. A physiologically stressful environment is created when such pathogens are introduced into low moisture foods, resulting in a higher bacterial reduction when the challenge study is performed immediately after the inoculation process (Jeong and Kang, 2014). Therefore, equilibration of the inoculated food product for few days is essential to allow the pathogen to adapt to the low moisture environment. Verma et al. (2021) reported that *Salmonella* and *E. faecium* population in dried basil leaves did not change significantly and was stable from day 5 to 15. Similarly, Wei et al. (2020) reported that inoculated whole black peppercorns were allowed to stabilize for at least five days before the ethylene oxide gas treatment was performed. Therefore, bacteria in the inoculated dried basil sample were allowed to stabilize in the relative humidity chamber for five

days before the chlorine dioxide treatment was performed. This may result in conservative estimate of antimicrobial efficacy chlorine dioxide gas, which is important for food safety applications.

7.5.2 Inactivation model

a) Primary model

The *D*-values for *Salmonella* and *E. faecium* NRRL B-2354 in dried basil leaves treated at different gas concentrations and relative humidities are presented in Table 7.2. Figure 7.2 shows the survival curves for *Salmonella* and *E. faecium* at different gas concentrations and relative humidities. It can be noted from Table 7.2 that as the gas concentration and relative humidity increase, the *D*-values for *Salmonella* and *E. faecium* decreases. For example, the *D*-value for *Salmonella* and *E. faecium* decreases from 68.5 to 46.5 min and 91.5 to 69.2 min, respectively, as the gas concentration increases from 5 to 15 mg/L at 70% relative humidity. Similarly, as the relative humidity increases from 60 to 80%, the *D*-values for *Salmonella* and *E. faecium* decreases from 70.5 to 41.8 min and 121.7 to 52.6 min, respectively, at a gas concentration of 10 mg/L. The effect of gas concentration on *D*-values has been very well studied in various high moisture foods. Mahmoud, Bhagat, and Linton (2007) reported that the *D*-values for *Salmonella* in strawberries decreased from 4.2 to 2.7 min as the chlorine dioxide gas concentration increased from 0.5 to 5.0 mg/L. The extremely low *D*-values for *Salmonella* in strawberries were due to the high moisture content of the product and high relative humidity (90-95%) at which the inactivation study was conducted.

Westphal et al. (2003) reported that higher humidity levels result in the hydration of microorganisms, making them sensitive to the chlorine dioxide treatment.

The results presented in Table 7.2 show that the log-linear model fits very well with adjusted R^2 values greater than 0.96 for *Salmonella* and 0.93 for *E. faecium*. Therefore, no other primary models were evaluated to fit the inactivation data for *Salmonella* and *E. faecium*. Other primary model such as Weibull have been used to explain the non-linear thermal inactivation of microorganisms in various low moisture foods, like peanut butter (Ma et al., 2009), almonds (Villa-Rojas et al., 2013), wheat flour (Smith et al., 2016), etc. In the case of the chlorine dioxide inactivation study, the log-linear model performed well to explain the inactivation kinetics of *Salmonella* in lettuce (Mahmoud and Linton, 2008) and strawberries (Mahmoud, Bhagat, and Linton, 2007). However, the studies on evaluating the effect of chlorine dioxide gas on the inactivation kinetics of *Salmonella* or other microorganisms in low moisture food are limited.

Wang et al. (2019) evaluated the effect of gaseous chlorine dioxide on the inactivation of *Salmonella* on almonds. Their study reported that only 1.46 log CFU/g reduction of *Salmonella* was achieved at a maximum gas concentration of 4.64 mg/L after 4 h of exposure time with relative humidity maintained at 95%. The authors reported that an additional mild heat treatment was required after chlorine dioxide to achieve a greater reduction of *Salmonella* in almonds. However, no such heat treatment was required for dried basil leaves treated with chlorine dioxide gas. A gas concentration of 5 mg/L and relative humidity of 80% resulted in more than 4 log

reduction of *Salmonella* in dried basil leaves when exposed to chlorine dioxide gas for 200 min (3.33 h). This could be due to antimicrobial compounds present in herbs and spices, which help reduce pathogens. Rane et al. (2020) studied the effect of chlorine dioxide gas treatment for *Salmonella* reduction in whole black peppercorns. The results from their study reported that 3.70 log reduction of *Salmonella* was achieved in whole black peppercorn when exposed at 0.40 mg chlorine dioxide/g peppercorn for 4 h, and relative humidity maintained at 80%.

b) Secondary models

The *D*-values for *Salmonella* and *E. faecium* calculated using the log-linear model were used to generate two secondary models: the response surface model and the modified Bigelow model. The purpose of the secondary models was to evaluate the effect of gas concentration and relative humidity on the *D*-values.

A response surface model was developed for *Salmonella* (Eqn. 7.5) and *E. faecium* (Eqn. 7.6) to evaluate the effect of relative humidity and gas concentration on the *D*-values.

***Salmonella*:** $D_{(RH,C)} = 323.1957 - 12.8602^{*}C - 3.3952^{*}RH + 0.1461^{*}C^{*}RH$

($R^2_{adjusted}$ = 0.95; AIC_c = 164.10; RMSE = 4.20 min) (7.5)

E. faecium: $D_{(RH,C)} = 1090.6411 - 28.1324^{*}C - 20.5884^{*}RH + 0.3460^{*}C^{*}RH + 0.0959^{*}RH^2$ ($R^2_{adjusted}$ = 0.96; AIC_c = 188.46; RMSE = 8.27 min) (7.6)

where C is the gas concentration (mg/L); RH is the relative humidity (%); D is the *D*-value (min). Only the significant terms were considered while developing the response surface model for the microorganisms. The response surface models fit well for both the microorganisms with adjusted $R^2 \geq 0.95$. For *Salmonella*, gas concentration and relative humidity showed a significant (*P < 0.0001*) linear effect on the *D*-value. Also, there was a significant (*P < 0.0001*) interactive effect of gas concentration and relative humidity seen in the final response surface model developed for *Salmonella*. For *E. faecium*, the final response surface model showed a significant (*P < 0.0001*) linear effect of gas concentration and relative humidity. Relative humidity also showed a significant (*P < 0.0001*) quadratic effect and interactive effect (*P < 0.0001*) with gas concentration. The contour plots were developed for *Salmonella* and *E. faecium* using the response surface model and are presented in Figure 7.3. It should be noted in Figure 7.3 that the contour lines are linear at a lower gas concentration (<10%). However, as the gas concentration increases from 10 to 15%, the contour lines became curvilinear, indicating the significant interaction effect of relative humidity and gas concentration as seen in the response surface equations for *Salmonella* and *E. faecium*.

The modified Bigelow model developed for *Salmonella* (Eqn. 7.7) and *E. faecium* (Eqn. 7.8) are given below:

Salmonella: $D_{(RH,C)} = 55.86 * 10^{\frac{10-C}{48.19}} * 10^{\frac{70-RH}{67.99}}$ ($R^2_{adjusted}$ = 0.96; AIC_c = 87.96; RMSE = 4.51 min) (7.7)

E. faecium: $D_{(RH,C)} = 78.70 * 10^{\frac{10-C}{44.56}} * 10^{\frac{70-RH}{49.66}}$ ($R^2_{adjusted}$ = 0.96; AIC_c = 120.70; RMSE = 6.35 min) (7.8)

where C is the gas concentration (mg/L); RH is the relative humidity (%); D is the *D*-value (min). The estimated D_{ref} value for *Salmonella* and *E. faecium* at C_{ref} of 10 mg/L and RH_{ref} of 70% were 55.9 and 78.7 min, respectively. These values were close to the experimental values, 59.7 min for *Salmonella* and 80.6 min for *E. faecium*. The z_C values for *Salmonella* (48.19 mg/L) and *E. faecium* (44.56 mg/L) were similar. The gas concentration tested in this study ranged from 5 to 15 mg/L. The chlorine dioxide gas concentration higher than 15 mg/L would have a deleterious effect on the quality of the dried basil leaves. The z_C values were about 4 times of test range (15-5 mg/L = 10 mg/L). When compared to the test range, z_C is much higher, indicating that the gas concentration has a lesser impact on *D*-values. The z_{RH} value for *Salmonella* (67.99%) was much higher than that for *E. faecium* (49.66%), indicating that increase in relative humidity had a higher antimicrobial efficacy against *E. faecium* than *Salmonella*. The z_{RH} values were ca. 2.48 to 3.40 times the tested range of relative humidity (80-60% = 20%), indicating that relative humidity is more effective than the gas concentration within the range of testing. This agrees with the experimental values shown in Table 7.2. For example, the *D*-values for *Salmonella* and *E. faecium* decreased by 40.7 and 56.8%, respectively as the relative humidity increased from 60 to 80% at a gas concentration of 10 mg/L. However, the *D*-values for *Salmonella* and *E. faecium* decreased by 32.1 and 24.4% as the gas concentration increased from 5 to 15 mg/L at 70% relative humidity.

Therefore, relative humidity had a more pronounced effect on *D*-values when compared to the gas concentration tested in this study.

The contour plots developed for *Salmonella* and *E. faecium* using the modified Bigelow model are presented in Figure 7.4. Overall, the contour plots from the response surface model and modified Bigelow model showed that the *D*-value of both microorganisms decreased with increased gas concentration and relative humidity. However, upon calculating the slope of the contour lines in Figure 7.4, it was found that the slope for *Salmonella* was steeper (1.5:1) than the slope of contour lines for *E. faecium* (1:1). This means that for every increase of 1.5% relative humidity, the gas concentration can be decreased by 1 mg/L to maintain the same antimicrobial efficacy for *Salmonella*. In the case of *E. faecium*, for every 1% increase in relative humidity, the gas concentration can be decreased by 1 mg/L to maintain the same antimicrobial efficacy. This indicates that relative humidity has a higher impact on *E. faecium* than *Salmonella*, which agreed with the z_{RH} value interpretation.

It can be noticed in the contour plots (Figure 7.4) that the distance between the contour lines was shorter at lower gas concentration and relative humidity, while the distance increased at higher gas concentration and relative humidity. At 60% relative humidity, increasing the gas concentration from 7.1 to 9.5 mg/L (ΔC = 2.4 mg/L) decreased the *D*-value of *Salmonella* by 10 min. To further decrease *D*-value by 10 min, the gas concentration has to be increased by 2.7 mg/L. This indicates that the *D*-values are not linearly related to the gas concentration and relative humidity. The modified

Bigelow model hypothesizes that log(*D*) values were linearly related to relative humidity and gas concentration. That non-linearity shows up in the non-linearity spacing of contour plot lines.

AIC_c values were used to compare the performance of both the secondary models. A lower value of AIC_c means that the selected model is correct for the data (Valdramidis et al., 2006). The results showed that the modified Bigelow model performed better than the response surface model due to the lower AIC_c values. Also, the modified Bigelow model provided a linear contour plot, which is easier to interpret by the food industries. The modified Bigelow model has been used in thermal inactivation studies to evaluate the effect of temperature and water activity on the *D*-values in various low moisture foods such as dried basil leaves (Verma et al., 2021), wheat flour (Smith et al., 2016), ground black pepper (Wei et al., 2021), and milk powders (Wei et al., 2020). The developed modified Bigelow model can be used by the herb and spice industry to understand the effect of gas concentration and relative humidity on *Salmonella* inactivation during the chlorine dioxide gas treatment of dried basil leaves.

Verma et al. (2021) reported that the *D*-values of *Salmonella* during thermal treatment ranged from 1.9-15.0 min for dried basil leaves at a water activity of 0.55 for the temperature range of 70-80°C. In comparison, the *D*-values of *Salmonella* during the chlorine dioxide gas treatment of dried basil leaves (a_w = 0.54) ranged from 32.8-104.9 min at different relative humidities and gas concentrations tested in this study. This shows that thermal treatments can be more effective than non-thermal treatment.

Verma et al. (2021) used radiofrequency (RF) heating for the pasteurization of dried basil leaves. The results showed that 65 s of RF heating was enough to inactivate the *Salmonella* population below the detection limit (<10 CFU/g). However, when compared to this study, a longer chlorine dioxide treatment (>2.5 h) was required to achieve a 5-log reduction of *Salmonella* depending on the gas concentration and relative humidity. Therefore, thermal method is more effective than the non-thermal method for pasteurizing the dried basil leaves. The advantage of the non-thermal method is the enhanced quality retention in the spices, especially total volatiles. However, Verma et al. (2021) did not find a significant reduction in the quality post RF treatment when compared to the untreated samples. It is important to note that the untreated dried basil leaves were steam-sterilized in both the studies. As steam treatment might have knocked out some volatiles, Verma et al. (2021) might have underestimated the quality loss. However, it is challenging to procure untreated raw samples for the study.

Because relative humidity showed a greater effect, the efficacy of the chlorine dioxide gas can be further improved by increasing the relative humidity during the treatment. When the samples were exposed at the highest relative humidity tested in this study (80%) for few hours, the moisture content increased by 1.0-1.5% in the treated sample. Because mild heat treatment (45-60°C for 4 h) has shown a synergistic effect (Wang et al., 2019), our future work will focus on treating the dried basil leaves with chlorine dioxide gas at higher relative humidity (>80%), which may further increase the moisture content. Following mild heat treatment should provide additional

microbial reduction and dry the food product to the desired moisture content if time-temperature combinations are optimized. This may allow the spice and herb industry to effectively inactivate *Salmonella* on dried basil leaves at lower gas concentrations while minimizing the loss of food product quality.

7.5.3 *E. faecium* as an appropriate surrogate for *Salmonella*

Identifying a suitable surrogate for *Salmonella* is necessary to assist the food industries in conducting an in-plant validation study related to the chlorine dioxide gas. For this study, *E. faecium* NRRL B-2354 was deemed an ideal surrogate for *Salmonella* during the chlorine dioxide treatment of dried basil leaves. The *D*-values of *E. faecium* at different gas concentrations and relative humidities are presented in Table 7.2. On comparing the *D*-values of *Salmonella* and *E. faecium*, it is clear that *E. faecium* exhibited a greater resistance at all the conditions tested in this study. At the lowest relative humidity of 60%, the *D*-value of *Salmonella* and *E. faecium* at 5, 10, and 15 mg/L was 104.9, 70.5, and 61.9 min and 170.6, 121.7, and 88.6 min, respectively. Overall, the *D*-values of *E. faecium* were approx. 1.2 to 1.9 times greater than that of *Salmonella* in chlorine dioxide treated dried basil leaves.

The suitability of *E. faecium* as an appropriate surrogate for *Salmonella* has not been evaluated for the different low moisture foods treated with chlorine dioxide gas. Rane et al. (2021) is the only study available in the literature which reported that *E. faecium* exhibited equal or higher resistance than *Salmonella* and thus can be used as a surrogate for *Salmonella* in chlorine dioxide treated almonds. In other gaseous studies

such as ethylene oxide, Wei et al. (2021) and Chen et al. (2021) reported that *E. faecium* is a suitable surrogate for *Salmonella* during the ethylene oxide fumigation of whole black peppercorn and cumin seeds, respectively. The suitability of *E. faecium* as a *Salmonella* surrogate has been well established in other thermally treated low moisture foods such as oat flour (Verma et al., 2018), wheat flour (Liu et al., 2018), egg powder (Pérez-Reyez et al., 2021), cocoa powder (Tsai et al., 2019), peanuts and pecans (Brar and Danyluk, 2019), and milk powders (Wei et al., 2021).

The surrogates are specific to the product and process type. The suitability of *E. faecium* as a suitable surrogate for *Salmonella* needs to be assessed in different low moisture foods treated with chlorine dioxide gas. This will allow the food industries to fully adopt the non-thermal gaseous technology and perform in-plant validation studies effectively. Because *E. faecium* has greater resistance than *Salmonella*, it can be used as a reasonable surrogate for *Salmonella* in chlorine dioxide treatment of dried basil leaves.

7.6 Conclusion

The current study demonstrated the efficacy of chlorine dioxide gas for the inactivation of *Salmonella* in dried basil leaves. A log-linear model was used to describe the inactivation kinetics of *Salmonella* and *E. faecium*. The results showed that the log-linear model fitted well for *Salmonella* and *E. faecium* with R^2 greater than 0.93. The increase in relative humidity and gas concentration resulted in a decrease in *D*-values for both the microorganisms. A longer chlorine dioxide treatment time (>2.5 hours) was

required to achieve a 5-log reduction of *Salmonella* in dried basil leaves, even at the highest gas concentration of 15 mg/L and relative humidity of 80%. The secondary models were developed and compared to assess relative humidity and gas concentration on *D*-values. The relative humidity had a bigger effect than gas concentration within the tested range; relative humidity also had a higher antimicrobial efficacy on *E. faecium* than *Salmonella*. The AIC_c values showed that the modified Bigelow model fared better than the response surface model. Also, *E. faecium* was demonstrated to be an appropriate surrogate for *Salmonella* in dried basil leaves during chlorine dioxide treatment. The results presented in this study will assist the herb and spice industry in using chlorine dioxide gas as an alternative treatment for controlling the microbial load in their food products.

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Table 7. 1 *Salmonella* serotypes and surrogate used in this study.

Microorganism	**Isolate #**	**Source**	**Associated recall**
Salmonella Agona	447967	FDA, ORA, Arkansas Regional Lab, Jefferson, AR (USA)	Oat cereal (CDC, 1998)
Salmonella Mbandaka	698538	FDA, ORA, Arkansas Regional Lab, Jefferson, AR (USA)	Sprouts (Jackson et al. (2013)
Salmonella Montevideo	488275	FDA, ORA, Arkansas Regional Lab, Jefferson, AR (USA)	Black and red pepper (CDC, 2010)
Salmonella Reading	Moff 180418	FDA culture collection, Bedford Park, IL (USA)	Alfalfa sprouts (CDC, 2016)
Salmonella Tennessee	K4643	Dr. L. Beuchet, University of Georgia, Griffin, GA (USA)	Peanut butter (CDC, 2007)
Enterococcus faecium	NRRL B-2354	USDA. ARS, Peoria, IL (USA)	-

Table 7. 2 *D*-values for *Salmonella* and *E. faecium* using the log-linear model.

Bacteria	Gas concentration (mg/L)	Relative humidity (%)	*D*-value (min)	95% Confidence interval		Adjusted R^2	RMSE (log CFU/g)
				Lower	Upper		
Salmonella	5	60	104.9	89.13	128.01	0.97	0.26
	10		70.5	56.63	93.02	0.96	0.31
	15		61.9	55.09	70.22	0.99	0.15
	5	70	68.5	61.50	77.46	0.99	0.15
	10		59.7	51.68	70.37	0.98	0.17
	15		46.5	39.11	56.91	0.97	0.24
	5	80	46.6	41.43	53.16	0.99	0.16
	10		41.8	37.02	47.73	0.98	0.14
	15		32.8	26.62	42.62	0.96	0.27
E. faecium	5	60	170.6	141.32	215.84	0.97	0.18
	10		121.7	97.46	161.52	0.96	0.18
	15		88.6	70.37	118.08	0.95	0.22
	5	70	91.5	69.49	134.62	0.93	0.31
	10		80.6	70.82	112.12	0.96	0.18
	15		69.2	46.97	89.13	0.94	0.30
	5	80	59.7	46.27	84.03	0.94	0.29
	10		52.6	43.25	66.80	0.97	0.18
	15		46.8	37.12	63.29	0.95	0.21

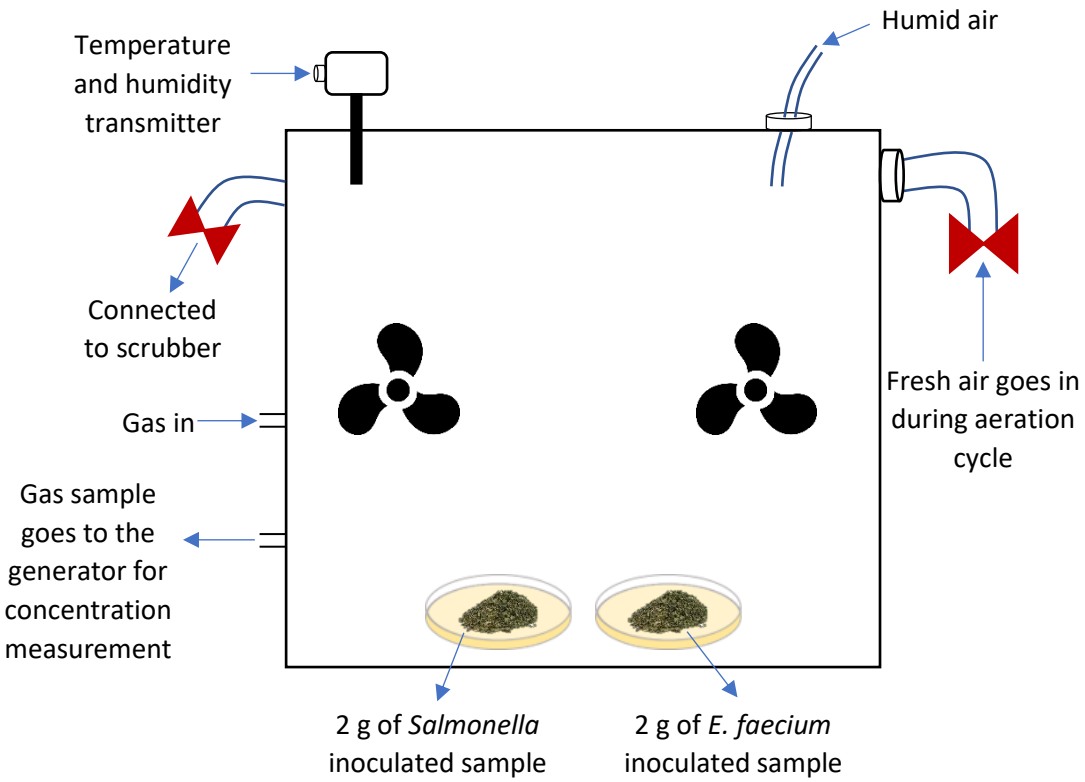


Figure 7. 1 Schematic of chlorine dioxide treatment chamber. (*Note: The red color valves remain closed during the decontamination cycle and were opened manually during the aeration cycle).

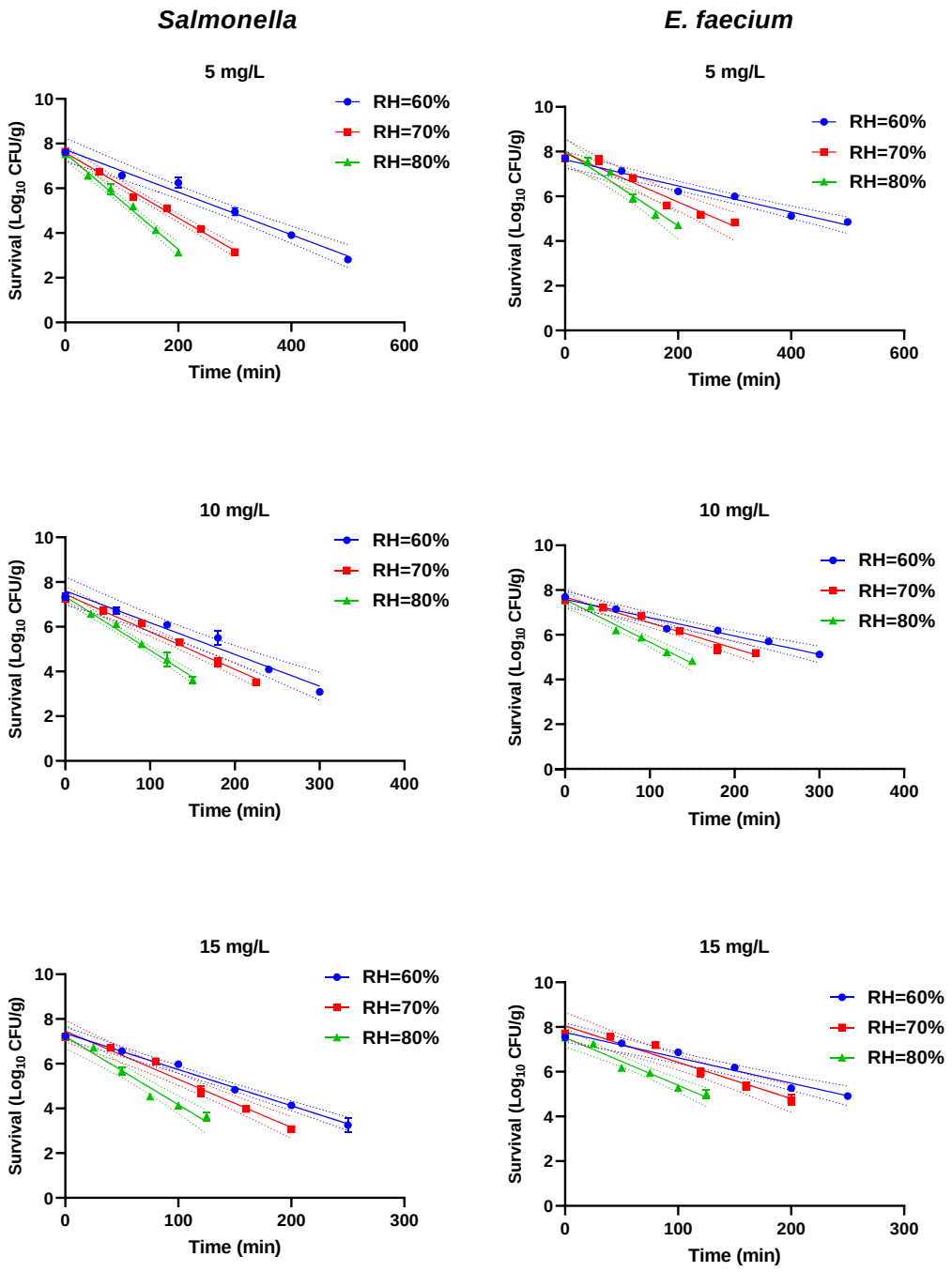
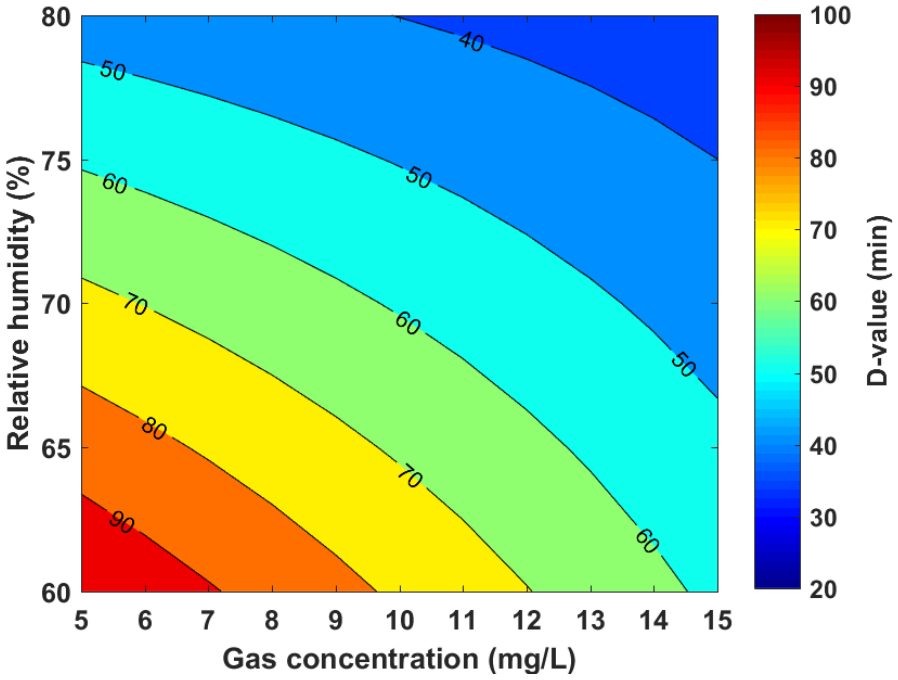
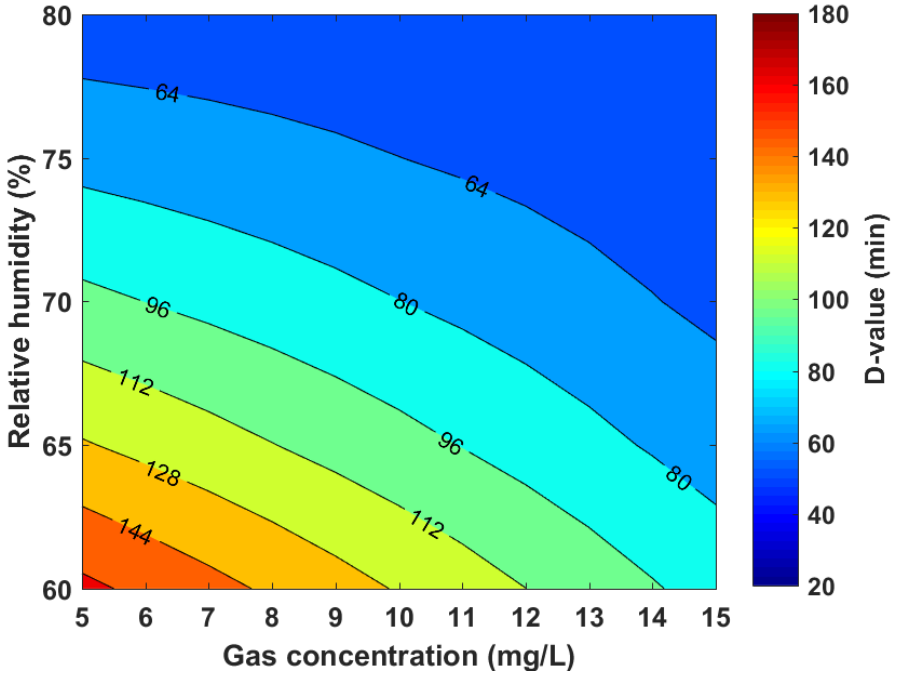


Figure 7. 2 Survival curves for *Salmonella* and *E. faecium* using the log-linear model at different relative humidites (RH) and gas concentrations. Error bars represent mean ± standard deviation (n=3). The dotted lines for each survival curve indicate the 95% confidence interval.

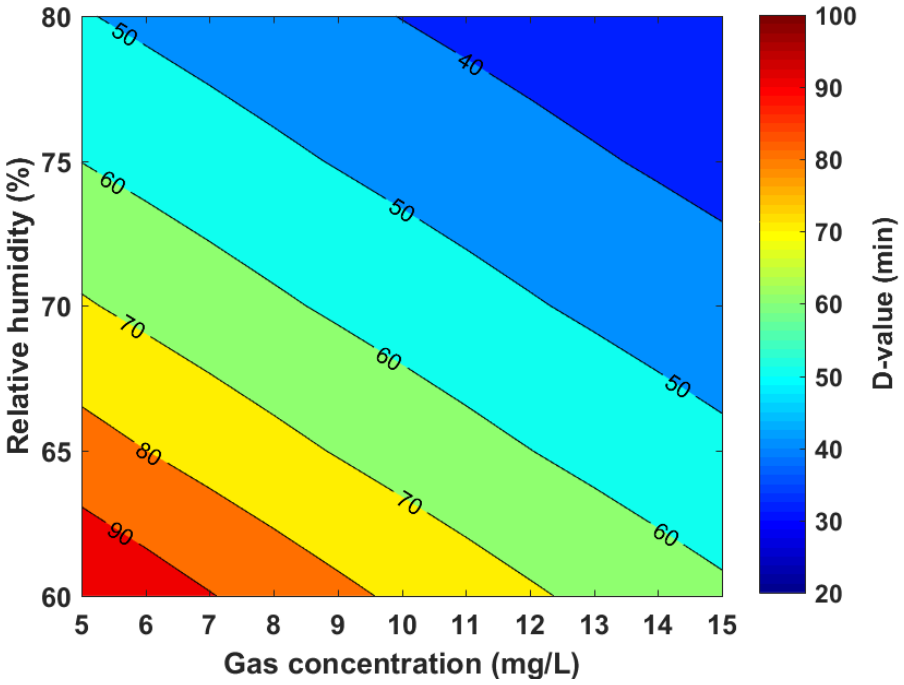


a) *Salmonella* ($R^2_{adjusted}$ = 0.95; AIC_c = 164.10; RMSE = 4.20 min)

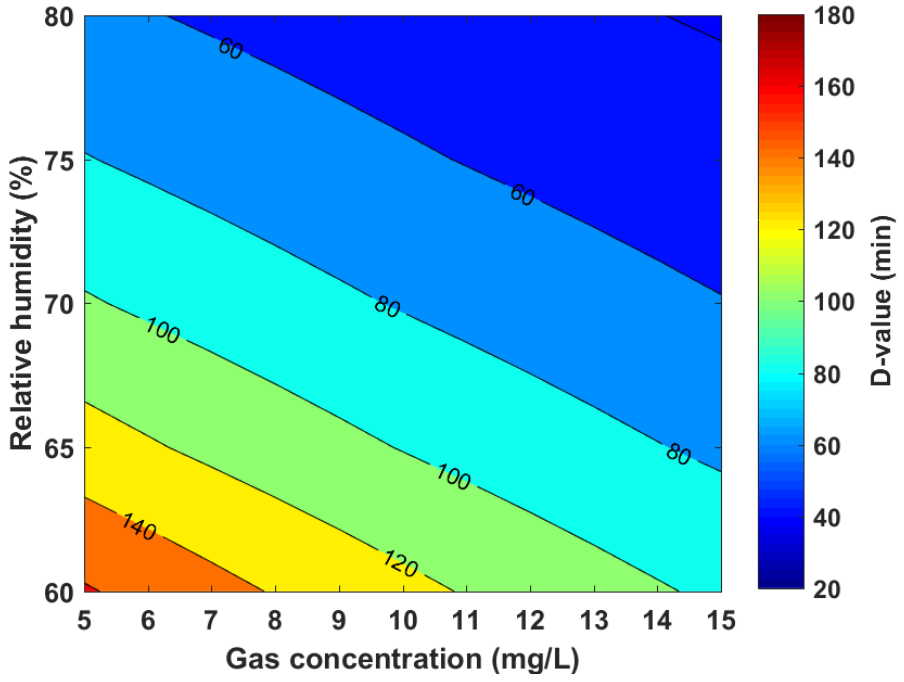


b) *E. faecium* ($R^2_{adjusted}$ = 0.96; AIC_c = 188.46; RMSE = 8.27 min)

Figure 7. 3 Contour plots showing the *D*-values of *Salmonella* and *E. faecium* using the response surface model.



a) *Salmonella* ($R^2_{adjusted}$ = 0.96; AIC_c = 87.96; RMSE = 4.51 min)



b) *E. faecium* ($R^2_{adjusted}$ = 0.96; AIC_c = 120.70; RMSE = 6.35 min)

Figure 7. 4 Contour plots showing *D*-values of *Salmonella* and *E. faecium* using the modified Bigelow model.

Chapter VIII: Conclusion and suggestions for future work

8.1 Conclusions

The overall goal of this dissertation was to evaluate different thermal and non-thermal technologies as tools for controlling the microbial load in various low moisture foods such as oat flour and dried basil leaves.

Chapter III evaluated the efficacy of conical twin-screw extrusion on the reduction of *Salmonella* in oat flour. A response surface model was developed to estimate the bacterial reduction over a range of moisture content, fat content, temperature, and screw speed. At temperature >65°C, the *Salmonella* population was below the detection limit (<10 CFU/g) at all the tested conditions. Therefore, the response surface model was developed at 55°C to evaluate the effect of moisture content, fat content, and screw speed on the reduction of *Salmonella*. Fat content showed a protective effect on the reduction of *Salmonella*. As the fat content increased from 5 to 15%, *Salmonella* reduction decreased from 8.0 to 0.8 log CFU/g at 14% moisture and 150 rpm. Moisture content showed a significant linear and quadratic effect on the bacterial reduction. As the moisture content increased from 14 to 22%, *Salmonella* reduction decreased from 8.0 to 4.8 log CFU/g and then increased from 4.8 to 5.6 log CFU/g as the moisture content increased from 22 to 26% at 5% fat and 150 rpm. Screw speed demonstrated a significant linear effect with higher screw speed resulted in a greater *Salmonella* reduction. In terms of *Salmonella* reduction, conical twin-screw extruder demonstrated to be an effective process compared to the single-screw extrusion. The response surface

model developed in this chapter would assist the food industry in planning an extrusion validation study in their processing plants. As industrial extrusion is often performed at temperatures much higher than 65°C, conical twin-screw extrusion is an effective inactivation process.

Chapter IV investigated the effect of various parameters such as moisture content, fat content, screw speed, and temperature on the mean residence time of oat flour in a single-screw extruder. Temperature did not show a significant effect on the mean residence time, in the range of 65-95°C. Therefore, temperature was not considered while developing the final response surface model. Screw speed had a significant linear as well as a slight quadratic effect on the mean residence time. As the screw speed increased from 100 to 200 rpm, the mean residence time dropped by approximately 22% at 5% fat content and 14% moisture content. The mean residence time was significantly affected by increasing the moisture content from 14 to 26%. The developed response surface equation showed a significant linear and quadratic effect of moisture content as well. As the moisture content increased from 14 to 19%, the mean residence time decreased slightly from 132 to 128 s at 100 rpm and 5% fat content. However, at same conditions, the mean residence time increased from 128 to 136 s as the moisture content increased from 19 to 26%. This was due to the quadratic effect of moisture content seen in the response surface equation. Fat content also showed a significant linear and quadratic effect on the mean residence time. As the fat content increased from 5 to 10%, the mean residence time increased from 132 to 136 s at 100 rpm and

14% moisture content. However, due to the quadratic effect, the mean residence time went back to 132 s when the fat content increased from 10 to 15%. The response surface model developed for the mean residence time was used to model the inactivation of *Salmonella* and *Enterococcus faecium* NRRL B-2354 as a function of mean residence time instead of screw speed along with other parameters. The purpose of this comparison was to evaluate the accuracy of the models by using mean residence time instead of screw speed for the microbial inactivation. On comparing the R^2 values from the response surface models (screw speed vs. mean residence time), the results showed that R^2 values improved from 0.83 to 0.85 for *Salmonella* and 0.84 to 0.89 for *E. faecium*. However, the slight improvement in accuracy of the models may not warrant the use of the mean residence time due to the complexity in the determination of mean residence time with high repeatability when compared to screw speed.

Chapter V investigated the effect of water activity on the thermal inactivation of *Salmonella* on dried basil leaves. Also, the suitability of *E. faecium* NRRL B-2354 as a surrogate for *Salmonella* was tested in this chapter. The inactivation data collected for *Salmonella* and *E. faecium* was fit to two different primary models, log-linear and Weibull. The results showed that as the temperature and water activity of the sample increased, the *D*-value of *Salmonella* and *E. faecium* decreased. At 75°C, the *D*-value of *Salmonella* and *E. faecium* at a_w= 0.40, 0.55, and 0.70 were 9.14, 6.64, and 3.30 min, and 14.07, 9.57, and 6.53 min, respectively. AIC_c was used as a parameter for evaluating the performance of both the primary models. Lower AIC_c values indicate a better model to

explain the thermal inactivation. According to the AIC_c values, the log-linear model fitted well for thermal inactivation of both *Salmonella* and *E. faecium* in dried basil leaves. Therefore, the data from the log-linear model was used to generate two secondary models, response surface and modified Bigelow model, to evaluate the effect of temperature and water activity on the *D*-values. Based on the AIC_c and RMSE, the modified Bigelow model performed better compared to the response surface model for the inactivation of *Salmonella* and *E. faecium*. Also, the *D*-values of *E. faecium* in dried basil leaves were approximately 1.4 to 2.8 times greater than *D*-values of *Salmonella* at all the conditions tested. Therefore, *E. faecium* can be used as a valid surrogate for *Salmonella* in dried basil leaves due to consistently higher thermal resistance. The output of this study may be used by the spice and herb industry in developing the thermal processes for improving the microbial safety of dried basil leaves.

In Chapter VI, radio frequency (RF) heating was demonstrated to be an effective process for inactivating *Salmonella* in dried basil leaves. Steam generation in the food product during RF heating enhanced the heating uniformity. The standard deviation of temperatures of 3 layers along the edge and center was 3.1°C and 2.8°C, respectively. The low temperatures indicated that the sample was uniformly heated during the RF heating. The container was covered with the plastic film, which helped reduce the moisture loss and improved the heating uniformity in the dried basil leaves. The top center location of the container was identified as the cold spot. Therefore, the pouches filled with *Salmonella* and *E. faecium* inoculated samples were placed in the cold spot

and subjected to RF heating for 45, 55, and 65 s. At 45 s, only 1.0 and 0.7 log reduction of *Salmonella* and *E. faecium* was achieved in dried basil leaves. A significant greater reduction (4.8 log CFU/g for *Salmonella*; 2.7 log CFU/g for *E. faecium*) was achieved when the dried basil leaves were heated for 55 s. Because the *Salmonella* reduction was below 5-log, the sample was further heated for additional 10 s which reduced the survivors to below the detection limit (<10 CFU/g). The reduction of *E. faecium* was significantly lower than *Salmonella* at all the three conditions (45, 55, and 65 s). Unlike products like wheat flour, milk powders, and egg white powder required holding in the oven for few hours post RF treatment, the dried basil leaves did not require additional holding time, probably due to the presence of antimicrobial components. This makes the RF process to be easily implemented in the spice and herb industry. Due to the higher thermal resistance, *E. faecium* can be used as a conservative surrogate for *Salmonella* during RF heating of dried basil leaves. The quality analysis was performed on the dried basil leaves treated for 65 s. The treated sample was allowed to cool down at the room temperature to represent the worst-case scenario for the quality analysis. The color values did not show any significant difference between untreated, and RF treated samples. The color difference (ΔE) of the RF treated sample was 0.51, which means that the human eye cannot notice any difference. The total phenolics content in the dried basil leaves was measured for untreated and RF treated samples. The results indicated that there was a slight decrease in the total phenolic content in the RF treated sample; however, the decrease was not significantly different from the untreated

sample. Also, the antioxidant activity was not significantly affected during the RF treatment of dried basil leaves. A total of 25 volatile compounds were identified in the untreated and RF treated dried basil leaves. A significant drop was seen in one minor volatile compound (camphor) post RF treatment. However, this volatile loss can be further reduced by cooling the food product immediately after the treatment. Overall, RF treatment effectively improved the microbial safety of dried basil leaves while maintaining the food quality. The results from this study can help the spice industry adopt RF as effective pasteurization method for dried basil leaves.

In Chapter VII, the antimicrobial efficacy of chlorine dioxide gas was evaluated for pasteurization of dried basil leaves. The non-thermal inactivation data collected for *Salmonella* and *E. faecium* was fit using the log-linear model. The results showed that that the log-linear model fits very well with adjusted R^2 values greater than 0.96 for *Salmonella* and 0.93 for *E. faecium*. Therefore, no other primary models were evaluated to fit the inactivation data for *Salmonella* and *E. faecium*. The increase in relative humidity and gas concentration resulted in a decrease in *D*-values for both the microorganisms. The *D*-value for *Salmonella* and *E. faecium* decreases from 68.5 to 46.5 min and 91.5 to 69.2 min, respectively, as the gas concentration increases from 5 to 15 mg/L at 70% relative humidity. Similarly, as the relative humidity increases from 60 to 80%, the *D*-values for *Salmonella* and *E. faecium* decreases from 70.5 to 41.8 min and 121.7 to 52.6 min, respectively, at a gas concentration of 10 mg/L. The data from the log-linear model was used to develop two secondary models: response surface model

and modified Bigelow model. The relative humidity had a bigger effect than gas concentration within the tested range; relative humidity also had a higher antimicrobial efficacy on *E. faecium* than *Salmonella*. The AIC_c values showed that the modified Bigelow model fared better than the response surface model. The *D*-values of *E. faecium* were approx. 1.2 to 1.9 times greater than that of *Salmonella* in chlorine dioxide treated dried basil leaves. Therefore, E. faecium can be used as an appropriate surrogate for *Salmonella* in dried basil leaves during chlorine dioxide treatment. The results presented in this study will assist the herb and spice industry in using chlorine dioxide gas as an alternative treatment for controlling the microbial load in their food products.

8.2 Suggestions for future research

While this dissertation focused on demonstrating the efficacy of various thermal and non-thermal technologies for improving the microbial safety of low moisture foods, there are several other interesting research opportunities that can be conducted in the future.

A laminated paper tray was used to hold the dried basil leaves for the RF treatment. Although RF heating was effective in reducing the microbial load from the dried basil leaves, but there are chances of post process contamination of the treated samples due to the involvement of human touch. To avoid post lethality contamination, it is imperative to evaluate the efficacy of RF heating of dried basil leaves when packaged in the steam vent retail pouches. The steam vent will help in releasing the excess moisture, avoid condensation, and the pressure generated in the product helps in heating

uniformity. The use of pouches will eliminate the post process contamination involved during packaging. Not just limited to dried basil leaves, other low moisture food products such as whole peppercorns, cumin seeds, chia seeds, walnuts etc. can also be evaluated.

Gaseous chlorine dioxide was used to inactivate microorganisms in dried basil leaves. It would be interesting to see how other gaseous technologies such as ozone, hydrogen peroxide, etc. are effective in reducing the microbial load in dried basil leaves. Even though the use ethylene oxide for dried basil leaves is prohibited, other low moisture foods such as chia seeds, walnuts can be used to test the efficacy of ethylene oxide for microbial reduction. A hurdle concept (combination of two different gases) can also be used for food products which cannot achieve desired level of pathogen reduction. Also, the suitability of *E. faecium* NRRL B-2354 as a *Salmonella* surrogate should be tested for other gaseous technologies and food products.