

University of Nebraska - Lincoln

DigitalCommons@University of Nebraska - Lincoln

Dissertations, Theses, & Student Research in Food
Science and Technology

Food Science and Technology Department

8-2018

Thermal Inactivation Kinetics of *Salmonella enterica* and *Enterococcus faecium* in Ground Black Pepper

Sabrina Vasquez

University of Nebraska - Lincoln, sabrina.vasquez22@gmail.com

Follow this and additional works at: <http://digitalcommons.unl.edu/foodscidiss>



Part of the [Food Microbiology Commons](#), and the [Food Processing Commons](#)

Vasquez, Sabrina, "Thermal Inactivation Kinetics of *Salmonella enterica* and *Enterococcus faecium* in Ground Black Pepper" (2018).
Dissertations, Theses, & Student Research in Food Science and Technology. 93.
<http://digitalcommons.unl.edu/foodscidiss/93>

This Article is brought to you for free and open access by the Food Science and Technology Department at DigitalCommons@University of Nebraska - Lincoln. It has been accepted for inclusion in Dissertations, Theses, & Student Research in Food Science and Technology by an authorized administrator of DigitalCommons@University of Nebraska - Lincoln.

Thermal inactivation kinetics of *Salmonella enterica* and *Enterococcus faecium* in ground
black pepper

by

Sabrina Vasquez

A THESIS

Presented to the Faculty of
The Graduate College at University of Nebraska
In Partial Fulfillment of Requirements
For the Degree of Master of Science

Major: Food Science and Technology

Under the Supervision of Professors Jeyamkondan Subbiah and Jayne Stratton

Lincoln, Nebraska

August 2018

Thermal inactivation kinetics of *Salmonella enterica* and *Enterococcus faecium* in ground
black pepper

Sabrina Vasquez, M.S

University of Nebraska, 2018

Advisors: Jeyamkondan Subbiah & Jayne Stratton

Food safety concerns of low- a_w foods have boosted, given the increased number of foodborne illnesses related to these products. Black pepper, among other spices, has been implicated in several outbreaks and recalls in recent years. *Salmonella enterica* has been identified as the causing agent of outbreaks of contaminated spices. Therefore, increased efforts should be conducted to ensure food safety of spices, including black pepper. Food Safety Modernization Act (FSMA) demands all food processing facilities to conduct proper validations of the decontamination technologies applied to food products. Complete thermal process validations require a deep understanding of the thermal inactivation kinetics of the pathogen identified in risk assessments. The objectives of this study were to validate a novel dry heating method, thermal-death-time (TDT) sandwiches, for determination of thermal inactivation kinetics of *Salmonella* spp. and *E. faecium* NRRL B-2354 in ground black pepper and to evaluate the effect of water activity on the thermal resistance of these microorganisms. Black pepper samples were inoculated with a 5-strain cocktail of *Salmonella* or *E. faecium* and equilibrated to the target water activity prior to thermal inactivation. D- and z-values were determined for 0.45 a_w at different heating rates and treatment temperatures using TDT sandwiches. Conventional heating method for thermal inactivation kinetics determination, TDT test cells, were used

to compare D-value results with those obtained using TDT sandwiches for 0.45 a_w samples. TDT sandwiches demonstrated to have operational benefits compared to TDT cells including lower log reductions during Come-up time, use of disposable sterile aluminum pouches with higher sample capacity, adjustable heating rates and dry heating method that eliminate possibility of leakage during treatment. Thermal resistance of *Salmonella* spp. in black pepper equilibrated to 0.45 a_w was influenced by the method used, whereas, the thermal resistance of *E. faecium* was not. Heating rates above 6.5 °C/min using TDT sandwiches did not change the D-values of both microorganisms at all temperatures evaluated. Later, TDT sandwiches were used at 600 °C/min for thermal inactivation of inoculated black pepper at 0.25 and 0.65 a_w . D- and z-values were also determined for these conditions. Response surface equations for log D-value determination of *E. faecium* and *Salmonella* spp. were developed.

In this study, TDT sandwiches were validated as a novel dry heating method for determination of thermal inactivation kinetics of *Salmonella* spp. and *E. faecium* in ground black pepper. *Salmonella*'s and *E. faecium*'s D-values at 75 °C for 0.25 a_w samples were 42.86 min and 58.35 min, respectively. These D-values decreased to 1.51 min for *Salmonella* spp. and 4.56 min for *E. faecium* for 0.65 a_w samples, at the same temperature. Furthermore, independent of the heating method used and for samples conditioned to 0.25, 0.45 and 0.65 a_w , *E. faecium* was identified as a suitable surrogate for *Salmonella* spp. in ground black pepper.

Keywords: thermal inactivation kinetics, process validation, heating rate, water activity, surrogate

To my family, friends and to my country, Panama!

Acknowledgements

This thesis is the fulfillment of a personal and professional goal that to be accomplished required several years of sacrifice and hard work. It even started with the vision of completing an undergraduate program in Panama with outstanding grades and continues on with a professional experience in the food industry for few years. With that in mind, the academic and professional experiences throughout the early stages of my career prepared me to the challenges involving studying abroad.

Foremost, I will like to thank the Fulbright Program and the Secretary of Science and Technology of Panama (SENACYT) for granting me with a scholarship for studying at the U.S. After more than 4 years of working in the food industry in Panama, it was a challenge to return to a life as a graduate student in a foreign country. Thanks to all the people that supported and guided me through the toughest decisions that needed to be made, including which program and university to choose from.

Ever since the beginning, I felt happy to be chosen to be part of the Food Engineering Lab, managed by Dr. Jeyamkondan Subbiah. I knew there would be challenges but certainly, I will have the opportunity to learn from the best. I appreciated Dr. Subbiah's interest in meeting me through Skype, while I was still in Panama deciding my future. That particular approach helped me feel welcome and part of a collaborative team. Additionally, I congratulate Dr. Subbiah for managing an excellent group of graduate students that were capable of working as a team to accomplish the objectives. This truly made the difference in my research experience at UNL.

Thank you Soon Kiat Lau, Tushar Verma, Xinyao Wei, Long Chen, Ryan Anderson, and Emily Bender, for been there for me, as friends and labmates. You all

helped me in different ways and gave me support, especially during the most difficult months of experiments. Thanks to professors and staff at the Department of Food Science and Technology who were always open to help me in any problem that I encountered.

Special thanks to the rest of the Committee members: Dr. Jayne Stratton, my co-advisor, who along with Dr. Andrea Bianchini guided me with the microbiological aspects of the research study. Also thanks to Dr. Sibel Irmak, who was always interested in guided me during any problem I could encountered. Thanks to Dr. Eskridge, who guide me with the statistic analysis of the study.

Finally, thanks to my parents and family who believed in me and supported me with every decision that I made. I dedicate this accomplishment to you all.

Declaration of Grant Information

This project is supported by funding from United States Department of Agriculture (USDA) - National Institute of Food and Agriculture (NIFA) Agriculture and Food Research Initiative (AFRI) grant 2015-68003-23415.

Table of contents

Acknowledgments.....	v
Declaration of Grant Information.....	vii
List of Tables.....	xiii
List of Figures.....	xiv
Chapter I: Introduction.....	1
1.1 Introduction.....	1
1.2 Objectives.....	3
1.3 Thesis Organization.....	4
1.4 References.....	5
Chapter II: Literature Review.....	7
2.1 Low- a_w or low-moisture foods.....	7
2.1.1 Food safety of low- a_w foods.....	7
2.2 <i>Salmonella</i> spp.....	8
2.2.1 Outbreaks and recalls of <i>Salmonella</i> spp. in low- a_w foods and spices.....	9
2.2.2 Survival of <i>Salmonella</i> spp. in low- a_w foods and desiccated environments.....	10
2.2.3 Influence of a_w thermal resistance of <i>Salmonella</i> in low- a_w foods.....	12
2.2.4 FDA's efforts to increase knowledge and improve food safety of spices.....	14
2.3 Black pepper production and its current decontamination technologies.....	15

2.4 Pasteurization process validation and use of <i>Enterococcus faecium</i> as a surrogate of <i>Salmonella</i> spp.....	18
2.5 Research gap of thermal inactivation processes and validation of low- a_w foods.....	19
2.6 References.....	21
Chapter III. Evaluation of a novel dry heating method for determination of thermal inactivation kinetics of microorganisms in low-a_w foods: <i>Salmonella</i> spp. and <i>E. faecium</i> in ground black pepper.....	
Abstract.....	28
3.1 Introduction.....	29
3.2 Materials and Methods.....	32
3.2.1 Low- a_w food: black peppercorns.....	32
3.2.2 Background flora.....	32
3.3.3 Bacterial strains.....	33
3.3.4 Inoculum preparation.....	33
3.2.5 Sample inoculation, grinding and equilibration.....	35
3.2.6 Homogeneity and stability tests.....	37
3.2.7 Thermal inactivation apparatus.....	38
3.2.7.1 TDT sandwiches design and development.....	38
3.2.7.2 Thermal inactivation using TDT sandwiches.....	38
3.2.7.3 Microbial enumeration procedure.....	40
3.2.7.4 Thermal inactivation using thermal-death time (TDT) test cells.....	40

3.2.7.5 Calculation of D- and z-values.....	41
3.2.8 Experimental design and Statistical Analysis.....	42
3.3 Results and Discussion.....	43
3.3.1 Homogeneity and stability of inoculated ground black pepper.....	43
3.3.2 Comparison of thermal inactivation kinetics of <i>Salmonella</i> spp. and <i>E. faecium</i> with different heating methods.....	44
3.3.3 Effect of heating rate on the thermal resistance of <i>Salmonella</i> and <i>E. faecium</i> NRRL-B2354 in ground black pepper when using TDT sandwiches.....	51
3.3.4 <i>E. faecium</i> NRRL B-2354 as a suitable surrogate for <i>Salmonella</i> spp.....	52
3.4 Conclusions.....	53
3.5 References.....	55
Chapter IV. Effect of water activity on thermal inactivation kinetics of <i>Salmonella</i> spp. and <i>E. faecium</i> in ground black pepper.....	70
Abstract.....	70
4.1 Introduction.....	71
4.2 Materials and methods.....	74
4.2.1 Low- a_w food: black peppercorns.....	74
4.2.2 Bacterial strains.....	74
4.2.3 Inoculum preparation.....	75
4.2.4 Sample preparation: inoculation, equilibration and grinding.....	76
4.2.5 Homogeneity and stability test.....	77

4.2.6. Thermal inactivation using TDT sandwiches.....	77
4.2.7. Microbial enumeration and D- and z-value determination.....	78
4.2.8 Experimental design and Statistical Analysis.....	79
4.3 Results and Discussion.....	80
4.3.1. Influence of water activity on the homogeneity and stability of inoculated ground black pepper.....	78
4.3.2. Influence of water activity on the microbial population before thermal treatments.	81
4.3.3. Influence of water activity on the thermal resistance of <i>Salmonella</i> spp. and <i>E. faecium</i> in ground black pepper.....	82
4.3.4 Response Surface Model to predict D-value at different water activities.....	84
4.3.5. Survival curves obtained after heat treatment using TDT sandwiches.....	85
4.3.6. z-value of <i>E. faecium</i> NRRL-B2354 and <i>Salmonella</i> in ground black pepper at 0.25, 0.45 and 0.65 a_w	86
4.3.7. <i>E. faecium</i> NRRL-B2354 as a suitable surrogate for <i>Salmonella</i> in ground black pepper.....	87
4.4 Conclusions.....	89
4.5 References.....	90
Chapter V: Conclusions and Suggestion for Future Research.....	108
5.1 Conclusions.....	108
5.2 Suggestions for Future Research.....	109

Appendices	112
Appendix A. <i>Salmonella</i> on mTSAYE an <i>E. faecium</i> on eTSAYE plates.....	112
Appendix B. Sample preparation and inoculation.....	113
Appendix C. Grinding process.....	114
Appendix D. TDT sandwiches components.....	115
Appendix E. TDT test cells.....	116
Appendix F. TDT test cells inside water bath during CUT determination.....	117

Lists of Tables

Table 2.1. Several <i>Salmonella enterica</i> serovars have been linked to outbreaks related to low- a_w foods.....	27
Table 3.1. D-values in minutes of <i>Salmonella</i> spp. and <i>E. faecium</i> NRRL B-2354 using different heating methods.....	59
Table 3.2. Come-up times (CUT) log reductions for <i>Salmonella</i> spp. and <i>E. faecium</i> NRRL B-2354 populations when treated with different heating methods and temperatures.....	60
Table 3.3. Repeatability Analysis for different heating methods.....	61
Table 3.4. D-values in minutes of <i>Salmonella</i> spp. and <i>E. faecium</i> NRRL B-2354 when using TDT Sandwiches with different heating rate.....	62
Table 4.1. Come-up times reductions for <i>Salmonella</i> spp. and <i>E. faecium</i> NRRL B-2354 populations in ground black pepper with different water activities treated with TDT sandwiches with heating rate of 600 °C/min at different temperatures.....	94
Table 4.2. D-values (minutes + SE) for <i>Salmonella</i> spp. and <i>E. faecium</i> NRRL B-2354 populations in ground black pepper with different water activities treated with TDT sandwiches with heating rate of 600 °C/min at different temperatures.....	95
Table 4.3. Observed and predicted D-values for <i>E. faecium</i>	96
Table 4.4. Observed and predicted D-values for <i>Salmonella</i> spp.....	97
Table 4.5. Correlation coefficient (R-square) of thermal inactivation curves of <i>E. faecium</i> and <i>Salmonella</i> spp. in ground black pepper.....	98
Table 4.6. z-values (°C) of <i>E. faecium</i> and <i>Salmonella</i> spp. in ground black pepper equilibrated to different water activities.....	99
Table 4.7. R-square of thermal death curves (z-value) of <i>E. faecium</i> and <i>Salmonella</i> spp. in ground black pepper equilibrated to different water activities.....	100

List of Figures

Figure 3.1. Homogeneity and stability of <i>E. faecium</i> and <i>Salmonella</i> spp. in ground black pepper equilibrated to 0.45 a_w , measured at 25°C.....	63
Figure 3.2. Survivor curves of <i>Salmonella</i> spp. in ground black pepper (0.45 $a_w \pm 0.025$ measured at 25°C) when treated with TDT sandwiches (filled color shape) and TDT test cells (unfilled color shapes) at different temperatures (• 65 °C, ♦ 70 °C, and ■ 75 °C).....	64
Figure 3.3. Survivor curves of <i>E. faecium</i> NRRL B-2354 in ground black pepper (0.45 $a_w \pm 0.025$ measured at 25°C) when treated with TDT sandwiches (filled color shape) and TDT test cells (unfilled color shapes) at different temperatures (• 70 °C, ■ 75 °C, and ♦ 80 °C).....	65
Figure 3.4. D-value comparisons for <i>Salmonella</i> spp. (a) and <i>E. faecium</i> NRRL B-2354 (b) when treated with different heating methods.....	66
Figure 3.5. Linear regression for z-value (°C) of ■ <i>Salmonella</i> spp. and • <i>E. faecium</i> NRRL B-2354 when determined by TDT sandwiches (filled color shapes) and TDT test cells (unfilled color shapes).....	67
Figure 3.6. Survivor curves of <i>Salmonella</i> spp. in ground black pepper (0.45 $a_w \pm 0.025$ measured at 25°C) when treated with TDT sandwiches at different heating rates (♦ HR1: 600 °C/min, ■ HR2: 17.10-24.05 °C/min, • HR3: 6.50-9.20°C/min) and temperatures (65 °C, 70 °C, 75 °C).....	68
Figure 3.7. Survivor curves of <i>E. faecium</i> NRRL B-2354 in ground black pepper (0.45 $a_w \pm 0.025$ measured at 25°C) when treated with TDT sandwiches at different heating rates (♦ HR1: 600 °C/min, ■ HR2: 17.10-24.05°C/min, • HR3:6.50-9.20°C/min) and temperatures (65 °C, 70 °C, 75 °C).....	69
Figure 4.1 Homogeneity and stability of <i>E. faecium</i> NRRL B-2354 (a) and <i>Salmonella</i> spp. (b) in ground black pepper equilibrated to different water activities (0.25,0.45, 0.65) and stored until 15 days.....	101
Figure 4.2. Correlation for Observed D-value (min) against Predicted D-value (min) using Response Surface Equation for <i>E. faecium</i>	102
Figure 4.3. Correlation for Observed D-value (min) against Predicted D-value (min) using Response Surface Equation for <i>Salmonella</i> spp.....	103
Figure 4.4 Survival curves of <i>Salmonella</i> spp. (unfilled shapes) and <i>E. faecium</i> NRRL B-2354 (filled shapes) in ground black pepper at 0.25 a_w (measured at room temperature) and different temperatures (■ 75°C, ♦ 80°C and ▲ 85°C).....	104

Figure 4.5 Survival curves of *Salmonella* spp. (unfilled shapes) and *E. faecium* NRRL B-2354 (filled shapes) in ground black pepper at 0.45 a_w (measured at room temperature) and different temperatures (• 70 °C and ■ 75°C) when using TDT sandwiches.....105

Figure 4.6. Survival curves of *Salmonella* spp. (unfilled shapes) and *E. faecium* (filled shapes) in ground black pepper at 0.65 a_w (measured at 25°C) and different temperatures. temperatures (•70°C and ■75°C).....106

Figure 4.7. Comparison between the D-values (min) at 75 °C of *Salmonella* spp. and *E. faecium* NRRL B-2354 influenced by water activity using TDT sandwiches.....107

Chapter I: Introduction

1.1. Introduction

Foodborne illnesses are a public health concern because they are a major cause of morbidity and mortality worldwide (World Health Organization, 2015). In 2010, 31 foodborne hazards including viruses, bacteria and protozoa caused 600,000,000 foodborne illnesses cases and 420,000 deaths worldwide (World Health Organization, 2015). It has been estimated that in the U.S., every year, 1 out of 6 individuals get sick, 128,000 are hospitalized and 3,000 die of foodborne illnesses (CDC, 2016a).

Traditionally, high moisture foods have been the most implicated foods in foodborne illnesses, due to their characteristics that promote bacterial survival and growth. On the contrary, there has been a general misconception that low-moisture foods, which inherently have low-water activity (a_w) (less than 0.70), are safe because bacteria are not able to grow in these products. However, there have been an increased number of recalls and outbreaks related to low- a_w foods over recent years. From 2010 to 2014, the FDA reported multiple recalls of low- a_w foods such as walnuts, nuts, pet food, pistachios, peanut butter, and products containing black pepper, red pepper or hydrolyzed vegetable protein contaminated with *Salmonella* (Gurtler, Doyle, & Kornacki, 2014). Additionally, several CDC investigations of multistate outbreaks have linked *Salmonella* to contaminated low- a_w foods such as black pepper (CDC, 2010), peanut butter (CDC, 2012), and pistachios (CDC, 2016b). *Salmonella* has become the pathogen of major concern in low- a_w foods because of its low infectious dose (<1 CFU/g) and survival in desiccated environments.

Spices are commonly used for seasoning of ready-to-eat (RTE) products that do not undergo further cooking. Therefore, the safety of spices must be ensured through proper

harvesting, storage and handling processes. In 2013, the FDA issued a draft risk profile to determine the presence of pathogens, such as *Salmonella*, and filth in spices. According to their results, approximately 6.6 % of the shipments offered to enter the U.S through 2007-2009 fiscal years were contaminated with *Salmonella*. Therefore, adequate decontamination processes are required to ensure the safety of spices.

Food processors have developed thermal and non-thermal technologies for the decontamination of spices. Most of these technologies have been used for many years but are not properly validated. With the Food Safety Modernization Act (FSMA) regulations, food-processing companies are required to validate their decontamination technologies. This requirement has become a great challenge especially for low- a_w foods processing facilities that apply thermal processes because of the knowledge gaps involving understating the effect of water activity, food matrix and heating methods for bacterial inactivation in these types of foods.

Determination of the thermal inactivation kinetics of pathogens is crucial for thermal decontamination process validations. Additionally, process validations are required to use surrogates because pathogens cannot be introduced inside processing facilities for validation. A surrogate is a non-pathogenic microorganism that behaves similarly to the pathogen under the same processing conditions. In low- a_w foods, *Enterococcus faecium* NRRL B-2354 has been validated as a suitable surrogate of *Salmonella* spp. in thermal processes for almonds (Almond Board of California, 2007), low-moisture pet foods (Ceylan & Bautista, 2015), wheat flour (Villa-Rojas, Zhu, Marks, & Tang, 2017), culinary seasoning, chicken meat powder and pet food (Rachon, Peñaloza, & Gibbs, 2016), oat flour (Verma et al., 2018) and whole and ground black pepper (Wei, 2017).

Currently, there are limited published data on the thermal inactivation kinetics of *Salmonella* spp. and *E. faecium* NRRL B-2354 in low- a_w foods. Among studies, it was reported that many factors such as strains tested, experimental conditions, growth phase of the cell and inoculation methodology, food composition or media used can affect the determination of thermal resistance of microorganisms (Hildebrandt et al., 2016; Keller et al., 2012; Rachon et al., 2016; Van Asselt & Zwietering, 2006). In addition, the heating method and heating rate can also influence the results (Stephens, Cole, & Jones, 1994; Yuk, Geveke, Zhang, & Jin, 2009). Therefore, it is difficult to compare the results among the available published data because of the variety of conditions used in different studies.

In this study, the thermal resistance of *Salmonella* spp. and *E. faecium* in ground black pepper was determined using a conventional heating method and a novel dry heating method and the results were compared. Additionally, the dry heating method was used to determine the thermal resistance of the microorganisms at different temperatures, heating rates and water activities.

1.2. Objectives

The main goal of this research is to increase knowledge related to the thermal resistance of *Salmonella* spp. and *E. faecium* in spices. The specific objectives of this study are:

- To validate a novel dry heating method for determination of thermal inactivation kinetics of microorganisms in low- a_w foods: *Salmonella* spp. and *E. faecium* in ground black pepper.
- To evaluate the effect of water activity on thermal inactivation kinetics of *Salmonella* spp. and *E. faecium* in ground black pepper.

1.3. Thesis organization

Chapter II includes a literature review with information regarding *Salmonella* and food safety in low- a_w foods, determination of thermal inactivation kinetics of microorganisms, current decontamination processes for spices and identification of requirements and knowledge gaps for development and validation of decontamination technologies for spices, including black pepper.

Chapter III describes the comparison between the traditional and the novel dry heating method, Thermal-Death-Time (TDT) sandwiches, while determining the thermal resistance of *Salmonella* and *Enterococcus faecium* NRRL B-2354 in black pepper as influenced by temperature. Additionally, the effect of heating rate on the thermal resistance of these microorganisms was evaluated using TDT sandwiches.

Chapter IV evaluates the suitability of *Enterococcus faecium* NRRL B-2354 as a potential surrogate for *Salmonella* spp. in black pepper at different water activities using TDT sandwiches as the dry heating method.

Chapter V includes general conclusions from the results of chapter III and IV and presents guidelines and recommendations for future work on related topics.

1.4. References

1. Almond Board of California. (2007). Guidelines for validation of dry roasting processes. Available from: <http://www.almonds.com/sites/default/files/content/attachments/dry-roast-validation-guidelines.pdf>. Accessed September, 2017
2. CDC. (2010). Multistate outbreak of human *Salmonella* Montevideo infections (Final Update). Available from: <http://www.cdc.gov/salmonella/2010/montevideo-5-4-2010.html>. Accessed January, 2018
3. CDC. (2012). Multistate outbreak of *Salmonella* Bredeney infections linked to peanut butter manufactured by Sunland, Inc. (Final Update). Available from: <http://www.cdc.gov/salmonella/bredeney-09-12/index.html>. Accessed January, 2018
4. CDC. (2016a). Burden of foodborne illness: Findings. Available from: <https://www.cdc.gov/foodborneburden/2011-foodborne-estimates.html>. Accessed January, 2018
5. CDC. (2016b). Multistate outbreak of *Salmonella* Montevideo and *Salmonella* Senftenberg infections linked to wonderful pistachios (Final Update). Available from: <http://www.cdc.gov/salmonella/montevideo-03-16/index.html>. Accessed January, 2018
6. Ceylan, E., & Bautista, D. A. (2015). Evaluating *Pediococcus acidilactici* and *Enterococcus faecium* NRRL B-2354 as thermal surrogate microorganisms for *Salmonella* for in-plant validation studies of low-moisture pet food products. *Journal of Food Protection*, 78(5), 934-939.
7. Gurtler, J. B., Doyle, M. P., & Kornacki, J. L. (2014). The microbiological safety of spices and low-water activity foods: Correcting historic misassumptions. In *The microbiological safety of low water activity foods and spices* (pp. 3-13) Springer, New York, NY.
8. Hildebrandt, I. M., Marks, B. P., Ryser, E. T., Villa-Rojas, R., Tang, J., Garces-Vega, F. J., & Buchholz, S. E. (2016). Effects of inoculation procedures on variability and repeatability of *Salmonella* thermal resistance in wheat flour. *Journal of Food Protection*, 79(11), 1833-1839.
9. Jeong, S., Marks, B. P., & Ryser, E. T. (2011). Quantifying the performance of *Pediococcus* sp. (NRRL B-2354: *Enterococcus faecium*) as a nonpathogenic surrogate for *Salmonella* Enteritidis PT30 during moist-air convection heating of almonds. *Journal of Food Protection*, 74(4), 603-609.

10. Keller, S. E., Grasso, E. M., Halik, L. A., Fleischman, G. J., Chirtel, S. J., & Grove, S. F. (2012). Effect of growth on the thermal resistance and survival of *Salmonella* Tennessee and Oranienburg in peanut butter, measured by a new thin-layer thermal death time device. *Journal of Food Protection*, 75(6), 1125-1130.
11. Rachon, G., Peñaloza, W., & Gibbs, P. A. (2016). Inactivation of *Salmonella*, *Listeria monocytogenes* and *Enterococcus faecium* NRRL B-2354 in a selection of low moisture foods. *International Journal of Food Microbiology*, 231, 16-25.
12. Stephens, P., Cole, M., & Jones, M. (1994). Effect of heating rate on the thermal inactivation of *Listeria monocytogenes*. *Journal of Applied Microbiology*, 77(6), 702-708.
13. Van Asselt, E. D., & Zwietering, M. H. (2006). A systematic approach to determine global thermal inactivation parameters for various food pathogens. *International Journal of Food Microbiology*, 107(1), 73-82.
14. Verma, T., Wei, X., Lau, S. K., Bianchini, A., Eskridge, K. M., & Subbiah, J. (2018). Evaluation of *Enterococcus faecium* NRRL B-2354 as a Surrogate for *Salmonella* During Extrusion of Low-Moisture Food. *Journal of Food Science*, 83(4), 1063-1072.
15. Wei, X. (2017). *Radiofrequency processing for inactivation of Salmonella spp. and Enterococcus faecium* NRRL B-2354 in whole black peppercorn and ground black pepper (Master's thesis). Available from: Digital Commons@ University of Nebraska-Lincoln. <https://digitalcommons.unl.edu/foodscidiss/88/> Accessed May, 2018
16. World Health Organization. (2015). *WHO estimates of the global burden of foodborne diseases: Foodborne disease burden epidemiology reference group 2007-2015* (). Available from: http://www.who.int/foodsafety/publications/foodborne_disease/fergreport/en/. Accessed January, 2018
17. Yuk, H., Geveke, D. J., Zhang, H. Q., & Jin, T. Z. (2009). Comparison of aluminum thermal-death-time disks with a pilot-scale pasteurizer on the thermal inactivation of *Escherichia coli* K12 in apple cider. *Food Control*, 20(11), 1053-1057.

Chapter II: Literature Review

2.1. Low- a_w foods or low-moisture foods

In recent years, there has been increased concern regarding the food safety of low-moisture foods or low-water activity (a_w) foods. Both terms are used interchangeably in published literature when referring to food products with low- a_w . Some authors have defined low-moisture foods to those with a_w less than 0.70 (Blessington, Theofel, & Harris, 2013; Farakos, Frank, & Schaffner, 2013). Interestingly, some high-moisture foods could have low- a_w due to water-binding solutes that bind the available water, therefore, reducing the a_w of the food (Gurtler et al., 2014).

Water activity (a_w) is defined as the ratio between the vapor pressure of the food and the vapor pressure of pure water at that same temperature (Gurtler et al., 2014). The a_w of a food material affects the growth and survival of microorganisms. Therefore, the measurement of a_w of food samples has become an important parameter when developing and processing foods products. Products within the low- a_w category might be used in food products that may or may not be further treated to inactivate pathogens. Therefore, if contaminated with foodborne pathogens, they may cause severe illness.

2.1.1. Food safety of low- a_w foods

For many years, low- a_w foods were inherently considered to be safe due to the unfavorable conditions for growth of either vegetative or spore-forming bacteria. Regardless of this misconception, foodborne pathogens such as *Cronobacter sakazakii*, *Clostridium botulinum*, *Bacillus cereus*, *Staphylococcus aureus* and *Salmonella* spp. have been reported to survive for a long time in low- a_w foods and environments (Syamaladevi, Tang et al., 2016). Specifically, some outbreaks related to *Salmonella* in low- a_w foods

have been caused by very low numbers of *Salmonella* present in the food. For example, low levels of *Salmonella* (0.04-0.05 CFU/g) were found in contaminated paprika and paprika-seasoned snacks that caused a nationwide outbreak in Germany with an estimate of 1000 cases with children below 14 mainly affected (Lehmacher, Bockemühl, & Aleksic, 1995).

During the last decade, several outbreaks caused by salmonellosis have been related to low- a_w foods including powdered infant formula, chocolate, peanut butter, nuts and spices (Grocery Manufacturers Association, 2009). For these reasons, numerous efforts have been made to prevent contamination of low- a_w foods during handling, processing and storage. For example, the Grocery Manufacturers Association (GMA) developed a guidance document for the control and prevention of *Salmonella* contamination in low-moisture food processing facilities. The guidance serves useful for processing of a variety of low-moisture foods including peanut butter, confectionary, snacks, cereals, spices, dried protein products, animal feeds, pet foods and treats (Grocery Manufacturers Association, 2009).

2.2. *Salmonella* spp.

Salmonella spp. is a genus of bacteria that causes the most cases of foodborne illnesses worldwide. It has been estimated that every year in the U.S, there are 1 million foodborne illnesses, with 19,000 hospitalizations and 380 deaths caused by salmonellosis alone (CDC, 2018). Symptoms of food poisoning by *Salmonella* include diarrhea, fever and abdominal cramps. *Salmonella* can be found almost everywhere in the environment, raw foods, human and animal gastrointestinal tract. Traditionally, the genus *Salmonella*

comprises two species, which are *S. enterica*, with six subspecies and *S. bongori*. In 2005, *S. subterranean* was added (ITIS, 2012).

Salmonella strains have an optimum growth temperature of 37 °C. Additionally, it is capable of tolerating stressful conditions and survives in low- a_w environments for long periods (Mattick et al., 2000; Podolak, Enache, Stone, Black, & Elliott, 2010). These specific characteristics contribute to its high risk for infection when present in low- a_w foods.

2.2.1. Outbreaks and recalls of *Salmonella* spp. in low- a_w foods and spices

Salmonella contamination in low- a_w foods can be caused by several factors including poor sanitation practices, inadequate equipment and facility design, and deficiency in operational practices and good manufacturing practices (GMP) (Podolak et al., 2010). Several *Salmonella enterica* serovars have been linked to outbreaks related to low- a_w foods including pistachios, peanut butter, salami and potato chips seasoned with paprika (Table 2.1).

Specifically, spice-related recalls and outbreaks caused by *Salmonella* have increased in recent years. Extensive reviews have attempted to elucidate the role of spices and herbs and sources of *Salmonella* infections (Beuchat et al., 2013; Finn, Condell, McClure, Amézquita, & Fanning, 2013a; Zweifel & Stephan, 2012). Results from an FDA review of spice-related recalls in the U.S caused by pathogen contamination revealed that 20 out of 21 monitored recalls were caused by *Salmonella* contamination (Vij, Ailes, Wolyniak, Angulo, & Klontz, 2006). From this FDA review in 2006, the majority of spice-related recalls occurred during 2001 and 2004.

Spice-related outbreaks might appear to be less frequent when compared to other low- a_w products but are considered high risk because spices are typically added to ready-to-eat products with no further killing step. In 1993, potato chips seasoned with paprika powders contaminated with *Salmonella* caused a major outbreak, with 1000 people affected in Germany (Lehmacher et al., 1995). The contaminated seasoning was added to roasted potatoes after the temperature of the food product had dropped below 60 °C (Lehmacher et al., 1995). More recently, black and red pepper contaminated with *Salmonella* Montevideo caused an outbreak involving 272 cases of infection in 44 states (CDC, 2010). The contaminated spices were added to Salami. This outbreak was unique because two spices (black and red pepper) tested positive for *Salmonella* and USDA-FSIS and FDA were both involved in this investigation (CDC, 2010).

2.2.2 Survival of *Salmonella* spp. in low- a_w foods and desiccated environments

Salmonella is the pathogen of the most concern in low- a_w foods because it possesses mechanisms that aid its survival in desiccated environments. Recent publications have reviewed the mechanisms of survival of *Salmonella* and the sources and risk factors contributing to contamination of low- a_w foods (Beuchat et al., 2013; Finn et al., 2013a; Podolak et al., 2010).

Studies have reported that the survival of *Salmonella* in reduced a_w environments is influenced by pH, presence of sucrose, product's composition and storage temperature (Finn, Condell, McClure, Amézquita, & Fanning, 2013b; Hiramatsu, Matsumoto, Sakae, & Miyazaki, 2005; Kieboom et al., 2006). Hiramatsu and others (2005) inoculated dried paper disks and foods containing sucrose and/or fat (chocolate, peanuts) with *Salmonella*

and compared both survival rates after 24 h of drying at 25 °C. The survival rates of *Salmonella* in chocolates and peanuts were much higher than the corresponding rates of inoculated strains in paper disks (Hiramatsu et al., 2005). These results suggested that the presence of sucrose and vegetable fat enhanced the survival of *Salmonella* (Hiramatsu et al., 2005). In other studies, it has also been reported, that the presence of fat in low- a_w foods, such as peanut butter, increases the survival and thermal resistance of *Salmonella* (Burnett, Gehm, Weissinger, & Beuchat, 2000; Li, Huang, & Chen, 2014). Additionally, high fat content in low- a_w food produces a protective effect for the bacteria while traveling through the gastrointestinal tract (Aviles, Klotz, Smith, Williams, & Ponder, 2013).

Storage of contaminated low- a_w foods in refrigerated temperature can become a food safety risk because of higher survival of microorganisms under these conditions. Hiramatsu and others (2005) reported *Salmonella* survival in dried paper disks for up to 24 months at 4 °C, while they have died after 35 and 70 days at 25 °C and 35 °C, respectively. Similarly, *Salmonella* in peanut butter and peanut butter spreads survived at higher levels at 5 °C than at 21 °C, when stored for 24 weeks (Burnett et al., 2000). When the storage temperature affects the quality of the food product, it is generally recommended to keep the product at refrigerated temperatures. For example, piperine content is an important quality characteristic of black pepper. In a study, the piperine content of black pepper samples stored at 4 °C was higher when compared to the samples stored at 20 °C (Waje, Kim, Kim, Todoriki, & Kwon, 2008). Therefore, it was recommended to store samples at refrigeration, but this recommendation enhances the survival of *Salmonella*.

Kieboom and others (2006) studied the morphological changes and cell viability of *Salmonella* Enteritidis at reduced a_w (from 0.99 to 0.94) on Tryptone Soy Agar (TSA) containing NaCl (4, 6 and 8%) and stored for 21 days. This study reported filamentation of cells (cells growing without dividing), which are more resistant to disinfection techniques. This survival characteristic becomes a food safety concern especially in food processing facilities with a high risk of cross-contamination because cell filaments can split and form numerous cells under favorable conditions (Kieboom et al., 2006). In another study, the viability of *S. enterica* in skim milk powder was evaluated at three levels a_w (0.33, 0.53 and 0.81) at 37 °C for 2 months (Lian, Zhao, Yang, Tang, & Katiyo, 2015). Log reductions were higher during the first month than the second month for all levels of a_w , while experiencing greater survivability with decreased a_w in skim milk powder (Lian et al., 2015).

2.2.3. Influence of a_w on the thermal resistance of *Salmonella* in low- a_w foods

Several studies have also demonstrated the influence of low- a_w on the thermal resistance of *Salmonella* in foods (Archer, Jervis, Bird, & Gaze, 1998; Jung & Beuchat, 1999). Protein denaturation is inhibited in environments and foods with reduced water available that are exposed to high temperatures during heating, because cells are less affected by vibrations of water molecules that cause breakage of disulfide and hydrogen bonds of intracellular proteins (Earnshaw, Appleyard, & Hurst, 1995). Archer and others (1998) reported this protective effect on the thermal destruction curves of *S. weltevreden* in wheat flour. Additionally, the results demonstrated that at any temperature, when the initial water activity of the sample prior to heating decreased, the heat resistance of *S. weltevreden* increases (Archer et al., 1998).

Recent publications have addressed concerns regarding the adequate determination of a_w of the food sample during thermal treatments and its influence on the thermal resistance of pathogens (Syamaladevi, Tadapaneni et al., 2016; Tadapaneni, Syamaladevi, Villa-Rojas, & Tang, 2017). Most of published studies have reported a_w of food samples measured at room temperature because of a lack of available instruments capable of measuring a_w at elevated pasteurization temperatures ($>60\text{ }^{\circ}\text{C}$) (Syamaladevi et al., 2016).

Syamaladevi and others (2016) generated adsorption and desorption isotherms for all-purpose flour and peanut butter at temperatures below $60\text{ }^{\circ}\text{C}$ using an Aqualab vapor sorption analyzer (VSA) and for temperatures above $80\text{ }^{\circ}\text{C}$ they used a newly developed thermal cell and relative humidity sensor. They later correlated the thermal resistance of *Salmonella* in selected foods with changes of a_w from $20\text{ }^{\circ}\text{C}$ to elevated treatment temperatures. In this study, water activity of peanut butter decreased and all-purpose flour increased when heated from $20\text{ }^{\circ}\text{C}$ to $80\text{ }^{\circ}\text{C}$. The thermal resistance of *Salmonella* in peanut butter was higher than all-purpose flour at $80\text{ }^{\circ}\text{C}$, partially attributed to the decrease in water activity in peanut butter compared to all-purpose flour (Syamaladevi et al., 2016).

In another study, a novel thermal-water activity (TAC) cell was designed to control the water activity of the organic wheat flour (OWF) at treatment temperatures to compare the thermal resistance of *Salmonella* determined by conventional thermal-death-time (TDT) test cells (Tadapaneni et al., 2017). *Salmonella* in OWF samples determined using TAC cells had higher thermal resistance than samples in TDT cells (Tadapaneni et al., 2017). The authors attributed this result to the ability of TAC cells with LiCl solution

to stabilize the relative humidity inside the TAC cells and therefore, controlled the a_w of the sample. Higher thermal resistance of *Salmonella* in TAC cells was related to the limited denaturation of key bacterial cells proteins as a result of low water molecules' availability and protein stabilization with compatible solutes (Tadapaneni et al., 2017). Adsorptions isotherms in this study, showed that the a_w of the OWF in TDT cells increased from 0.45 ± 0.02 at room temperature to 0.73 ± 0.02 when heated to 80 °C. Therefore, the increased water activity reduced the thermal resistance of *Salmonella* (Tadapaneni et al., 2017).

2.2.4. FDA's efforts to increase knowledge and improve food safety of spices

The Food and Drug Administration (FDA) have recently increased its efforts to fully understand the source and prevalence of *Salmonella* in spices. An analysis of FDA sampling and testing data for imported spice shipments offered entry into the U.S during 2007-2009 fiscal years, reported 6.6% of prevalence of *Salmonella* (Van Doren, Kleinmeier, Hammack, & Westerman, 2013). Additionally, more than 92% of spice and seasoning-related entries reported to the FDA Reportable Food Registry during 2009-2011 had a prevalence of *Salmonella* (Pinkas & Keller, 2014). Similarly, FDA examined spices offered entry during 2011-2015 and results were not significantly different from those reported previously (U.S Food and Drug Administration, 2017).

In 2013, the FDA issued a Draft Risk Profile: Pathogens and Filth in Spices. In this draft risk profile, it was identified the lack of information regarding the prevalence of *Salmonella* contamination in spices offered for sale to consumers (U.S Food and Drug Administration, 2013). For this reason, in 2016 the FDA initiated a survey of prevalence of *Salmonella* in packaged spices offered in retail establishments in the U.S and posted

Questions and Answers on Improving Food Safety of Spices (U.S Food and Drug Administration, 2017). The 2017 Final Update Risk Profile document presented the results from this survey indicating a prevalence of *Salmonella* of less than 1% for all 11 types of spices studied, including basil, black pepper, paprika, oregano and others) (Zhang et al., 2017). The estimates of prevalence of *Salmonella* in imported spices at retail establishments revealed that they have significantly smaller prevalence than the estimates of *Salmonella* in spices that were monitored at points of entry in U.S (U.S Food and Drug Administration, 2017). Despite the reduction in prevalence of *Salmonella*, suggesting that decontamination treatments applied in the U.S are in some way effective, there are still some shipments that are not receiving an effective decontamination or are being contaminated post-lethality treatment contamination (U.S Food and Drug Administration, 2017).

2.3. Black pepper production and its current decontamination technologies

Black pepper is mainly produced in countries such as Vietnam, Indonesia, India, Brazil, China, Cambodia and others (Van Gulick, 2017). Black peppers are harvested from the *Piper nigrum* plants when the berries are green and are further sundried. During this process, fermentation causes browning reactions, turning the berries dark. Later the berries are ranked for uniform drying (Tainter & Grenis, 2001). Finally, the black pepper is placed into bags and stored until it is shipped to spice processors for further cleaning, grinding and decontamination procedures.

The practices described above and improper hygienic conditions have contributed to the high level of exposure to contamination sources such as insects, rodents and dust, among others. A study assessing the microbiological quality of different spices reported

black peppercorns to have the highest level of contamination of *Escherichia coli*, coliforms, *Bacillus cereus*, presumptive *C. perfringens*, molds and *Salmonella* (Pafumi, 1986). Therefore, a number of methods have been used to reduce the microbial load on spices, including black pepper. The most common technologies used are fumigation with ethylene oxide, steam treatment and irradiation.

Fumigation with ethylene oxide (ETO) has been proven to effectively reduce the microbial population of spices. In the United States, its use is approved for microbial and insect infestations decontamination with a residue of no more than 50 ppm in the spice after treatment. There are health concerns about its use due to the fact that ETO is a known carcinogen. Therefore, the use of ETO has been banned in the European Union as a decontamination technology for spices (Song et al., 2014).

Steam treatment is commonly used as it does not add any chemicals to the product and is widely accepted by consumers. However, steam application causes degradation of color and bioactive components due to the high-temperature steam applied on whole or ground spices. In addition, it has also been reported that an increase in moisture content of the spice occurs after treatment, decreasing the shelf life of the product (Almela, Nieto-Sandoval, & Fernández López, 2002a). To solve this, additional treatments are required to remove the water from the surface of the spice.

Finally, irradiation has widely been proven to be an effective method to decontaminate spices while preserving its physicochemical properties (Song et al., 2014; Waje et al., 2008). The spices are exposed to irradiation sources on its final package, reducing possibility for post packaging contamination. In addition, irradiation does not cause any rise in the product's temperature. The main disadvantage of this technology is

the poor consumer acceptance, given that consumers relate irradiated products with a risk of being irradiated.

Thermal treatments such as radiofrequency (RF) heating have been proposed for decontamination of low- a_w foods, including spices (Jeong & Kang, 2014; Kim, Sagong, Choi, Ryu, & Kang, 2012; Ozturk, Kong, Trabelsi, & Singh, 2016). RF technology applies electromagnetic waves with a frequency range of 3 kHz to 300 MHz. Frequencies of 13.56, 27.12 and 40.68 MHz are approved by the U.S Federal Communications Commission (FCC) to be used for industrial, scientific and medical (ISM) applications. RF energy generated in these frequencies has deeper penetration depths in dielectric materials, which makes the technology applicable for packaged food products (Y. Wang, Wig, Tang, & Hallberg, 2003). During RF-heating, volumetric heat is generated within the product due to ionic movement and dipolar rotation caused by the alternating electromagnetic field (Michael et al., 2014).

Only a few publications have reported microbial challenge studies using pathogens for RF heating of spices. Kim and others (2012) inoculated black pepper and red pepper of different sizes with cocktails of *S. Typhimurium* and *Escherichia coli* 0157:H7. They reported that RF heating could meet the requirement of a 5-log reduction of pathogens in samples without affecting the color of the product. Similarly, Jeong and others (2014) inoculated the same pathogens into powdered black pepper and red pepper and equilibrated the samples to different moisture content before RF treatment. Similar to previous studies by Kim and others (2014), Jeong and others (2014) reported no significant color differences after RF treatment.

Food processing companies interested in applying thermal process such as

radiofrequency heating as an alternative decontamination technology for black pepper require more information about the thermal inactivation kinetics of *Salmonella* spp. and the possible surrogate, *Enterococcus faecium* NRRL-B2354, to develop and validate the thermal processes.

2.4. Pasteurization process validation and use of *Enterococcus faecium* as a surrogate for *Salmonella* spp.

The FSMA was signed into law in 2011. FSMA's objective is to prevent contamination rather than responding to it. With its implementation, all food processing facilities, including low- a_w food processing facilities, are required to develop and validate preventive controls, which include the validation of pathogen reduction methods to acceptable limits to ensure the safety of foods. The thermal inactivation characteristics of the surrogate microorganisms may vary at different process conditions and product compositions. Therefore, verification of the thermal inactivation characteristics of target pathogens and surrogate should be conducted (Ceylan & Bautista, 2015).

An appropriate surrogate should be nonpathogenic with similar growth, inactivation and survival characteristics to those of target pathogen in the pasteurization or decontamination process conditions (Bianchini et al., 2014). Adequate surrogate microorganisms should be easily cultivated and enumerated following standard microbiology methods and equipment (Bianchini et al., 2014; Ceylan & Bautista, 2015). In addition, the use of surrogates for in-plant thermal processes validations is recommended to ensure workers safety and avoid introducing pathogens to processing areas.

The Almond Board of California used *Enterococcus faecium* as a surrogate to validate dry-roasting process to kill *Salmonella* in almonds (Almond Board of California, 2007). In addition, several authors have investigated the use of *Enterococcus faecium* as a potential non-pathogenic surrogate for thermal treatments including extrusion of balanced carbohydrate-protein meal (Bianchini et al., 2014), oat flour (Verma et al., 2018), pet foods and almonds (Ceylan & Bautista, 2015; Jeong, Marks, & Ryser, 2011). Few studies have assessed the efficacy of *Enterococcus faecium* as a surrogate for *Salmonella* spp. on black peppercorns treated with vacuum steam pasteurization (Shah, Asa, Sherwood, Graber, & Bergholz, 2017) and radiofrequency (Wei, 2017). In previous studies, the thermal resistance of *Enterococcus faecium* was higher than *Salmonella* spp. suggesting that it is an adequate surrogate. On the contrary, it has also been reported that *Enterococcus faecium* has lower thermal resistance than *Salmonella* in confectionary formulations (high sugar content) (Rachon et al., 2016). In another study, *E. faecium* had a D_{75} -value of one-tenth the D_{75} -value of *S. Typhimurium* PT42 when these microorganisms were inoculated to flour (Fudge, Dunn, Pike, Robison, & Steele, 2016). More studies are needed to validate the use of *Enterococcus faecium* as a surrogate for *Salmonella* spp. in different food matrices and under different process conditions.

2.5. Research gap of thermal inactivation processes and validation of low- a_w foods

Currently, low- a_w food processing facilities lack the tools and methods to validate their process control methods (Syamaladevi et al., 2016). Determination of thermal resistances of pathogens and surrogates are required for adequate process validations. Most of available thermal inactivation kinetics studies related to *Salmonella* in low- a_w foods have been conducted with foods including peanut butter, peanut spread, and flours

(Keller et al., 2012; Li et al., 2014; Syamaladevi et al., 2016; Tadapaneni et al., 2017).

Available published data for decontamination of spices have focused on the efficiency of technologies and quality of thermal treated samples rather than determining the thermal inactivation kinetics of *Salmonella* (Almela, Nieto-Sandoval, & Fernández López, 2002b; Jeong & Kang, 2014; Kim et al., 2012; Suhaj, Rácová, Polovka, & Brezová, 2006).

Therefore, there is a need for more extensive research on thermal resistance of *Salmonella* and *Enterococcus faecium*, in low- a_w foods such as spices. It is necessary to understand the different parameters including food matrix, water activity, temperature, inoculation and treatment method that might influence the thermal resistance.

2.6 References

1. Almela, L., Nieto-Sandoval, J. M., & Fernández López, J. A. (2002a). Microbial inactivation of paprika by a high-temperature short-X time treatment. influence on color properties. *Journal of Agricultural and Food Chemistry*, 50(6), 1435-1440.
2. Almond Board of California. (2007). Guidelines for validation of dry roasting processes. Available from: <http://www.almonds.com/sites/default/files/content/attachments/dry-roast-validation-guidelines.pdf>. Accessed September, 2017
3. Archer, J., Jervis, E. T., Bird, J., & Gaze, J. E. (1998). Heat resistance of *Salmonella* Weltevreden in low-moisture environments. *Journal of Food Protection*, 61(8), 969-973.
4. Aviles, B., Klotz, C., Smith, T., Williams, R., & Ponder, M. (2013). Survival of *Salmonella enterica* serotype Tennessee during simulated gastric passage is improved by low water activity and high fat content. *Journal of Food Protection*, 76(2), 333-337.
5. Beuchat, L. R., Komitopoulou, E., Beckers, H., Betts, R. P., Bourdichon, F., Fanning, S., . . . Ter Kuile, B. H. (2013). Low–water activity foods: Increased concern as vehicles of foodborne pathogens. *Journal of Food Protection*, 76(1), 150-172.
6. Bianchini, A., Stratton, J., Weier, S., Hartter, T., Plattner, B., Rokey, G., . . . Eskridge, K. M. (2014). Use of *Enterococcus faecium* as a surrogate for *Salmonella enterica* during extrusion of a balanced carbohydrate-protein meal. *Journal of Food Protection*, 77(1), 75-82.
7. Blessington, T., Theofel, C. G., & Harris, L. J. (2013). A dry-inoculation method for nut kernels. *Food Microbiology*, 33(2), 292-297.
8. Burnett, S., Gehm, E., Weissinger, W., & Beuchat, L. (2000). Survival of *Salmonella* in peanut butter and peanut butter spread. *Journal of Applied Microbiology*, 89(3), 472-477.
9. CDC. (2010). Multistate outbreak of human *Salmonella* Montevideo infections (Final Update). Available from: <http://www.cdc.gov/salmonella/2010/montevideo-5-4-2010.html>. Accessed January, 2018
10. CDC. (2018). *Salmonella*. Available from: <https://www.cdc.gov/salmonella/index.html>. Accessed February, 2018

11. Ceylan, E., & Bautista, D. A. (2015). Evaluating *Pediococcus acidilactici* and *Enterococcus faecium* NRRL B-2354 as thermal surrogate microorganisms for *Salmonella* for in-plant validation studies of low-moisture pet food products. *Journal of Food Protection*, 78(5), 934-939.
12. Earnshaw, R., Appleyard, J., & Hurst, R. (1995). Understanding physical inactivation processes: Combined preservation opportunities using heat, ultrasound and pressure. *International Journal of Food Microbiology*, 28(2), 197-219.
13. Farakos, S. S., Frank, J., & Schaffner, D. (2013). Modeling the influence of temperature, water activity and water mobility on the persistence of *Salmonella* in low-moisture foods. *International Journal of Food Microbiology*, 166(2), 280-293.
14. Finn, S., Condell, O., McClure, P., Amézquita, A., & Fanning, S. (2013). Mechanisms of survival, responses and sources of *Salmonella* in low-moisture environments. *Frontiers in Microbiology*, 4, 331.
15. Fudge, J., Dunn, M., Pike, O., Robison, R., & Steele, F. (2016). The isolation and identification of *Pantoea dispersa* strain JFS as a non-pathogenic surrogate for *Salmonella* Typhimurium phage type 42 in flour. *International Journal of Food Microbiology*, 219, 1-6.
16. Grocery Manufacturers Association. (2009). Control of *Salmonella* in low-moisture foods. Available from: <https://www.gmaonline.org/downloads/technical-guidance-and-tools/SalmonellaControlGuidance.pdf>. Accessed January, 2018
17. Gurtler, J. B., Doyle, M. P., & Kornacki, J. L. (2014). The microbiological safety of spices and low-water activity foods: Correcting historic misassumptions. In *The microbiological safety of low water activity foods and spices* (pp. 3-13) Springer, New York, NY.
18. Hiramatsu, R., Matsumoto, M., Sakae, K., & Miyazaki, Y. (2005). Ability of shiga toxin-producing *Escherichia coli* and *Salmonella* spp. to survive in a desiccation model system and in dry foods. *Applied and Environmental Microbiology*, 71(11), 6657-6663.
19. ITIS. (2012). *Salmonella*. Available from: https://www.itis.gov/servlet/SingleRpt/SingleRpt?search_topic=TSN&search_value=302#null. Accessed February, 2018
20. Jeong, S., Marks, B. P., & Ryser, E. T. (2011). Quantifying the performance of *Pediococcus* sp. (NRRL B-2354: *Enterococcus faecium*) as a nonpathogenic

surrogate for *Salmonella* Enteritidis PT30 during moist-air convection heating of almonds. *Journal of Food Protection*, 74(4), 603-609.

21. Jeong, S., & Kang, D. (2014). Influence of moisture content on inactivation of *Escherichia coli* O157: H7 and *Salmonella enterica* serovar Typhimurium in powdered red and black pepper spices by radio-frequency heating. *International Journal of Food Microbiology*, 176, 15-22.
22. Jung, Y., & Beuchat, L. (1999). Survival of multidrug-resistant *Salmonella* Typhimurium DT104 in egg powders as affected by water activity and temperature. *International Journal of Food Microbiology*, 49(1), 1-8.
23. Keller, S. E., Grasso, E. M., Halik, L. A., Fleischman, G. J., Chirtel, S. J., & Grove, S. F. (2012). Effect of growth on the thermal resistance and survival of *Salmonella* Tennessee and Oranienburg in peanut butter, measured by a new thin-layer thermal death time device. *Journal of Food Protection*, 75(6), 1125-1130.
24. Kieboom, J., Kusumaningrum, H. D., Tempelaars, M. H., Hazeleger, W. C., Abee, T., & Beumer, R. R. (2006). Survival, elongation, and elevated tolerance of *Salmonella enterica* serovar Enteritidis at reduced water activity. *Journal of Food Protection*, 69(11), 2681-2686.
25. Kim, S., Sagong, H., Choi, S. H., Ryu, S., & Kang, D. (2012). Radio-frequency heating to inactivate *Salmonella* Typhimurium and *Escherichia coli* O157: H7 on black and red pepper spice. *International Journal of Food Microbiology*, 153(1), 171-175.
26. Lehmacher, A., Bockemühl, J., & Aleksic, S. (1995). Nationwide outbreak of human salmonellosis in Germany due to contaminated paprika and paprika-powdered potato chips. *Epidemiology and Infection*, 115(3), 501-512.
27. Li, C., Huang, L., & Chen, J. (2014). Comparative study of thermal inactivation kinetics of *Salmonella* spp. in peanut butter and peanut butter spread. *Food Control*, 45, 143-149.
28. Lian, F., Zhao, W., Yang, R., Tang, Y., & Katiyo, W. (2015). Survival of *Salmonella enteric* in skim milk powder with different water activity and water mobility. *Food Control*, 47, 1-6.
29. Mattick, K. L., Jorgensen, F., Legan, J. D., Cole, M. B., Porter, J., Lappin-Scott, H. M., & Humphrey, T. J. (2000). Survival and filamentation of *Salmonella enterica* serovar Enteritidis PT4 and *Salmonella enterica* serovar Typhimurium DT104 at low water activity. *Applied and Environmental Microbiology*, 66(4), 1274-1279.

30. Michael, M., Phebus, R., Thippareddi, H., Subbiah, J., Birla, S., & Schmidt, K. (2014). Validation of radio-frequency dielectric heating system for destruction of *Cronobacter sakazakii* and *Salmonella* species in nonfat dry milk. *Journal of Dairy Science*, 97(12), 7316-7324.
31. Ozturk, S., Kong, F., Trabelsi, S., & Singh, R. K. (2016). Dielectric properties of dried vegetable powders and their temperature profile during radio frequency heating. *Journal of Food Engineering*, 169, 91-100.
32. Pafumi, J. (1986). Assessment of the microbiological quality of spices and herbs. *Journal of Food Protection*, 49(12), 958-963.
33. Pinkas, J. M., & Keller, S. E. (2014). Spices. In J. B. Gurtler, M. P. Doyle & J. L. Kornacki (Eds.), *The microbiological safety of low water activity foods and spices, food microbiology and food safety* (pp. 99).
34. Podolak, R., Enache, E., Stone, W., Black, D. G., & Elliott, P. H. (2010). Sources and risk factors for contamination, survival, persistence, and heat resistance of *Salmonella* in low-moisture foods. *Journal of Food Protection*, 73(10), 1919-1936.
35. Rachon, G., Peñaloza, W., & Gibbs, P. A. (2016). Inactivation of *Salmonella*, *Listeria monocytogenes* and *Enterococcus faecium* NRRL B-2354 in a selection of low moisture foods. *International Journal of Food Microbiology*, 231, 16-25.
36. Shah, M. K., Asa, G., Sherwood, J., Graber, K., & Bergholz, T. M. (2017). Efficacy of vacuum steam pasteurization for inactivation of *Salmonella* PT 30, *Escherichia coli* O157: H7 and *Enterococcus faecium* on low moisture foods. *International Journal of Food Microbiology*,
37. Song, W., Sung, H., Kim, S., Kim, K., Ryu, S., & Kang, D. (2014). Inactivation of *Escherichia coli* O157:H7 and *Salmonella* Typhimurium in black pepper and red pepper by gamma irradiation. *International Journal of Food Microbiology*, 172, 125-129.
38. Suhaj, M., Ráková, J., Polovka, M., & Brezová, V. (2006). Effect of γ -irradiation on antioxidant activity of black pepper (*Piper nigrum* L.). *Food Chemistry*, 97(4), 696-704.
39. Syamaladevi, R. M., Tadapaneni, R. K., Xu, J., Villa-Rojas, R., Tang, J., Carter, B., . . . Marks, B. (2016). Water activity change at elevated temperatures and thermal resistance of *Salmonella* in all purpose wheat flour and peanut butter. *Food Research International*, 81, 163-170.
40. Syamaladevi, R. M., Tang, J., Villa-Rojas, R., Sablani, S., Carter, B., & Campbell, G. (2016). Influence of water activity on thermal resistance of

microorganisms in Low-Moisture foods: A review. *Comprehensive Reviews in Food Science and Food Safety*, 15(2), 353-370.

41. Tadapaneni, R. K., Syamaladevi, R. M., Villa-Rojas, R., & Tang, J. (2017). Design of a novel test cell to study the influence of water activity on the thermal resistance of *Salmonella* in low-moisture foods. *Journal of Food Engineering*, 208, 48-56.
42. Tainter, D. R., & Grenis, A. T. (2001). *Spices and seasonings: A Food Technology Handbook*. John Wiley & sons.
43. U.S Food and Drug Administration. (2013). FDA Draft Risk Profile: Pathogens and Filth in Spices. Available from: <https://www.fda.gov/downloads/Food/FoodScienceResearch/RiskSafetyAssessment/UCM367337.pdf>. Accessed February, 2018
44. U.S Food and Drug Administration. (2017). Risk Profile: Pathogens and Filth in Spices. Available from: <https://www.fda.gov/downloads/Food/FoodScienceResearch/RiskSafetyAssessment/UCM581362.pdf>. Accessed February, 2018
45. Van Gulick, A. (2017). Pepper crop report. ESA Conference. Available from: https://www.nedspice.com/upload/docs/170601_Nedspice_-_ESA_Pepper_Crop_Report_vFinal.pdf. Accessed May, 2018
46. Van Doren, J. M., Kleinmeier, D., Hammack, T. S., & Westerman, A. (2013). Prevalence, serotype diversity, and antimicrobial resistance of *Salmonella* in imported shipments of spice offered for entry to the United States, FY2007–FY2009. *Food Microbiology*, 34(2), 239-251.
47. Verma, T., Wei, X., Lau, S. K., Bianchini, A., Eskridge, K. M., & Subbiah, J. (2018). Evaluation of *Enterococcus faecium* NRRL B-2354 as a Surrogate for *Salmonella* During Extrusion of Low-Moisture Food. *Journal of Food Science*, 83(4), 1063-1072.
48. Vij, V., Ailes, E., Wolyniak, C., Angulo, F. J., & Klontz, K. C. (2006). Recalls of spices due to bacterial contamination monitored by the U.S Food and Drug Administration: The predominance of *Salmonellae*. *Journal of Food Protection*, 69(1), 233-237.
49. Waje, C. K., Kim, H., Kim, K., Todoriki, S., & Kwon, J. (2008). Physicochemical and microbiological qualities of steamed and irradiated ground black pepper (*Piper nigrum* L.). *Journal of Agricultural and Food Chemistry*, 56(12), 4592-4596.

50. Wang, Y., Wig, T., Tang, J., & Hallberg, L. (2003). Sterilization of foodstuffs using radio frequency heating. *Journal of Food Science*, 68(2), 539-544.
51. Wei, X. (2017). Radiofrequency processing for inactivation of *Salmonella* spp. and *Enterococcus faecium* NRRL B-2354 in whole black peppercorn and ground black pepper (Master's thesis). Available from: Digital Commons@ University of Nebraska-Lincoln. <https://digitalcommons.unl.edu/foodscidiss/88/> Accessed May, 2018
52. Zhang, G., Hu, L., Pouillot, R., Tatavarthy, A., Doren, J. M. V., Kleinmeier, D., . . . Brown, E. W. (2017). Prevalence of *Salmonella* in 11 spices offered for sale from retail establishments and in imported shipments offered for entry to the United States. *Journal of Food Protection*, 80(11), 1791-1805.
53. Zweifel, C., & Stephan, R. (2012). Spices and herbs as source of *Salmonella*-related foodborne diseases. *Food Research International*, 45(2), 765-769.

Table 2.1. Several *Salmonella enterica* serovars have been linked to outbreaks related to low- a_w foods.

Year	Food Product	People affected/ Country	Serotype	Comments	References
2016	Pistachios	11, USA	Montevideo, Senftenberg	The organisms were isolated from infected patients and production facility.	CDC, 2016
2012	Peanut butter	42, USA	Bredeney	FDA inspection indicated that company's facility conditions, manufacturing processes and testing program may have allowed the distribution of contaminated peanut butter.	CDC, 2012
2009-2010	Salami	272, USA	Montevideo	Crushed black and red peppers were added to Salami after the cooking step. <i>Salmonella</i> was isolated from these products.	CDC, 2010
2008	Raw flour	75, New Zealand	Typhimurium	Consumption of uncooked baking mixture containing flour contaminated with <i>Salmonella</i> was associated as the cause of outbreak.	McCallum et al., 2013
2000	Powdered infant formula	30, Korea	London	The organism was isolated from an open package. It is unclear if contamination occurred during manufacturing or after the package was opened.	Cahill et al., 2008
1996	Peanut butter	15, Australia	Mbandaka	<i>Salmonella</i> Mbandaka was isolated from open and closed peanut butter jars from households and retailers, respectively. Roasted peanuts from another Austrian state were implicated as the source of contamination.	Scheil et al., 1998
1993	Potato chips	1000, Germany	Saintpaul, Rubislaw, Javiana	Organisms were isolated from paprika powders, spice mixtures snacks and patients. Paprika powders were applied to roasted potatoes at the end of production when the temperature had dropped to 60 °C.	Lechmache r et al., 1995

Chapter III: Evaluation of a novel dry heating method for determination of thermal inactivation kinetics of microorganisms in low- a_w foods: *Salmonella* spp. and *E. faecium* in ground black pepper.

Abstract

Black pepper is the most used spice in the world. Similar to many other spices, black pepper is commonly added to products without further cooking. *Salmonella* spp. has been found in a variety of spices. *Salmonella*'s known ability to survive low water activity (a_w) environments and high thermal resistance increase the risk factor for foodborne illnesses. The objectives of this study were: 1) to evaluate a novel dry heating method for determination of the thermal inactivation kinetics of *Salmonella* spp. and *E. faecium* in ground black pepper at 0.45 a_w and compare it with a conventional heating method (Thermal Death Time test cells), 2) to evaluate *E. faecium* as an adequate surrogate for *Salmonella* spp. in ground black pepper and 3) to investigate the effect of heating rates on the thermal inactivation parameters of *Salmonella* and *E. faecium* when using TDT sandwiches. The use of TDT sandwiches showed operational benefits over conventional heating method. Benefits include lower log reductions (<0.16 log CFU/g) were observed in TDT sandwiches during Come-up-time, when compared to TDT test cells (0.6 log CFU/g). Additionally, TDT sandwiches had larger sample capacity (~ 1.5 g) compared to TDT test cell (~ 0.7 g), no risk of leakage during treatment and adjustable heating rates. Results demonstrated that thermal resistance of *Salmonella* spp. was influenced by the method used when treated at 65 °C and 75 °C, whereas, the thermal resistance of *E. faecium* was not for all three temperatures. *E. faecium* was identified as a suitable surrogate for *Salmonella* spp. in black pepper, independently of the heating

method used. Finally, heating rates above 6.5 °C/min using TDT sandwiches did not affect the thermal resistance determination of both microorganisms. Future process validation studies for thermal treatments could benefit from these results, which could be utilized as guidelines for related industry applications.

3.1. Introduction

Black pepper is commonly known as the “King of Spices”, because it is the most widely used spice in the world. It is mainly used as a flavor enhancer, by improving products’ taste and aroma. Black pepper and other spices are mainly grown in humid and warm areas, which are also conditions that promote microbial growth (Banerjee & Sarkar, 2003). Additionally, improper hygienic conditions during handling, drying and storage of black pepper increase the risk of contamination with spoilage and pathogenic bacteria.

Assessments of the microbiological quality of black pepper have reported microbial loads greater than 10^7 CFU/g for total aerobic counts (McKee, 1995; Pafumi, 1986). Additionally, *Salmonella* spp. has been found in a variety of spices, including black pepper (Vij et al., 2006; Zweifel & Stephan, 2012). *Salmonella*’s ability to survive in low-water activity (a_w) environments and its high thermal resistance increase the risk factor for foodborne illnesses. Therefore, spices such as black pepper could become a food safety threat when added as ingredients to foods with no further heat treatment. In 2010, salami products prepared with black and red pepper contaminated with *Salmonella* spp. caused a multistate outbreak in the USA (Zweifel & Stephan, 2012). This outbreak confirmed the importance of increasing efforts in developing and validating current and novel decontamination technologies for spices, including black pepper.

The Food Modernization Act (FSMA) requires all food-processing facilities to validate the decontamination processes chosen to control hazards. Facilities that apply thermal decontamination processes require an understanding of the thermal resistance of pathogenic microorganisms identified in their processes' risk assessment. An adequate surrogate of the pathogenic microorganism should also be identified. Additionally, it is required that process validation should be conducted for each product and processing conditions.

Traditionally, thermal inactivation kinetics of microorganisms was determined using high levels of organisms inoculated into buffers or foods that were heat treated inside capillary tubes or vials. These methods are limited to small amounts of liquid (~0.01g) or semisolid foods (~0.25 g), and are not feasible for spices and other powdered products. Therefore, it limited the study of thermal resistance of microorganisms in these food matrices.

Later, other alternative methods were developed using aluminum tubes and test cells that permitted testing higher amounts (~1g) of liquids, semisolids and solids foods (Chung, Birla, & Tang, 2008; Chung, Wang, & Tang, 2007; Jin, Zhang, Boyd, & Tang, 2008). When using aluminum tubes, non-isothermal conditions were achieved during the heating-up period, resulting in the preconditioning of microorganisms and thus increasing thermal resistance (Chung et al., 2007). In contrast, thermal-death-time (TDT) test cells were proven to have shorter heating-up time, improving isothermal heating conditions (Chung et al., 2008). Therefore, TDT cells have been used in studies for determination of the thermal resistance of *Salmonella* spp. in low- a_w foods, such as wheat flour and peanut butter (Hildebrandt et al., 2016; Syamaladevi et al., 2016; Tadapaneni et al., 2017).

Additionally, another study evaluated the inactivation of *Salmonella* spp., *Listeria monocytogenes* and *Enterococcus faecium* in a selection of low- a_w foods (chicken meat powder, pet food, confectionary and culinary products) using solid aluminum chambers (thermal cells) (Rachon et al., 2016). However, there are difficulties when comparing the thermal resistances of microorganisms with available data on literature due to the variability of strains tested, sample holders, heating methods, temperatures applied and food matrix tested.

With regard to black pepper and other spices, only a few studies have evaluated the effectiveness of thermal decontamination of *Salmonella enterica* serovar Typhimurium and Enteritidis PT 30 using radio frequency (Jeong & Kang, 2014; Kim et al., 2012), vacuum steam pasteurization (Shah et al., 2017) and irradiation (Song et al., 2014). Therefore, to date, there is a lack of information on the thermal inactivation kinetics of *Salmonella* spp. and *E. faecium*, as a surrogate in ground black pepper.

In this study, the use of a novel dry heating method was evaluated for thermal-death-time (TDT) determinations. TDT sandwiches, developed at the University of Nebraska-Lincoln, consisted of a system in which the temperature of the heating elements were precisely controlled by an Arduino Uno-compatible microcontroller that communicated with a custom-written C# program on a computer. The inoculated samples were placed in between the heating elements covered with an insulating material. The objectives of this study were to evaluate a novel dry heating method, TDT sandwiches, for determination of the thermal inactivation kinetics of *Salmonella* spp. and *E. faecium* through a comparison with conventional heating method (TDT test cells) using ground black pepper at 0.45 a_w and then, to determine if heating rates have an influence on the

thermal inactivation parameters of *Salmonella* and *E. faecium* using TDT sandwiches.

The use of *E. faecium* as an adequate surrogate for *Salmonella* spp. in ground black pepper was evaluated.

The results of this study expanded the available information regarding the thermal resistance of *Salmonella* spp. and *E. faecium* in black pepper and how it is influenced by the method used for its determination. Researchers in academia and food industry will be able to use the information as a guide in the development and validation of decontamination technologies for spices and other low- a_w foods.

3.2. Materials and methods

3.2.1. Low- a_w food: black peppercorns

Several batches of black peppercorns were obtained from McCormick & Co. Inc. (Hunt Valley, MD, U.S.A). Upon arrival, the water activity (a_w) of the black peppercorns was measured using an AquaLab dew point water activity meter (Model: 4TE, METER Group, Inc., Pullman, WA, U.S.A). The black peppercorns were placed in polypropylene bags, double sealed and placed at -20 °C until use.

3.2.2. Background flora

To evaluate the background flora of the black peppercorns, three 10-g samples were each added to 90 mL of 0.1% peptone water and then serially diluted. Samples were plated on 3M™ Petrifilm™ plates (St. Paul, MN) for total Aerobic Plate Counts and generic *E. coli* and coliform counts, as well in Dichloran Rose Bengal Chloramphenicol (DRBC) (Neogen, Lansing, MI) plates for yeast and molds counts. The generic *E. coli* and total coliform plates were incubated at 35 °C for 48 ± 3 h; while the DRBC plates were incubated at 25 °C for 5 days. These experiments were repeated for each batch of black

peppercorns received. The total microbial populations for all batches of black peppercorns tested were below the detection limit (<10 CFU/g).

3.2.3. Bacterial strains

A cocktail of 5 different strains of *Salmonella enterica* was used throughout this study: Agona 447967, Montevideo 48827, Mbandaka 698538 (FDA, ORA Regional Lab, Jefferson, AK, U.S.A), Reading (ATTC BAA-1045), and Tennessee K4643 (Dr. Larry Beuchat, University of Georgia, Griffin, GA., U.S.A). Additionally, *Enterococcus faecium* NRRL B-2354 (Agriculture Research Service, USDA) was selected as the surrogate microorganism. These bacterial strains were stored at -80 °C as frozen stocks cultures in 80% glycerol. Selection of the strains was based on thermal resistance, frequency of occurrence in low- a_w foods and association with recalls and outbreaks: *S.* Agona (puffed rice cereal recall), *S.* Montevideo (Black pepper outbreak), *S.* Mbandaka (sesame paste), *S.* Reading (cumin), *S.* Tennessee (peanut butter outbreak).

3.2.4. Inoculum preparation

Prior to culture, the bacterial strains were re-activated from cryopreservation conditions. The individual vials were thawed at 37 °C and the entire content was transferred to 10 ml of tryptic soy broth with 0.6% yeast extract (TSBYE; Becton, Dickinson and Company, Sparks, MD, U.S.A) and incubated at 37 °C for 24 ± 2 h. 10- μ l loops were used to streak colonies for isolation and plates were incubated at 37 °C for 24 ± 2 h. When the growth of isolated colonies were confirmed, the plates were wrapped with parafilm and stored at 4 °C. These plates were called “permanent plates” and were limited to five monthly transfers to prepare future working plates. To prepare working plates, one isolated colony from the permanent plates was picked using a 10- μ l loop and

streaked onto tryptic soy agar plates with 0.6% yeast extract (TSAYE; Becton, Dickinson and Company, Sparks, MD, U.S.A) and incubated at 37 °C for 24 ± 2 h. When the growth of isolated colonies was confirmed, the working plates were wrapped with parafilm and stored at 4 °C for up to a month.

Previous studies have shown that lawn based cells used for inoculation of low moisture food, are capable of producing more stable populations before thermal inactivation treatments (Hildebrandt et al., 2016). In this study, an isolated colony was aseptically harvested from a working plate using a 10-µl loop and transferred to 10 mL TSBYE. The broth was incubated at 37 °C for 24 ± 2 h and 100 µl of the overnight culture was spread on the surface of TSAYE agar plates to form lawns. The plates were then incubated at 37 °C for 24 ± 2 h. To harvest the lawns, 3 ml of 0.1% buffered peptone water (BPW; Becton, Dickinson and Company, Sparks, MD, U.S.A) was added to the surface of TSAYE plates. An L-shaped spreader was used to gently displace the cells into suspension. From each plate, 2 ml of cell suspension was drawn using a sterile pipette and poured into conical centrifugal sterile tube. This procedure was repeated for each *Salmonella* strain and *E. faecium*. To prepare the *Salmonella* cocktail equal amounts of cell suspension from each strain were added to a single conical centrifugal sterile tube.

To test the initial concentration of each inoculum, they were serially diluted (1:10) and spread plated on TSAYE supplemented with differential agents 0.05% ammonium iron (III) citrate (Sigma Aldrich, St. Louis, MO, U.S.A) and 0.03 % sodium thiosulfate (Fisher Chemical, Fair Lawn, N.J, U.S.A) (mTSAYE) for *Salmonella*, and TSAYE with 0.05% ammonium iron (III) citrate and 0.025% of 97% esculin hydrate (Acros Organics, NJ, U.S.A) (eTSAYE) for *E. faecium* and incubated at 37 °C for 24 ± 2

h. Black precipitate in the center of colonies differentiated either *Salmonella* or *E. faecium*, from other contaminating microorganisms (Smith & Marks, 2015). See Appendix A. The inoculum concentrations were 10-11 log CFU/g for *Salmonella* spp. and 9-10 log CFU/g for *E. faecium*.

3.2.5. Sample inoculation, grinding and equilibration

For the inoculation procedure, black peppercorn samples were removed from the freezer 24 h before inoculation and poured onto aluminum trays inside a controlled relative humidity chamber set up to achieve $0.45 \pm 0.025 a_w$ in the peppercorns. Preliminary tests demonstrated that the samples reached the desired water activity after 48 h.

The relative humidity controlled chambers consisted of a humidity sensor (AM2303, Aosong Electronics Co., Ltd., Guangzhou, China), an air pump (Fusion 700, JW Pet, Teterboro, NJ, U.S.A) a fan, solenoid valves, a wet column consisting of water and humidifier wicks, a dry column filled with silica beads (640SGO55, Sorbent Systems, Los Angeles, CA, U.S.A), and a microcontroller (Mega 2560 R3, SainSmart Technology, Inc., Lenexa, KS, U.S.A). The microcontroller receives humidity readings from the humidity sensor and switches the appropriate solenoid valves to pump air through the wet or dry column. The system maintains humidity within 0.3% of the humidity set point.

Once equilibrated to the desired water activity, the black peppercorns were spray inoculated with *Salmonella* spp. cocktail (2 ml per 100 g of black peppercorns) or *E. faecium* NRRL B-2354 and hand-massaged in a polypropylene bag for 10 minutes inside a biosafety cabinet. After an hour, the water activity of the inoculated, whole black

pepper was measured. Contaminated supplies were discarded in a biohazard bag and the water activity meter was properly disinfected. As expected, the water activity of the black peppercorns increased upon inoculation. The inoculated product was poured onto aluminum trays and once again placed inside of a controlled relative humidity chamber set up to achieve the final desired water activity. The samples were kept under these conditions for 48 h before grinding. For this study, black peppercorns were chosen instead of ground black pepper, to simulate possible contamination that occurs to black peppercorns during harvesting, storage and production. Additionally, it is preferred to spray inoculate as opposed to pouring the inoculum onto the sample, to ensure a more homogenous distribution of the inoculum. Forty-eight hours after inoculation, the black peppercorns were ground for 30 s using a 3-cup Power Grinder, 120 V, 750 W (Waring Commercial, Torrington, CT, U.S.A) placed in a biosafety cabinet.

For the purpose of the experiments, after grinding the samples, they were passed through a U.S 20-mesh sieve to achieve a uniform size before the heat treatment. Additionally, the U.S 20-mesh was selected to permit the evaluation of treated samples, by chemical analysis, such as piperine content, without requiring additional grinding (American Trade Spice Trade Association, 1997). See Appendix B.

The grinding process produced fine (pass-through the 20-mesh sieve, P_1 , in grams) and coarse particles (retained over the 20-mesh sieve, R_1 , in grams). The coarse particles (R_1) may come from the pericarp (outer portion of the drupe), which may contain most of the inoculation. The seed (inner portion of the drupe) may contain the majority of the antimicrobials components; therefore, to avoid losing significant portions of inoculated particles and antimicrobials components in the final ground black pepper

sample (S), the coarse particles (R_1) were further ground and passed again through a U.S 20-mesh sieve to obtain (P_2). Further, the second pass-through fine black pepper (P_2 , in grams) was mixed with the P_1 . In order to mix the second pass-through material (P_2) in the same proportion as the first pass-through (P_1), the following equation was used: S (in grams) = $P_2 * (1 + P_1/R_1)$. See Appendix C.

The finely ground black pepper samples (S) were then poured on the aluminum trays and placed back inside the equilibrium chamber for at least 24 h before using it for thermal inactivation treatments or packaging for storage. This was done because previous studies have reported the importance of sample equilibration and stabilization of microorganisms before heat treatments (Beuchat & Scouten, 2002; Fudge et al., 2016; Jeong, Marks, Ryser, & Harte, 2012). The grinding process described above was also followed for the preparation of non-inoculated ground black pepper used for come-up time determinations.

3.2.6. Homogeneity and stability tests

To assess the homogeneity and stability of *Salmonella* spp. and *E. faecium* NRRL B-2354 in black pepper at 0.45 a_w before and after grinding, five 3-g samples of inoculated black pepper were taken randomly from the tray after inoculation (day 0), after grinding on day 2 and subsequent days of storage (3, 6, 9, 12 and 15). Each subsample was serially diluted using 0.1% BPW and 3 dilutions were plated on mTSAYE for *Salmonella* spp. and eTSAYE for *E. faecium*. The plates were incubated at 37 °C for 24 ± 2 h.

The inoculated black pepper samples were considered to be homogenous when the standard deviations associated with microbial counts from the subsamples were no

more than 0.3 log CFU/g. These experiments were performed using three biological replicates, meaning samples were inoculated with bacteria prepared from independent working plates.

3.2.7. Thermal inactivation apparatus

3.2.7.1. TDT sandwiches design and development

A novel dry heating method for thermal-death-time (TDT) determination was needed, because the current available devices suffer from limitations when used with low- a_w foods, as explained previously in the introduction. This novel system was composed of independent units of TDT sandwiches. The system had a capacity for 18 units to be used at a given time. TDT sandwiches consisted of two 3'' (76.2 mm) by 3'' (76.2 mm) flat heating elements (KH-303/10-P, Omega Engineering, Inc., Stamford, CT, U.S.A) backed-up with two insulating ceramic fiber blocks (height: 25.4 mm, width: 127 mm, length: 127 mm). See Appendix D. The heating elements were controlled by an Arduino Uno-compatible microcontroller (Elegoo UNO R3, Elegoo, Shenzhen, China) which constantly communicates with a custom-written C# program on a computer. The system was developed to allow the user to record the experimental parameters of the program, including temperature set point, duration of heating, and control settings.

3.2.7.2. Thermal inactivation using TDT sandwiches

The come-up time (CUT) is defined as the time required for the sample to reach 0.5 °C from the desired temperature. TDT sandwiches were configured to three different heating rates ranging from 6.5 °C/min (slowest) to 600 °C/min (fastest). The heating rates were selected to achieve CUT as fast as possible within less than a minute, to approximately 2.5 min and 6 min, at different temperatures. These heating rates were

selected to further compare the thermal inactivation and the effect of the applied method (sandwiches against TDT cells) and come-up times (CUT).

To determine the CUT associated with each heating rate in the TDT sandwiches, 18 water-impermeable aluminized plastic pouches (PAKVF4C, IMPAK Corp., Los Angeles, CA, U.S.A) with a width approximately 2.7" to 2.8" (68 mm to 73 mm) and height of 2.8" (73 mm) were filled with 1.5 ± 0.2 g of non-inoculated ground black pepper equilibrated to 0.45 a_w (measured at 25 °C). The pouches were heat-sealed with a heat sealer (IPKHS-606T, IMPAK Corp., Los Angeles, CA, U.S.A) in relative humidity controlled chambers with closed air circulation. The thickness of the pouches filled with the ground black pepper was approximately 1.3 mm. Later, a thermocouple was placed inside each aluminum pouch to record the temperature of the samples. The pouches were placed in between the 2 heating elements and further sandwiched by 2 insulation blocks. The average time among all TDT sandwiches required to achieve the desired temperature plus 2 standard deviations was taken as the CUT to account for variability in various TDT sandwiches.

For thermal inactivation studies using TDT sandwiches, 14-16 aluminum pouches were filled with inoculated sample, heated sealed and sandwiched as described above. Inoculated samples of ground black pepper (without any heat treatment) were retained for determination of initial microbial counts.

During the thermal inactivation study, 2 aluminum pouches were removed at the predetermined CUT for each temperature. Then, the total isothermal treatment time was divided in equal time intervals. The treatment time was determined empirically with trial and error. The thermal treatment was applied to achieve 3-5 log CFU/g reduction from

time zero (CUT) and the limit of detection (LOD) of 2.4 log CFU/g. For each time point, 2 aluminum pouches were removed from the corresponding TDT sandwiches. Once removed from the TDT sandwiches, the aluminum pouches were immersed into an ice-water mixture for 1 min to stop the thermal inactivation.

3.2.7.3. Microbial enumeration procedure

The aluminum pouches containing the treated or untreated samples (initial microbial counts) were opened, weighed and diluted with 0.1% buffered peptone water (BPW) to achieve a 1:10 dilution. The aluminum pouches were disposed immediately after opening. The diluted samples were mixed for 30 s using a stomacher. The mixed sample was serially diluted (1:10) and at least 3 dilutions were plated in duplicate on mTSAYE and eTSAYE, for the enumeration of *Salmonella* spp. and *E. faecium*, respectively. The plates were incubated at 37 °C for 24 ± 2 h. The raw plate count data were converted to log CFU/g. These experiments were done with 3 biological replicates.

3.2.7.4. Thermal inactivation using thermal-death time (TDT) test cells

To compare results from TDT sandwiches with the conventional heating method, TDT test cells designed by Washington State University (Chung et al., 2008; Jin et al., 2008) were used. See Appendix E. These TDT test cells have also been used in several other low moisture foods inactivation studies (Hildebrandt et al., 2016; Syamaladevi et al., 2016; Tadapaneni et al., 2017).

The CUT at each temperature was determined by filling 18 TDT test cells with 0.70 ± 0.1 g non-inoculated equilibrated to 0.45 a_w (measured at 25 °C before treatment) ground black pepper inside a controlled relative humidity equilibrium chamber. One TDT test cell had a T-type thermocouple inserted. A detailed description of this cell can be

found in Chung and others (2008). The TDT test cell with a thermocouple was positioned in the middle of the 6 racks containing all 18 TDT test cells. The TDT test cells were immersed in a circulating water bath (Neslab RTE17, Thermo Fisher Scientific, Newington, NH, U.S.A), then the start time was recorded and the temperature monitored for 5 minutes. See Appendix F. The tests were repeated 6 times for each temperature and the average time plus 2 standard deviations was calculated as the CUT, to account for variability in the circulating water bath.

For thermal inactivation treatments, 18 TDT test cells were filled with 0.7 ± 0.1 g of inoculated ground black pepper inside a controlled relative humidity equilibrium chamber with closed air circulation. The 18-TDT test cells were placed in 6 aluminum racks. The 18-TDT test cells were placed inside the circulating water bath that was previously preheated to the desired treatment temperature (65 °C, 70 °C, 75 °C and 80 °C). One rack with 3 TDT test cells was pulled out at the predetermined CUT for each temperature. Total time treatment was determined empirically. The other 5 racks were removed from the water bath at equal intervals to achieve a total of 3-5 log CFU/g reductions. Once removed from the water bath, the TDT test cells were immersed into an ice-water mixture for 1 minute, for stopping thermal inactivation. Once the TDT test cells were opened and weighed, the microbial enumeration procedure described previously was used to determine microbial enumeration in each sample. Inoculated samples, without heat treatment, were also plated for determining initial microbial counts.

3.2.7.5. Calculation of D- and z-values

To obtain survivor curves, the averages of \log_{10} numbers of survivors of the three subsamples (log CFU/g) were plotted against the heat treatment time, in minutes.

Survival counts for time points between time zero (CUT) and the limit of detection (LOD) of 2.4 log CFU/g were used. The lines of best fit for survivor plots were determined by linear regression and the D-values were calculated from the negative inverse of the slope of the linear survival curves for each run using Microsoft Excel.

Plotting the log D-value obtained against the tested heating temperature depicted thermal death time curves. The negative inverse slope of the linear regression line provided by Microsoft Excel was used to calculate the z-value for each replicate. The average z-value of all replicates was reported. Differences among mean parameter values were tested by paired student's t-test, using Microsoft Excel.

3.2.8. Experimental design and Statistical Analysis

To compare the D-values obtained when using TDT sandwiches using medium heating rate (HR2) against TDT test cells, the D-values were statistically analyzed by Analysis of Variance, using SAS software. A split plot design with temperature as the split plot factor, and the heating method as a whole plot treatment in a randomized complete block design was utilized for analysis. Differences among mean parameter values between methods, microorganisms and temperatures were tested by a two tail paired student's t-test, using Microsoft Excel.

To evaluate the performance of TDT test cells against TDT sandwiches, the D-values at each temperature obtained with the medium heating rate (HR2) on TDT sandwiches and the D-values obtained using TDT test cells were statistically analyzed by Analysis of Variance, using SAS software for a randomized complete block design with each replicate as a block and temperature as treatment. The R-square, coefficient of variation (CV) and Root Square Mean Error (RMSE) of each heating method were used

to compare the performance of the heating method for each microorganism.

D-values for each bacteria and temperature combination were obtained from the mean of 3 biological replicates. To determine if heating rates used by TDT sandwiches influenced the D-value, the D-values were statistically analyzed by Analysis of Variance, using SAS version 9.4 (SAS Institute, Cary, NC, U.S.A). Split plot design with heating rate as the split plot factor, and temperature as a whole plot treatment in a randomized complete block was utilized for analysis.

3.3. Results and Discussion

3.3.1. Homogeneity and stability of inoculated ground black pepper

Homogeneity and stability tests demonstrated that inoculated black pepper samples were homogenous (variability of samples ≤ 0.3 log CFU/g). After inoculation the microbial populations of *Salmonella spp.* and *E. faecium* were 8.0 ± 0.13 log CFU/g and 7.9 ± 0.04 log CFU/g, respectively. A sharp drop in microbial load (0.43 to 1.07 log CFU/g) was observed after grinding on day 2 for both microorganisms. Previous studies have reported reductions of about 1 log CFU/log of *Salmonella* after the inoculation of black peppercorns due to the antimicrobial compounds present, change in environment and the dehydration of the cells at lower water activities (Keller, VanDoren, Grasso, & Halik, 2013; Sun, Anderson, & Keller, 2014).

The microbial load was stable after the third day after inoculation (<0.3 log CFU/g differences) from day 3 to day 15 (Figure 3.1). Bowman et al., (2015) reported that inoculation procedures affected the recovery of *Salmonella* in black peppercorns after 28 days. However, during the first 28 days of storage, a <0.2 log reduction of *Salmonella* was observed in black peppercorns when TSA-grown cells were used to

inoculate the sample (Bowman, Waterman, Williams, & Ponder, 2015). These results are comparable to the inoculation levels of *Salmonella* and *E. faecium* obtained in this study.

During the stability test, *E. faecium* had a significantly higher microbial population (7.42 ± 0.12 log CFU/g) compared to *Salmonella* (6.74 ± 0.07 log CFU/g) ($p < 0.05$) after the grinding process from day 2 to day 15 (Figure 3.1). Similarly, the microbial populations before heat treatments for inoculated samples of *Salmonella* spp. and *E. faecium* were 6.44 ± 0.24 log CFU/g and 7.51 ± 0.12 log CFU/g, respectively. These results confirmed that the inoculated samples maintained a stable population throughout the experiments with *E. faecium* being more resistant to the grinding and equilibration process when compared to *Salmonella* spp.

3.3.2. Comparison of thermal inactivation kinetics of *Salmonella* spp. and *E. faecium* with different heating methods

Survivor curves for *Salmonella* spp. and *E. faecium* depict log linear inactivation kinetics for both heating methods (Figure 3.2 and Figure 3.3). It was found that sandwiches always provided higher D-values than TDT test cells for both bacteria at all temperatures (65 °C, 70 °C, 75 °C, 80 °C). Those differences were statistically significant ($p < 0.05$) for *Salmonella* spp. at 65 °C and 75 °C but not significantly different for *Salmonella* spp. at 70 °C and *E. faecium* at all three temperatures evaluated (70 °C, 75 °C, 80 °C) (Table 3.1). Many factors could contribute to the difference in response of both microorganisms to the heating method applied.

The heating method applied influences how the sample is initially heated to reach the desired isothermal conditions for thermal inactivation treatments. Some studies have suggested that longer come-up times (CUT) influenced by the heating rate of the system

affects the determination of thermal resistance of bacteria (Chung et al., 2008; Chung et al., 2007). In this study, when comparing both heating methods, a similar heating rate and CUTs was established for both methods. Despite this, the results shown here indicated a higher population reduction during CUT for *Salmonella* spp. and *E. faecium* using TDT test cells compared to TDT sandwiches at temperatures above 70 °C (Table 3.2). When using TDT test cells the initial population was reduced to more than 0.6 log CFU/g for *Salmonella* spp. at 70 °C and 75 °C, and for *E. faecium* at 80 °C. In contrast, when using TDT sandwiches the log reduction achieved at all temperatures for both microorganisms were less than 0.16. These results suggest an advantage of TDT sandwiches versus TDT test cells when validating processes. Higher initial populations in food samples are required when there is too much population reduction during CUT in thermal inactivation determinations.

Studies have suggested that the method used to determine the thermal resistance of microorganisms may influence the accuracy of the results due to the effect of the heating rate of the apparatus (Kou et al., 2016). Previous studies have compared sample holding devices with traditional ones such as glass capillary tubes and aluminum tubes to determine the thermal resistance of vegetative cells and spores in liquid and semi solid foods, respectively (Chung et al., 2008; Chung et al., 2007; Loss & Hotchkiss, 2004). Loss and Hotchkiss (2004) reported the thermal resistance of *P. fluorescens* in buffer and milk, using a continuous microflow submerged microcoil (CSMC) apparatus to be comparable to results obtained using glass capillary tubes. The time required for the milk sample to reach 1 °C of the set temperature (71.3 °C) were 3 and 0.29 s for microcoils and 9 s for glass capillary tubes, which are relatively short times, indicating very high

heating rates (1,406 -14,544 °C/min). Loos and Hotchkiss (2004) reported comparable surviving ratios were obtained when similar heating rates were achieved, independently of the method. These results are congruent with those presented here for thermal inactivation kinetics of *E. faecium*, but not for *Salmonella* spp.

In another study, D-values for *Escherichia coli* K-12 in mashed potato (semisolid food) when treated at temperatures between 57 °C and 63 °C were 1.6 to 4.5 times lesser ($p < 0.05$) when using glass capillary tubes (3 mm) than with 2 different diameter aluminum tubes (13 mm and 20 mm) (Chung et al., 2007). Capillary tubes had faster heating rates (13,222 °C/min) compared to aluminum tubes (19.83 and 8.5 °C/min) (Chung et al., 2007). Isothermal heating conditions obtained during capillary tubes (short CUT) reflect the intrinsic thermal resistance of the bacterial culture while long CUT can affect the accuracy of results (Chung et al., 2007).

Another study compared the thermal resistance of *C. sporogenes* PA 3679 in mashed potato when treated also with capillary tubes, aluminum tubes and novel aluminum test cells (Chung et al., 2008). The thermal resistance of *C. sporogenes* PA 3679 tested using capillary tubes and the novel aluminum test cells were not statistically different; but were different to the results obtained using aluminum tubes (Chung et al., 2008). The CUT for aluminum test cells was approximately half the time of those obtained for aluminum tubes. The authors have attributed the difference of thermal resistance to a possible preconditioning of the bacteria during the long come-up times in the internal layers of the large tubes.

Additionally, differences in the thermal resistance of microorganisms obtained from different studies can also be attributed to the water activity inside the sample holder

during the thermal treatment. Thermal resistance of bacteria decreases with increased water activity (Syamaladevi et al., 2016). In a recent study, researchers tested a novel test cell (TAC) to study the influence of water activity on the thermal resistance of *Salmonella* in organic wheat flour (Tadapaneni et al., 2017). In this study, the water activity of the sample was controlled during the heat treatment using Lithium chloride (LiCl) as a water activity-controlling agent. They compared the $D_{80^{\circ}\text{C}}$ of *Salmonella* in wheat flour using TAC cells and the same type of TDT test cells used in this study. Their results for $D_{80^{\circ}\text{C}}$ using TAC (7.3 ± 0.7 min) were significantly higher compared to TDT test cells (4.3 ± 0.2 min). The authors reported that the difference of thermal resistance was due to the ability of TAC to control the water activity ($0.45 a_w$) at treatment temperatures, compared to TDT test cells which allowed the change of water activity from 0.45 at 20°C to 0.73 at 80°C . In this study, inoculated black pepper samples were placed in aluminum pouches and treated them using TDT sandwiches without any water activity-controlling agent. Notwithstanding, when using TDT sandwiches, the D-value for *Salmonella* was also higher compared to TDT test cells. However, the heating method did not influence the D-values for *E. faecium*. These results were not expected since for both heating methods the CUT was close to 2.5 minutes and based on published data the difference in thermo resistance is mainly influenced by CUT and changes in water activity caused by increased temperature. These results suggest that there are other possible factors contributing to the response of the microorganisms to the heating method. One factor could be that, if water vapors leak into the product, it should increase water activity and reduce the D-value in TDT test cells, while TDT sandwiches gave higher D-value, which is a more conservative estimate for food safety applications.

This study hypothesized that the heating method applied, TDT test cells (wet method) versus TDT sandwiches (dry method), should not have an effect on D-values if the heating rate was kept constant. However, results in this study indicated differences in thermal resistance of *Salmonella* spp. while no difference in thermal resistance of *E. faecium*. TDT test cells have been used in recent studies as a traditional method for thermal inactivation in low moisture foods (Hildebrandt et al., 2016; Syamaladevi et al., 2016; Tadapaneni et al., 2017). They were designed to have hermetic seal with the use of a rubber O-ring (Jin et al., 2008). However, in this study, visible leakage was found in some time point's cells during thermal inactivation treatments. Leakage during treatments might have attributed to the increase the water activity of the sample, therefore changing the environment conditions during treatments. Heating in moist conditions is known to be more efficient lethal treatment than dry heat (Goepfert, Iskander, & Amundson, 1970). Samples from the test cells that showed leakage were plated and included into the statistical analysis to account for the variability of results obtained using this method.

TDT sandwiches used heat sealed aluminum pouches in a dry heating system, which eliminated the risk of any water/oil entering the sample, as it could occur in water/oil bath systems. From the results' comparison of this study, it was not clear if leakage influenced considerably the determination of thermal resistance since only *Salmonella* spp. seems to be affected by heating method.

There have been few attempts to develop dry heating methods used for thermal death kinetics determinations. In one study, a heating block system was designed to kill larvae, which consisted of "aluminum heating blocks, a sandwiching plate, heating pads, an insulation box, and a data acquisition/control unit" (Ikediala, Tang, & Wig, 2000). The

elements of this system were similar to the novel dry heating system, TDT sandwiches, used in this study. Recently, another study developed a Heating Block System (HBS) to determine the thermal resistance of bacteria in several food products (Kou et al., 2016). In this particular study, the $D_{57^{\circ}\text{C}}$ for *E. coli* ATCC 25922 in mashed potato determined when using the HBS was in agreement with values obtained with aluminum tubes with different diameters in another study (Kou et al., 2016). Therefore, it appears that the response to the heating method applied for the determination of thermal resistance of bacteria in low-moisture foods varies among microorganisms. A deeper understanding of the response of *Salmonella* spp. (and other organisms) to the application of dry heat is required to compare the thermal resistance of different microorganisms in other low moisture foods and their response to dry heating methods versus traditional methods that use water or oil bath.

As expected for *Salmonella* spp. and *E. faecium*, the thermal resistance decreased with increased temperature when using both heating methods (Figure 3.2 and Figure 3.3). Additionally, the D-values obtained using TDT sandwiches were higher compared to those obtained using TDT test cells for both microorganisms at all temperatures (Figure 3.4). These results suggest that although the influence of temperature on microorganisms was similar, the use of TDT sandwiches provided more conservative D-values compared to those obtained with TDT test cells.

Surprisingly, when comparing z-values obtained using different heating methods it was found that when using TDT sandwiches, *Salmonella* spp. and *E. faecium* values were smaller than those obtained from TDT test cells by 25.47% and 1.82%, respectively but were not statistically different ($p > 0.05$) (Table 3.1). These results suggest that

despite the significant differences of D-values at specific temperatures, the z-value calculated was not affected by the method.

Variability of methods used for determination of thermal resistance of microorganisms can affect the comparison of results among studies. Therefore, methods used to determine thermal resistance should provide the least variability to reduce deviations that can be attributed to experimental artifacts. In this study, the performance of the novel dry heating method, TDT sandwiches, and TDT test cells in water bath were evaluated. The R-square from Analysis of Variance for both microorganisms with TDT sandwiches was 1.0, while with TDT test cells was 0.98 and 0.96 for *Salmonella* spp. and *E. faecium*, respectively (Table 3.3). This suggests that the data obtained from both heating methods fit well into the log-linear regression line.

The coefficients of variation (CV) of *Salmonella* spp. using TDT sandwiches and TDT test cells were similar, 7.21% and 9.71%, respectively. In contrast, the CV for *E. faecium* obtained from TDT test cells was much higher, 24.35%, than the values obtained for TDT sandwiches, 4.76%. For *E. faecium*, these results suggest that D-values obtained from TDT sandwiches have less variation from the estimated mean. Additionally, the Root Mean Square Errors (RSME) for D-values of *Salmonella* spp. were 1.67 and 1.05 min, for TDT sandwiches and TDT test cells, respectively. In contrast, the RMSE for D-values of *E. faecium* from TDT sandwiches was much lower (0.80 min) compared to the results obtained from TDT test cells (3.27 min) (Table 3.3). Smaller RMSE are preferred because they indicate a better fit and more accurate prediction of the response in the model. Overall, these results suggest that TDT sandwiches produced more reproducible results, when evaluating the thermal resistance of *E. faecium*.

3.3.3. Effect of heating rate on the thermal resistance of *Salmonella* and *E. faecium* NRRL-B2354 in ground black pepper using TDT sandwiches

In this study, the heating rate on TDT sandwiches were adjusted to three different heating rates (HR1: 600 °C/min – the fastest possible heating rate with the system, HR2: 17.10-24.05 °C/min – the heating rate that matches the come-up time of the TDT test cells immersed in hot water bath and HR3: 6.50-9.20 °C/min – a slower heating rate) to achieve different CUT (less than a minute, 2.5 minutes and 6 minutes). These different CUT would allow for further comparisons for both microorganisms at three different temperatures.

In this study, the heating rate did not have a significant effect ($p>0.05$) on the thermal resistance of *Salmonella* spp. and *E. faecium* in ground black pepper tested at all three temperatures (Table 3.4). These results indicated that the determination of thermal resistance of *Salmonella* and *E. faecium* was not affected by heating rates higher than 6.50 °C/min, when using TDT sandwiches. For all heating rates testes in TDT sandwiches, as temperature increased from 65 °C to 75 °C, for *Salmonella* spp., and from 70 °C to 80 °C for *E. faecium*, the thermal resistance decreased (Figure 3.6 and Figure 3.7).

It has been reported in several studies that the heating rate has an effect on the thermal inactivation kinetics of microorganisms (Kou et al., 2016; Stephens et al., 1994). *Listeria monocytogenes* cells exposed to slow heating rates (<5.0 °C/min) developed an increased thermo tolerance when further treated to temperatures of 50-64 °C (Stephens et al., 1994). In contrast, *Listeria monocytogenes* cells exposed to heating rates ≥ 5.0 °C/min did not showed increased thermo tolerance (Stephens et al., 1994). Similar to the results

obtained in this study for *Salmonella* spp. and *E. faecium*, In another study the thermo resistance at 57 °C of *E. coli* ATCC 25922 in mashed potato increased when slow heating rates were applied (0.1 and 0.5 °C/min) (Kou et al., 2016). In this study, similar D-values at 57 °C were obtained when the sample was treated with heating rates of 1.5 and 10 °C/min. Therefore, a very slow heating rates increases the D-value of microorganisms.

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

In this study, the D-values of *E. faecium* and *Salmonella* spp. were compared to determine whether *E. faecium* had an equal or higher resistance than *Salmonella* spp. in ground black pepper. Irrespective of the heating method used, *E. faecium* had significantly higher D-values ($p < 0.05$) than *Salmonella* spp. at 70 °C and 75 °C (Table 3.1). D-values of *E. faecium* obtained with TDT sandwiches were more than twice the D-value of *Salmonella* at the same temperature and method. These results indicated *E. faecium* as a suitable surrogate for use in validating thermal pasteurization processes for reducing and/or eliminating *Salmonella* spp. in ground black pepper at 70 °C and 75 °C.

E. faecium NRRL-2354 has been considered as a suitable surrogate of *Salmonella* in almonds, dairy products, juice and meats (Kopit, Kim, Siezen, Harris, & Marco, 2014). In recent years, there has been an increased interest in validating its use as surrogate for *Salmonella* in a variety of low moisture foods and technologies. Verma et al., (2018) demonstrated *E. faecium* NRRL-2354 has a higher thermal resistance than *Salmonella* in extrusion processes of oat flour. Also, Wei (2017) demonstrated *E. faecium* NRRL-2354 to be a suitable surrogate for *Salmonella enterica* in whole and ground black pepper treated with radiofrequency. Ceylan and Bautista (2015) determined the thermal inactivation kinetics of *E. faecium* NRRL-2354, *P. acidilactici* and *Salmonella* using

water bath. They confirmed *E. faecium* NRRL-2354 and *P. acidilactici* as suitable surrogates of *Salmonella* for validation studies of low moisture pet foods. They suggest the use of *P. acidilactici* since it has less thermal resistance than *E. faecium* but still higher enough compared to *Salmonella*.

Thermal treatments to pasteurize ground black pepper could deteriorate their physical and chemical properties. Temperatures applied should be enough to reduce/eliminate the pathogen of concern, such as *Salmonella* spp., without considerably affecting the quality of the final product. According to the results reported here, when validating a thermal process to inactivate *Salmonella* in ground black pepper (0.45 a_w), *E. faecium* NRRL-2354 can be used as a suitable surrogate. However, further studies should evaluate other possible surrogate with less thermal resistance than *E. faecium* NRRL-2354 but higher than *Salmonella* to avoid over processing.

3.4. Conclusions

Thermal inactivation kinetics of *Salmonella* spp. and *E. faecium* were determined using TDT sandwiches, which is a novel dry heating device. This method demonstrated operational benefits compared to TDT test cells such as lower log reductions (<0.16 log CFU/g) during CUT, when compared to TDT test cells (0.6 log CFU/g). Additionally, TDT sandwiches had larger sample capacity (1.5 g) compared to TDT test cell (~0.7 g) and no risk of leakage during treatment and adjustable heating rates. D-values of *Salmonella* spp. were influenced by the method used for 65 °C and 75°C but not for 70 °C. In contrast, the D-values of *E. faecium* were not at all three temperatures evaluated. Surprisingly, z-values of *Salmonella* spp. and *E. faecium* were similar for both heating methods applied. Additionally, it was proven that heating rates above 6.5 °C/min using

TDT sandwiches did not affect the thermal resistance of both microorganisms.

Independent of the method used, *E. faecium* was identified as a suitable surrogate for *Salmonella* spp. in black pepper.

The results demonstrated in this study increased the understanding of the factors that might influence the thermal inactivation kinetics of *Salmonella* in spices, such as black pepper. Future process validation studies for thermal treatments could benefit from these results, which could be utilized as guidelines for related industry applications.

3.5 References

1. American Trade Spice Trade Association. (1997). *Official analytical methods of the American Spice Trade Association*. Englewood Cliffs, N.J.: American Spice Trade Association.
2. Banerjee, M., & Sarkar, P. K. (2003). Microbiological quality of some retail spices in India. *Food Research International*, 36(5), 469-474.
3. Beuchat, L., & Scouten, A. (2002). Combined effects of water activity, temperature and chemical treatments on the survival of *Salmonella* and *Escherichia coli* O157: H7 on alfalfa seeds. *Journal of Applied Microbiology*, 92(3), 382-395.
4. Bowman, L. S., Waterman, K. M., Williams, R. C., & Ponder, M. A. (2015). Inoculation preparation affects survival of *Salmonella enterica* on whole black peppercorns and cumin seeds stored at low water activity. *Journal of Food Protection*, 78(7), 1259-1265.
5. Chung, H., Birla, S., & Tang, J. (2008). Performance evaluation of aluminum test cell designed for determining the heat resistance of bacterial spores in foods. *LWT-Food Science and Technology*, 41(8), 1351-1359.
6. Chung, H., Wang, S., & Tang, J. (2007). Influence of heat transfer with tube methods on measured thermal inactivation parameters for *Escherichia coli*. *Journal of Food Protection*, 70(4), 851-859.
7. Fudge, J., Dunn, M., Pike, O., Robison, R., & Steele, F. (2016). The isolation and identification of *Pantoea dispersa* strain JFS as a non-pathogenic surrogate for *Salmonella* Typhimurium phage type 42 in flour. *International Journal of Food Microbiology*, 219, 1-6.
8. Goepfert, J. M., Iskander, I. K., & Amundson, C. H. (1970). Relation of the heat resistance of *Salmonellae* to the water activity of the environment. *Applied Microbiology*, 19(3), 429-433.
9. Hildebrandt, I. M., Marks, B. P., Ryser, E. T., Villa-Rojas, R., Tang, J., Garces-Vega, F. J., & Buchholz, S. E. (2016). Effects of inoculation procedures on variability and repeatability of *Salmonella* thermal resistance in wheat flour. *Journal of Food Protection*, 79(11), 1833-1839.
10. Ikediala, J., Tang, J., & Wig, T. (2000). A heating block system for studying thermal death kinetics of insect pests. *Transactions of the ASAE*, 43(2), 351.
11. Jeong, S., Marks, B. P., Ryser, E. T., & Harte, J. B. (2012). The effect of X-ray irradiation on *Salmonella* inactivation and sensory quality of almonds and walnuts

- as a function of water activity. *International Journal of Food Microbiology*, 153(3), 365-371.
12. Jeong, S., & Kang, D. (2014). Influence of moisture content on inactivation of *Escherichia coli* O157: H7 and *Salmonella enterica* serovar Typhimurium in powdered red and black pepper spices by radio-frequency heating. *International Journal of Food Microbiology*, 176, 15-22.
 13. Jin, T., Zhang, H., Boyd, G., & Tang, J. (2008). Thermal resistance of *Salmonella* Enteritidis and *Escherichia coli* K12 in liquid egg determined by thermal-death-time disks. *Journal of Food Engineering*, 84(4), 608-614.
 14. Keller, S. E., VanDoren, J. M., Grasso, E. M., & Halik, L. A. (2013). Growth and survival of *Salmonella* in ground black pepper (*Piper nigrum*). *Food microbiology*, 34(1), 182-188.
 15. Kim, S., Sagong, H., Choi, S. H., Ryu, S., & Kang, D. (2012). Radio-frequency heating to inactivate *Salmonella* Typhimurium and *Escherichia coli* O157: H7 on black and red pepper spice. *International Journal of Food Microbiology*, 153(1), 171-175.
 16. Kopit, L. M., Kim, E. B., Siezen, R. J., Harris, L. J., & Marco, M. L. (2014). Safety of the surrogate microorganism *Enterococcus faecium* NRRL B-2354 for use in thermal process validation. *Applied and Environmental Microbiology*, 80(6), 1899-1909.
 17. Kou, X., Li, R., Hou, L., Huang, Z., Bo, L., & Wang, S. (2016). Performance of a heating block system designed for studying the heat resistance of bacteria in foods. *Scientific Reports*, 6, 30758.
 18. Loss, C., & Hotchkiss, J. (2004). Determination of thermal inactivation kinetics of microorganisms with a continuous microflow apparatus. *Journal of Food Protection*, 67(11), 2560-2564.
 19. McKee, L. (1995). Microbial contamination of spices and herbs: A review. *LWT-Food Science and Technology*, 28(1), 1-11.
 20. Pafumi, J. (1986). Assessment of the microbiological quality of spices and herbs. *Journal of Food Protection*, 49(12), 958-963.
 21. Rachon, G., Peñaloza, W., & Gibbs, P. A. (2016). Inactivation of *Salmonella*, *Listeria monocytogenes* and *Enterococcus faecium* NRRL B-2354 in a selection of low moisture foods. *International Journal of Food Microbiology*, 231, 16-25.
 22. Shah, M. K., Asa, G., Sherwood, J., Graber, K., & Bergholz, T. M. (2017). Efficacy of vacuum steam pasteurization for inactivation of *Salmonella* PT 30,

Escherichia coli O157: H7 and *Enterococcus faecium* on low moisture foods. *International journal of food microbiology*, 244, 111-118.

23. Smith, D. F., & Marks, B. P. (2015). Effect of rapid product desiccation or hydration on thermal resistance of *Salmonella enterica* serovar Enteritidis PT 30 in wheat flour. *Journal of Food Protection*, 78(2), 281-286.
24. Song, W., Sung, H., Kim, S., Kim, K., Ryu, S., & Kang, D. (2014). Inactivation of *Escherichia coli* O157:H7 and *Salmonella* Typhimurium in black pepper and red pepper by gamma irradiation. *International Journal of Food Microbiology*, 172, 125-129.
25. Stephens, P., Cole, M., & Jones, M. (1994). Effect of heating rate on the thermal inactivation of *Listeria monocytogenes*. *Journal of Applied Microbiology*, 77(6), 702-708.
26. Sun, S., Anderson, N. M., & Keller, S. (2014). Atmospheric pressure plasma treatment of black peppercorns inoculated with salmonella and held under controlled storage. *Journal of Food Science*, 79(12).
27. Syamaladevi, R. M., Tadapaneni, R. K., Xu, J., Villa-Rojas, R., Tang, J., Carter, B., . . . Marks, B. (2016). Water activity change at elevated temperatures and thermal resistance of salmonella in all purpose wheat flour and peanut butter. *Food Research International*, 81, 163-170.
28. Syamaladevi, R. M., Tang, J., Villa-Rojas, R., Sablani, S., Carter, B., & Campbell, G. (2016). Influence of water activity on thermal resistance of microorganisms in Low-Moisture foods: A review. *Comprehensive Reviews in Food Science and Food Safety*, 15(2), 353-370.
29. Tadapaneni, R. K., Syamaladevi, R. M., Villa-Rojas, R., & Tang, J. (2017). Design of a novel test cell to study the influence of water activity on the thermal resistance of salmonella in low-moisture foods. *Journal of Food Engineering*, 208, 48-56.
30. Verma, T., Wei, X., Lau, S. K., Bianchini, A., Eskridge, K. M., & Subbiah, J. (2018). Evaluation of *Enterococcus faecium* NRRL B-2354 as a Surrogate for *Salmonella* During Extrusion of Low-Moisture Food. *Journal of Food Science*, 83(4), 1063-1072.
31. Vij, V., Ailes, E., Wolyniak, C., Angulo, F. J., & Klontz, K. C. (2006). Recalls of spices due to bacterial contamination monitored by the U.S Food and Drug Administration: The predominance of *Salmonellae*. *Journal of Food Protection®*, 69(1), 233-237.

32. Wei, X. (2017). *Radiofrequency processing for inactivation of Salmonella spp. and Enterococcus faecium NRRL B-2354 in whole black peppercorn and ground black pepper* (Master's thesis). Available from: Digital Commons@ University of Nebraska-Lincoln. <https://digitalcommons.unl.edu/foodscidiss/88/> Accessed May, 2018
33. Zweifel, C., & Stephan, R. (2012). Spices and herbs as source of *Salmonella*-related foodborne diseases. *Food Research International*, 45(2), 765-769.

Table 3.1

D-values in minutes of *Salmonella* spp. and *E. faecium* NRRL B-2354 using different heating methods

Temperature (°C)	Microorganism	D-value \pm SE (min)		z-value (°C)	
		sandwiches	test cells	sandwiches	test cells
65	<i>Salmonella</i>	44.47 \pm 1.18 ^a	17.49 \pm 1.18 ^b	12.64 ^a	15.86 ^a
70	<i>Salmonella</i>	17.68 \pm 1.18 ^{a,x}	10.87 \pm 1.18 ^{a,x}		
70	<i>E. faecium</i>	34.12 \pm 1.86 ^{a,y}	27.54 \pm 1.86 ^{a,x}		
75	<i>Salmonella</i>	7.21 \pm 1.18 ^{a,x}	4.09 \pm 1.18 ^{b,x}		
75	<i>E. faecium</i>	12.01 \pm 1.86 ^{a,y}	9.23 \pm 1.86 ^{a,x}	10.97 ^a	11.17 ^a
80	<i>E. faecium</i>	4.18 \pm 1.86 ^a	3.50 \pm 1.86 ^a		

When comparing between methods (rows), value followed by different letters (ab) are significantly different ($p < 0.05$).

When comparing between microorganisms (columns), values with different letters (xy) are significantly different ($p < 0.05$).

Table 3.2

Come-up times (CUT) log reductions for *Salmonella* spp. and *E. faecium* NRRL B-2354 populations when treated with different heating methods and temperatures

Microorganism	Heating method	CUT (s)	CUT reductions (log CFU/g)			
			65°C	70°C	75°C	80°C
<i>Salmonella</i>	sandwiches	155	-0.19	0.01	0.16	
	test cells	128-175	0.0	0.61	0.94	
<i>E. faecium</i>	sandwiches	154-155		0.05	-0.02	0.10
	test cells	128-175		0.18	0.12	0.53

Table 3.3

Repeatability Analysis for different heating methods

Microorganisms	Heating method	R-Square	Coefficient Variation (CV), %	Root MSE (min)
<i>E. faecium</i>	sandwiches	0.998	4.760	0.798
	test cells	0.959	24.354	3.269
<i>Salmonella</i> spp.	sandwiches	0.995	7.213	1.667
	test cells	0.984	9.713	1.051

Table 3.4

D-values in minutes of *Salmonella* spp. and *E. faecium* NRRL B-2354 when using TDT sandwiches with different heating rates

Temperature (°C)	Microorganism	HR1 (fastest)	HR2 (medium)	HR3 (slowest)
65	<i>Salmonella</i>	42.21±2.18 ^a	44.47±2.18 ^a	43.38±2.18 ^a
70	<i>Salmonella</i>	18.07 ± 2.18 ^{a,x}	17.68 ± 2.18 ^{a,x}	19.87±2.18 ^{a,x}
70	<i>E. faecium</i>	31.98 ± 0.85 ^{a,y}	34.12± 0.85 ^{a,y}	33.49±0.85 ^{a,y}
75	<i>Salmonella</i>	6.97 ± 2.18 ^{a,x}	7.21 ± 2.18 ^{a,x}	7.82±2.18 ^{a,x}
75	<i>E. faecium</i>	12.01 ± 0.85 ^{a,y}	12.01 ± 0.85 ^{a,y}	12.29±0.85 ^{a,y}
80	<i>E. faecium</i>	4.31 ± 0.85 ^a	4.18 ± 0.85 ^a	4.60±0.85 ^a

When comparing between heating rates (rows) values followed by different letters (ab) are significantly different (p<0.05).

When comparing between microorganisms (columns) values with different letters (xy) are significantly different (p<0.05) at the same temperature.

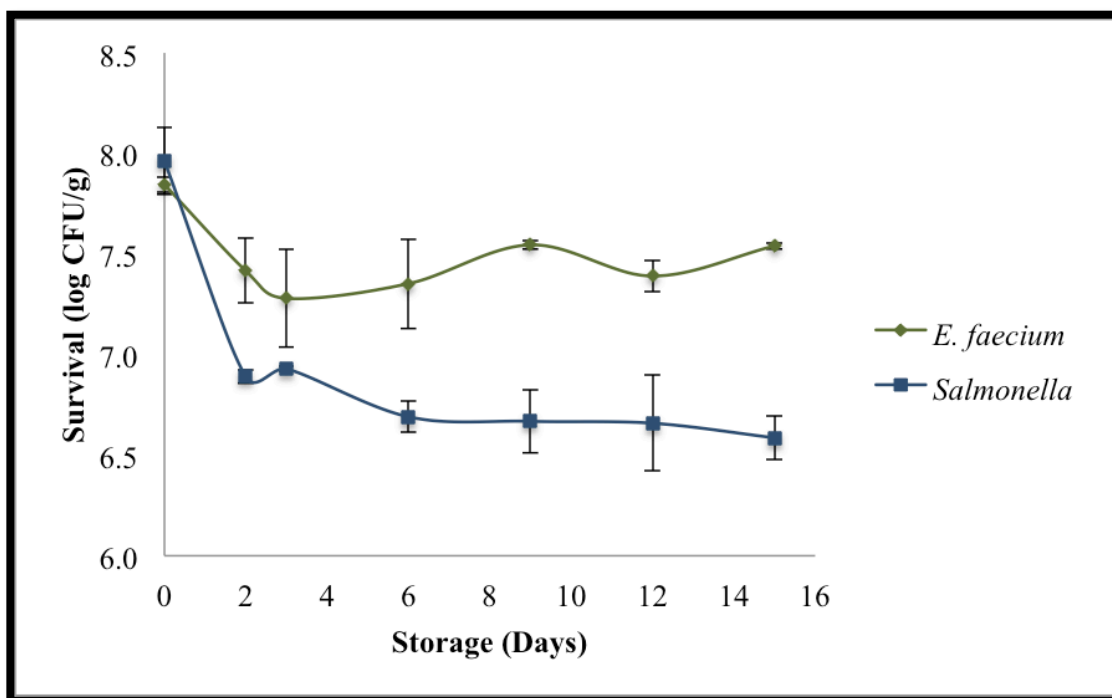


Figure 3.1 Homogeneity and stability of *E. faecium* and *Salmonella* spp. in ground black pepper equilibrated to 0.45 a_w , measured at 25 °C. Error bars indicate standard deviations.

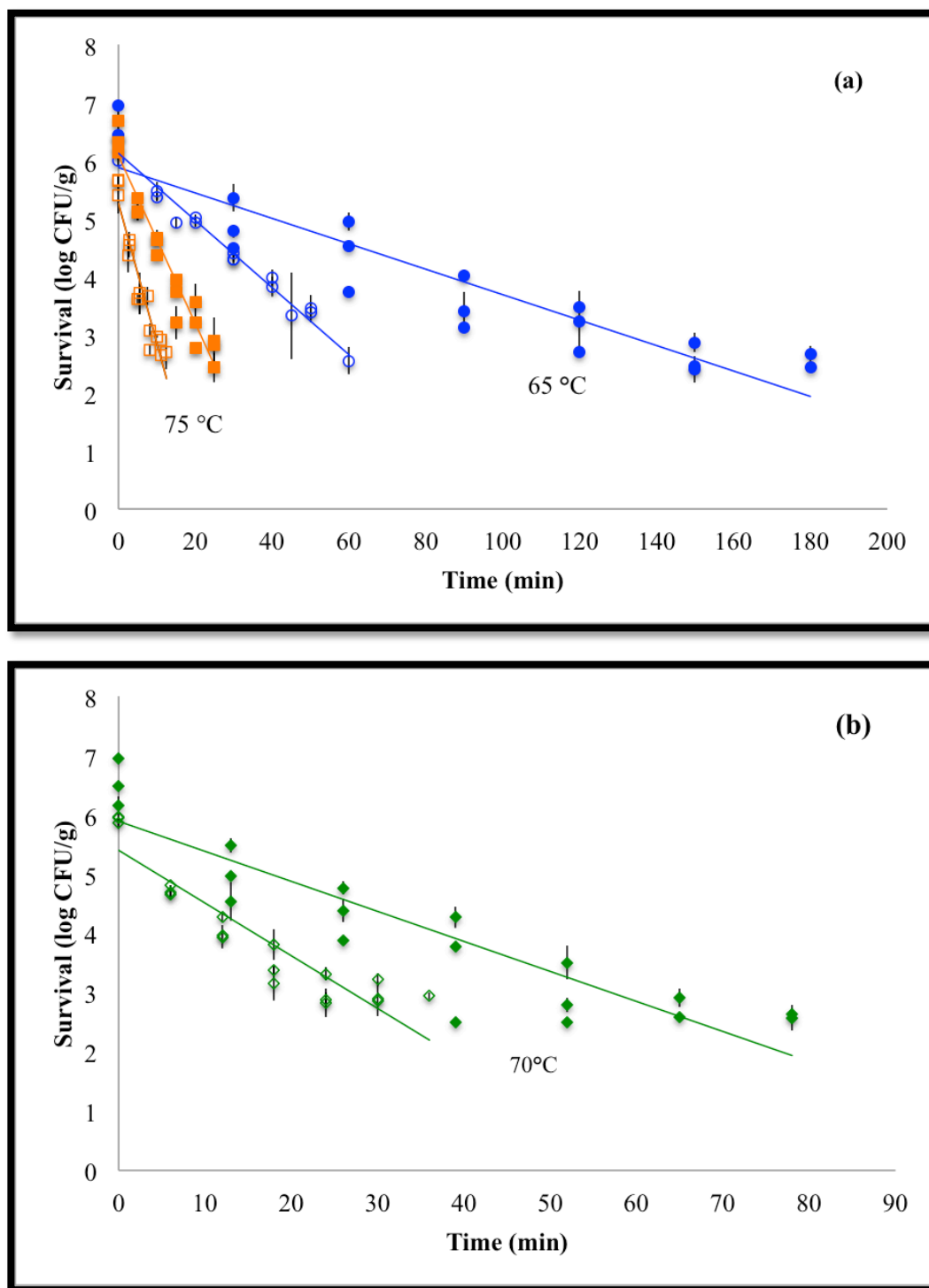


Figure 3.2 Survivor curves of *Salmonella* spp. in ground black pepper ($0.45 a_w \pm 0.025$ measured at 25 °C) when treated with TDT sandwiches (filled color shape) and TDT test cells (unfilled color shapes) at different temperatures (• 65 °C, ♦ 70 °C, and ■ 75 °C). Data points were from the average of subsamples ($n=2$ for TDT sandwiches and $n=3$ for TDT test cells) for all three biological replicates plotted. Error bars indicate standard deviations.

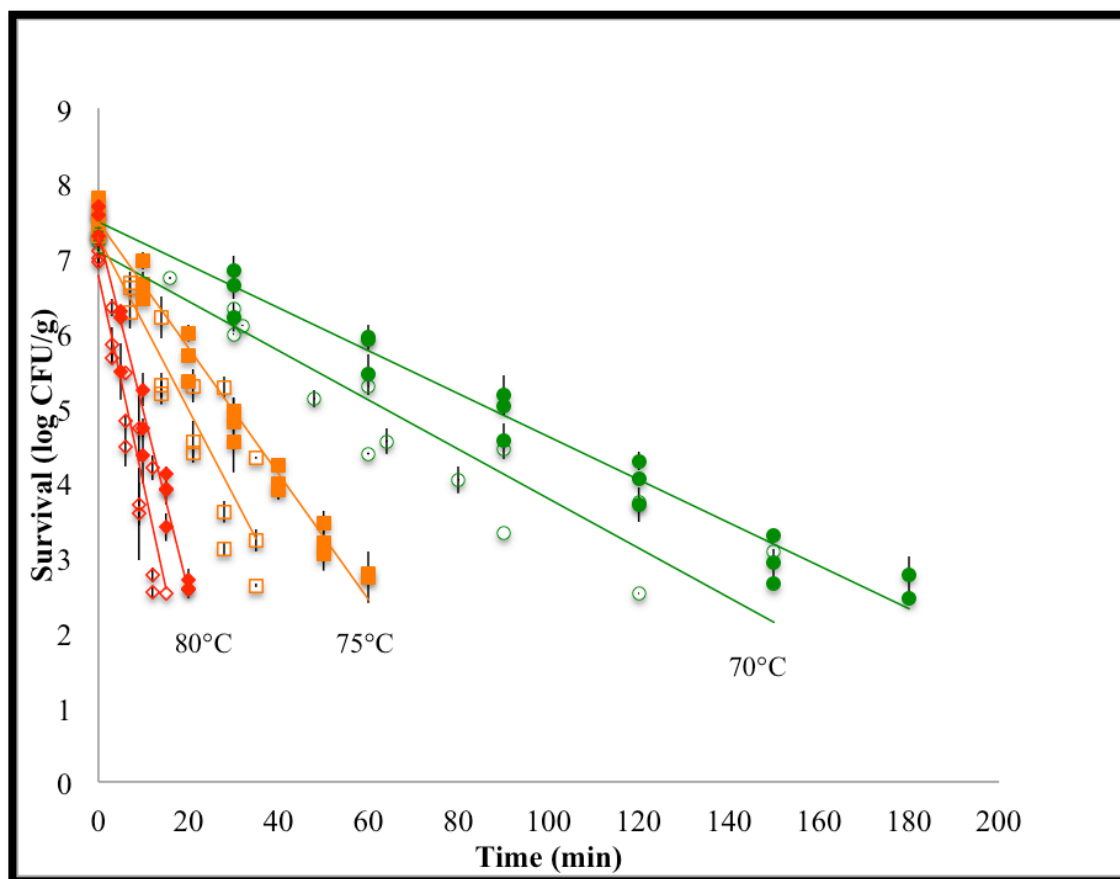


Figure 3.3 Survivor curves of *E. faecium* NRRL B-2354 in ground black pepper ($0.45 a_w \pm 0.025$ measured at 25°C) when treated with TDT sandwiches (filled color shape) and TDT test cells (unfilled color shapes) at different temperatures ($\bullet 70^\circ\text{C}$, $\blacksquare 75^\circ\text{C}$, and $\blacklozenge 80^\circ\text{C}$). Data points were from the average of subsamples ($n=2$ for TDT sandwiches and $n=3$ for TDT test cells) for all three biological replicates plotted. Error bars indicate standard deviation.

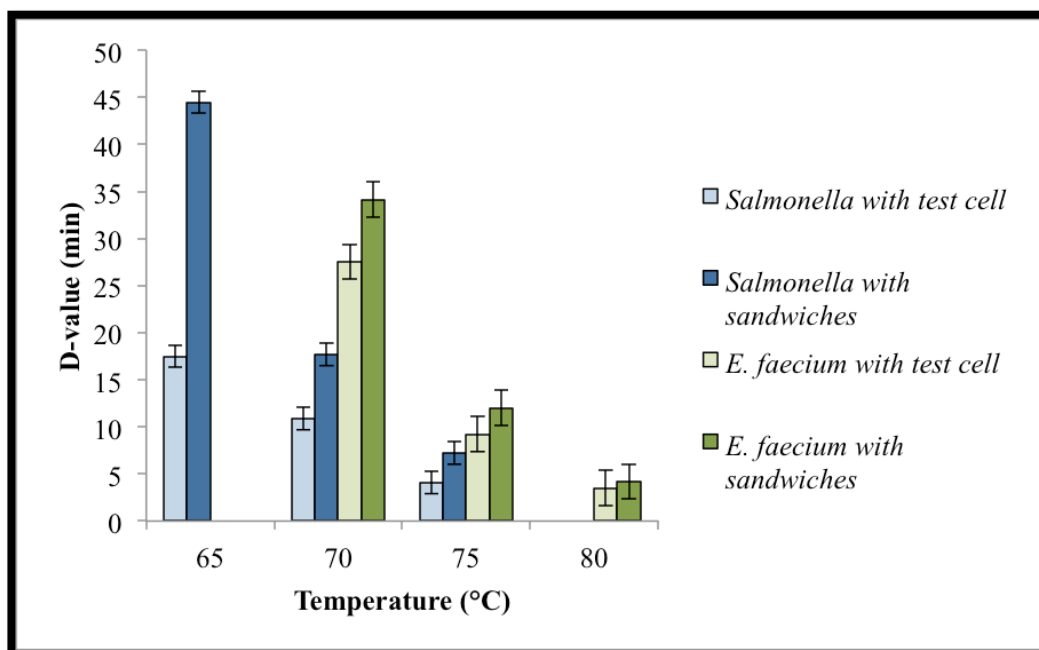


Figure 3.4 D-value comparisons for *Salmonella* spp. and *E. faecium* NRRL B-2354 when treated with different heating methods. Error bars indicate standard error.

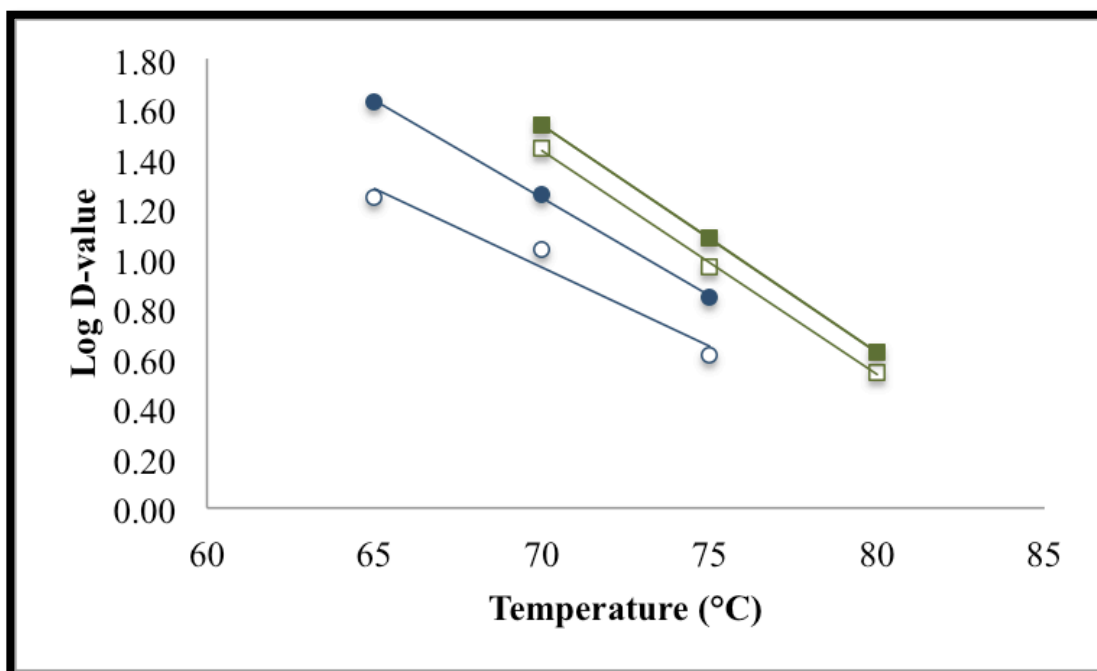


Figure 3.5 Linear regression for z-value (°C) of ■ *Salmonella* spp. and • *E. faecium* NRRL B-2354 when determined by TDT sandwiches (filled color shapes) and TDT test cells (unfilled color shapes).

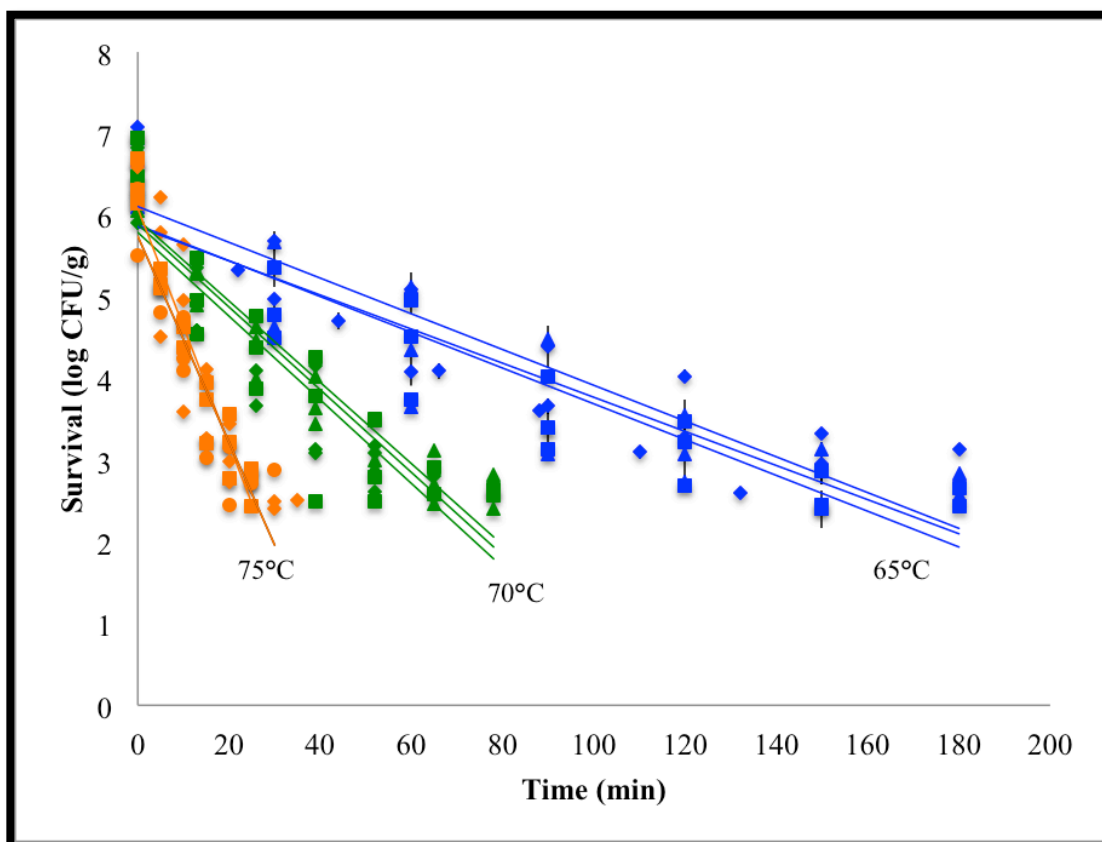


Figure 3.6 Survivor curves of *Salmonella* spp. in ground black pepper ($0.45 a_w \pm 0.025$ measured at 25 °C) when treated with TDT sandwiches at different heating rates (♦ HR1: 600 °C/min, ■ HR2: 17.10-24.05 °C/min, • HR3: 6.50-9.20 °C/min) and temperature (65 °C, 70 °C, 75 °C). Data points were from the average of subsamples ($n=2$) for all three biological replicates plotted. Error bars indicate standard deviation.

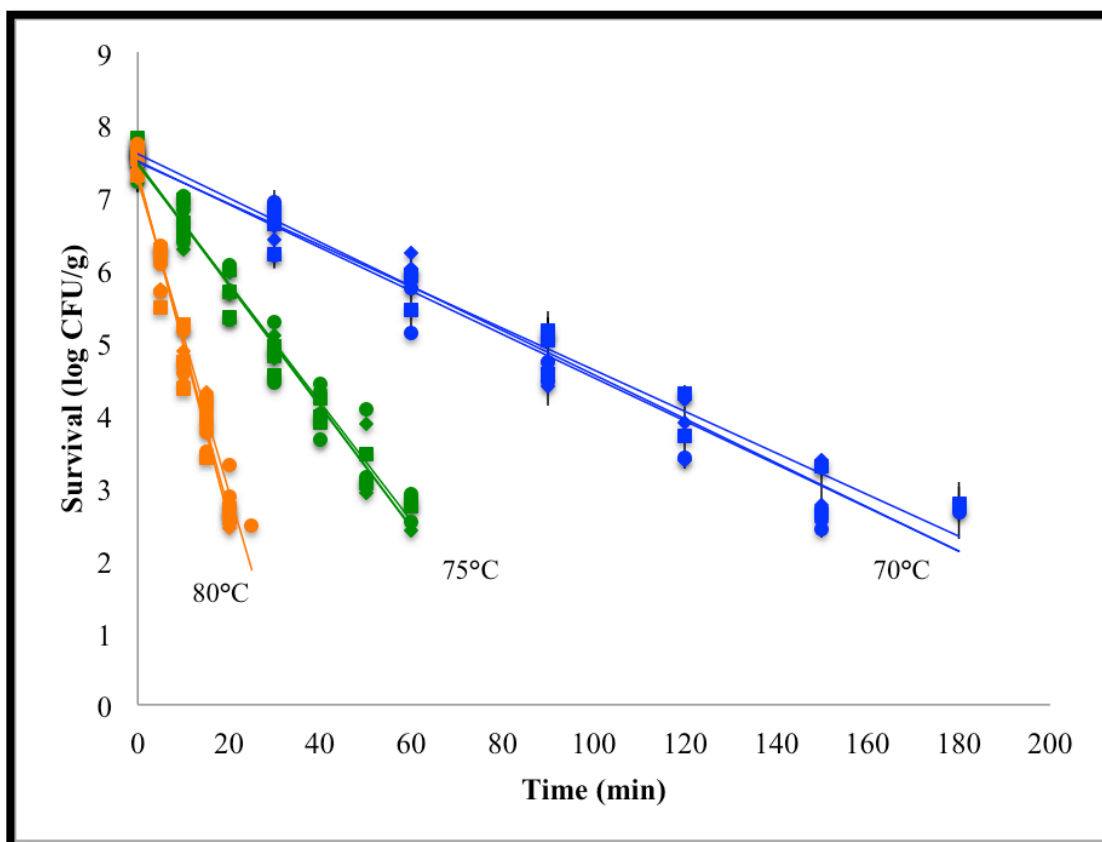


Figure 3.7 Survivor curves of *E. faecium* NRRL B-2354 in ground black pepper (0.45 ± 0.025 measured at 25 °C) when treated with TDT sandwiches at different heating rates (\blacklozenge HR1: 600 °C/min, \blacksquare HR2: 17.10-24.05 °C/min, \bullet HR3: 6.50-9.20 °C/min) and temperature (65 °C, 70 °C, 75 °C). Data points were from the average of subsamples ($n=2$) for all three biological replicates plotted. Error bars indicate standard deviations.

Chapter IV: Effect of water activity on thermal inactivation kinetics of *Salmonella* spp. and *E. faecium* NRRL-B2354 in ground black pepper

Abstract

Food safety of low-water activity (a_w) foods has become of great interest due to the increased foodborne illness outbreaks related to these products. *Salmonella enterica* has been identified as the causing agent of outbreaks of contaminated spices. With the Food Safety Modernization Act (FSMA) regulations in place, low- a_w processing facilities are required to validate their decontamination technologies. The objective of this study was to evaluate the suitability of *E. faecium* NRRL-B2354 as a potential surrogate of *Salmonella* spp. in ground black pepper at different water activities (0.25, 0.45 and 0.65 a_w) using Thermal-Death-Time (TDT) sandwiches. This dry-heating method provided short come-up-times (CUT) (less than a minute), producing ideal isothermal conditions. Black pepper samples were equilibrated to three different a_w and inoculated with either a 5-strain *Salmonella* spp. cocktail or *E. faecium*. Inoculated samples were grounded and heat-treated, at three different temperatures. D- and z-values were determined at different water activities. The thermal resistances of both microorganisms were affected by a_w and temperature. D-values decreased with increased a_w and treatment temperature. Results indicated that *E. faecium* could be used as a suitable surrogate for *Salmonella* spp. in thermal decontamination of ground black pepper at a range of 0.25-0.65 a_w . The response surface equations for both microorganisms were developed with the log D-values obtained experimentally. These equations could be used to estimate the log D-value required for decontamination of ground black pepper with other a_w and treatment temperatures.

4.1 Introduction

Low-moisture foods are those with a_w less than 0.70 (Blessington et al., 2013; Farakos et al., 2013). The expressions low-moisture foods and low- a_w foods are commonly found in published literature to refer to products with low- a_w . Food safety of low- a_w foods has become a major topic of study in recent years, given the increased amount of outbreaks related to this group of foods. In a previous review of foodborne illness outbreaks related to microbial contamination in spices during 1970 to 2010, *Salmonella enterica* subspecies enterica was revealed as the causing agent in 71% (10/14) of outbreaks, representing 87% of reported illnesses (Van Doren et al., 2013).

Spices are commonly added to products with no further cooking. Therefore, when applied decontamination technologies have not been efficient, spices can become a major food safety threat. Additionally, spices have long shelf life and can be available to consumers for long periods while causing illness.

In recent years, increased efforts have been made to expand the knowledge on food safety of spices in the U.S market. The U.S Food and Drug Administration (FDA) monitored 21 recalls involving 12 spices contaminated with bacterial pathogens from 1970 to 2003 (Vij et al., 2006). In that study, all but one recall was related to *Salmonella*. In a more recent study, 11 spices examined from shipments of imported spices offered to enter the United States between October 2011 and September 2015, showed a *Salmonella* prevalence of 1.7 to 18% (Zhang et al., 2017). Specifically, black pepper offered to enter the U.S had a prevalence of *Salmonella* of 6.7%. Surprisingly, from November 2013 to March 2015, the 11 samples of spices from retail establishments were examined and the prevalence of *Salmonella* was significantly lower ($p < 0.05$) to the results obtained from

imported spices (Zhang et al., 2017). Inefficient pasteurization techniques for some spices such as black pepper, basil, coriander, and red pepper found in retail establishments might explain the presence of *Salmonella* in these samples (Zhang et al., 2017).

With the Food Safety Modernization Act (FSMA) regulations in place, low- a_w foods processing facilities are required to validate their processes to ensure food safety of their products. For plants that apply thermal process, information related to the thermal inactivation kinetics of pathogen of high risk, such as *Salmonella* spp., and a surrogate, are important while designing process validation of pasteurizations conditions. In a previous study, *E. faecium* has been proven to be a suitable surrogate for *Salmonella* spp. in low- a_w foods including a carbohydrate-protein meal (Bianchini et al., 2014), almonds (Jeong et al., 2011), chicken meat powder (Rachon et al., 2016) and pet food (Ceylan & Bautista, 2015).

It has been previously reported that the thermal resistance of *Salmonella* varies with a_w since a decrease in a_w provides a protective effect against thermal inactivation (Farakos et al., 2013; S. Wang et al., 2013). Adequate pasteurization processes development and validation require a deeper understanding of the different factors that might affect the thermal resistance of the pathogen. Product composition, water activity, strain type and growth conditions may influence the determination of the thermal resistance of microorganisms.

Different studies have developed methods that include aluminum test tubes (Chung et al., 2007), Thermal-Death-Time (TDT) disks (Chung et al., 2008; Jin et al., 2008) and Thermal Water Activity Cell (TAC) (Tadapaneni et al., 2017) to determine the thermal inactivation kinetics of microorganisms in foods. In all of these studies, the heat

treatments were conducted either in water or oil bath. Overall, in these studies researchers aimed to clarify the effect of different factors including sample holder type and food-sample a_w on the thermal resistance of microorganisms. However, there are difficulties in comparing the available data due to the variability of sample preparation and inoculation methods, strains, food products, a_w of food samples and inactivation technology studied.

Available data for thermal inactivation kinetics of *Salmonella* and *E. faecium* in low- a_w foods under same conditions are limited to almonds (Jeong et al., 2011), wheat flour (Villa-Rojas et al., 2017), whole flaxseed, quinoa, sunflower kernels (Shah et al., 2017). Some studies have evaluated the inactivation of *Salmonella* spp. in black pepper using different pasteurization technologies including radiofrequency (Jeong & Kang, 2014; Kim et al., 2012), gamma irradiation (Song et al., 2014), cold atmospheric pressure plasma applications (Hertwig, Reineke, Ehlbeck, Knorr, & Schlüter, 2015), vacuum steam pasteurization (Shah et al., 2017) and ozone (Emer, Akbas, & Ozdemir, 2008). However, there are limited data about the inactivation of *Salmonella* and *E. faecium* in black pepper influenced by water activity during thermal processes.

Therefore, the objective of this study is to investigate the effect of water activity on thermal inactivation kinetics of *Salmonella* spp. and *E. faecium* using a dry-heating method (TDT sandwiches).

TDT sandwiches, a novel dry heating method for the determination of thermal inactivation kinetics, were developed at the University of Nebraska-Lincoln. This system consisted of hermetically heat-sealed aluminum pouches (1.1 mm thickness) containing the inoculated samples that were sandwiched between two heating elements surrounded by fiber block insulators. See Appendix D. The system was designed to achieve

isothermal conditions inside the pouches within less than a minute without the use of water or oil bath.

Thermal inactivation data was fitted to first order kinetics to describe the thermal inactivation curves. Additionally, response surface equations for log D-value determination of *E. faecium* and *Salmonella* spp. in ground black pepper were developed based on the results from this study.

4.2 Materials and methods

4.2.1 Low- a_w food: black peppercorns

Black peppercorns from different production lots were obtained from McCormick & Co. Inc. (Hunt Valley, MD, U.S.A). The water activity of all lots was measured using an AquaLab Dew Point Water activity meter 4TE (METER Group, Inc., Pullman, WA, U.S.A). For easy storage and handling, the black peppercorns were transferred to approximately 1-kg double sealed polypropylene bags and stored at -20 °C until use. The background flora was evaluated as described in Chapter 3. All lots of black pepper tested had total aerobic counts below the detection limit (<10 CFU/g).

4.2.2 Bacterial strains

The selections of strains used in this study were based on thermal resistance and frequency of occurrence in low-moisture foods and association with recalls and outbreaks. Therefore, 5 different strains of *Salmonella enterica* were used to prepare a cocktail for inoculation: Agona 447967 (puffed rice cereal recall), Montevideo 48827 (black pepper outbreak), Mbandaka 698538 (sesame paste) (FDA, ORA Regional Lab, Jefferson, AK, U.S.A), Reading (cumin) (ATTC BAA-1045) and Tennessee K4643 (peanut butter outbreak) (Dr. Larry Beuchat, University of Georgia, Griffin, GA, U.S.A).

Additionally, *Enterococcus faecium* NRRL B-2354 (Agriculture Research Service, USDA) was selected as a surrogate microorganism. Upon arrival, these bacterial strains were stored at -80 °C as frozen stocks cultures in 80% glycerol.

4.2.3 Inoculum preparation

Each bacterial strain was re-activated from cryopreservation. Each vial was thawed at 37 °C and transferred to 10 ml of tryptic soy broth with 0.6% yeast extract (TSBYE; Becton, Dickinson and Company, Sparks, MD, U.S.A) and incubated at 37 °C for 24 ± 2 h. Isolation was achieved by using 10- μ l loops to streak colonies. Streaked plates were incubated at 37 °C for 24 ± 2 h. When the growth of isolated colonies was achieved, the plates were wrapped with parafilm and stored at 4 °C. These plates were named “permanent plates” and were restricted to five monthly transfers to prepare future working plates. Working plates were prepared by picking one isolated colony from the permanent plates using 10- μ l loop and streaking onto tryptic soy agar plates with 0.6% yeast extract (TSAYE; Becton, Dickinson and Company, Sparks, MD, U.S.A). Plates were incubated at 37 °C for 24 ± 2 h. When the growth of isolated colonies was definite, the working plates were wrapped with parafilm and kept at 4 °C for a maximum of one month.

In this study, lawn-based cells suspensions were preferred for the inoculation because they produce more stable populations before thermal inactivation of low-moisture foods (Hildebrandt et al., 2016). A 10- μ l loop was used to aseptically harvest an isolated colony from a working plate and transfer it to 10 mL of tryptic soy broth with 0.6% yeast extract (TSBYE; Becton, Dickinson and Company, Sparks, Md., U.S.A). Tubes were incubated at 37 °C for 24 ± 2 h. After incubation, 100 μ l of the overnight

culture was spread on the surface of tryptic soy agar with 0.6% yeast extract (TSAYE; Becton, Dickinson and Company, Sparks, MD, U.S.A) and incubated at 37 °C for 24 ± 2 h. To each plate, after incubation, 3 mL of 0.1% BPW was added to gently displace the cells into suspension using a L-shaped spreader. Sterile pipettes were used to draw 2 ml of cell suspension from each plate and transfer it into a conical centrifugal sterile tube. This procedure was repeated for each *Salmonella* strain and *E. faecium* NRRL-B2354. When preparing the *Salmonella* spp. cocktail, equal amounts of cell suspension from each strain were added to a single conical centrifugal sterile tube and homogenized using a vortex. The inoculums were serially diluted (1:10) and plated on TSAYE that was additionally supplemented with differential agents. For *Salmonella* spp., TSAYE was supplemented with 0.05% ammonium iron (III) citrate (Sigma Aldrich, St. Louis, MO, U.S.A) and 0.03 % sodium thiosulfate (Fisher Chemical, Fair Lawn, NJ, U.S.A) (mTSAYE). For *E. faecium*, TSAYE was supplemented with 0.05% ammonium iron (III) citrate and 0.025% of 97% esculin hydrate (Acros Organics, NJ, U.S.A) (eTSAYE). The plates were then incubated at 37 °C for 24 ± 2 h. See Appendix A. The inoculum concentrations were 10-11 log CFU/g for *Salmonella* spp. and 9-10 for *E. faecium*.

4.2.4 Sample preparation: inoculation, equilibration and grinding

Twenty-four hours before inoculation, black peppercorns samples were removed from the freezer and poured onto aluminum trays inside a controlled relative humidity chamber (as described in Chapter 3). The chamber was set up to an appropriate relative humidity to equilibrate the black peppercorns to one of each of the desired water activity (0.25, 0.45 and 0.65 a_w) to be tested for thermal inactivation. After equilibration, the black peppercorns were spray inoculated (2 ml of inoculum per 100 g of black

peppercorns) and hand-massaged in a polypropylene bag for 10 minutes inside a biosafety cabinet. The inoculated sample had an initial concentration of 10^7 CFU/g. The inoculated samples were placed onto aluminum trays inside the relative humidity chamber for approximately 48 hours to equilibrate the sample back to the desired water activity and allow the microorganisms to adapt to these conditions. Once equilibrated, the black peppercorns were ground for 30 s using a 3-cup Power Grinder, 120 V, 750 W (Waring Commercial, Torrington, CT, U.S.A) placed in a biosafety cabinet. The ground-inoculated samples were mixed as described in Chapter 3. See Appendices B and C. The final mixture of finely ground black pepper samples were poured onto aluminum trays and placed back inside the equilibrium chamber for 24 h, for further conditioning. After this, the inoculated samples were packed in aluminum pouches and stored at room temperature or used for thermal inactivation treatments immediately.

4.2.5 Homogeneity and stability test

The homogeneity and stability of black pepper inoculated with *Salmonella* spp. and *E. faecium* NRRL B-2354 and equilibrated to each of the three different water activities (0.25, 0.45 and 0.65) were evaluated as described in Chapter 3 (section 3.2.6).

4.2.6 Thermal inactivation using TDT sandwiches

TDT sandwiches were set up to the fastest heating rate (600 °C/min) for all thermal inactivation treatments for all 3 water activities. Subsamples of 1.5 ± 0.2 g of inoculated ground black pepper equilibrated to each of the desired water activities (0.25, 0.45 and 0.65 a_w , measured at 25 °C) were added to water-impermeable aluminized plastic pouches (PAKVF4C, IMPAK Corp., Los Angeles, Calif.). A heat sealer (IPKHS-606T, IMPAK Corp., Los Angeles, Calif., U.S.A) was used to heat-seal the pouches (1.3

mm of thickness). Before the thermal inactivation began, the pouches were placed in between 2 heating elements and further sandwiched by 2 insulation blocks. For come-up times (CUT) determinations (time required to reach within 0.5 °C of set-point temperature), non-inoculated ground samples were equilibrated and ground as described in section 4.2.4. The CUT was determined following the procedure described in Chapter 3 (Section 3.2.7.2). Once the thermal inactivation began, two aluminum pouches were removed at the predetermined CUT. Following, 2 aluminum pouches were removed from the corresponding TDT sandwiches for each time point interval to achieve a total of 3-5 log reductions. The pouches were then submerged in an ice-water bath for 1 minute to stop the thermal inactivation.

4.2.7 Microbial enumeration and D- and z-value determination

For control microbial counts, inoculated samples of ground black pepper without any heat treatment, were serially diluted and plated on mTSAYE and eTSAYE for *Salmonella* spp. and *E. faecium*, respectively. After the thermal inactivation, the aluminum pouches were opened and the treated samples were added to a sterile sampling bag. The used aluminum pouch was immediately disposed in a biosafety waste container. The treated sample was diluted with 0.1% buffered peptone water (BPW) and mixed using a stomacher to obtain a decimal dilution. This mixture was further serially diluted (10:1) and at least 3 dilutions were plated in duplicate on mTSAYE and eTSAYE, for the survival enumeration of *Salmonella* spp. and *E. faecium*, respectively. The plates were incubated at 37 °C for 24 ± 2 h. The raw plate counts were converted to log CFU/g for each subsample. These experiments were repeated with 3 biological replicates. The averages of \log_{10} numbers of survivors of the two subsamples (log CFU/g) for each time

points between time zero (CUT) and the limit of detection (LOD) of the method (2.4 log CFU/g) were plotted to obtain survival curves against the heat treatment time (in minutes). Linear regression was applied to the survival curves using Microsoft Excel (Microsoft Corp., Richmond, WA, U.S.A). The negative inverse of the slope was used to calculate the D-value (in minutes) for each experiment. Similarly, to calculate the z-value, each log D-value obtained previously was plotted against the treatment temperature (°C). Thermal death time curves were obtained and the negative inverse slope from the linear regression was used to calculate the z-value for each replicate. The average value plus the standard deviation of z-value of all replicates was reported. Differences among mean parameter values were tested by pair student's t-test, using Microsoft Excel.

4.2.8 Experimental design and Statistical Analysis

The mean D-values for each bacteria and temperature combination were obtained by three independent replicates. To determine the influence of water activity on D-values, these were statistically analyzed by Analysis of Variance using SAS version 9.4 (SAS Institute, Cary NC, USA) for a split plot design with temperature as the split plot factor and water activity as a whole plot treatment in a randomized complete block. D-values were obtained for each replicate-water activity-temperature combination. Regression statistics using Microsoft Excel was used to determine the coefficients for the response surface equations for log D-value determination of both microorganisms. The response surface equation can be used to predict the log D-values at different temperatures and water activities in between 0.25-0.65 a_w .

4.3 Results and Discussion

4.3.1 Influence of water activity on the homogeneity and stability of inoculated ground black pepper

Whole black pepper inoculated with *Salmonella* spp. and *E. faecium* were homogeneous (variability of samples ≤ 0.3 log CFU/g) after inoculation at all water activities evaluated (0.25, 0.45 and 0.65) (Figure 4.1). After grinding the black pepper samples, the microbial populations were reduced by 0.43 to 1.08 log CFU/g, with black pepper inoculated with *Salmonella* spp. having more reduction than *E. faecium* (Figure 4.1). After equilibrating black pepper to a reduced water activity, previous studies have reported a drop of about 1 log CFU/g of *Salmonella* spp. which was attributed to the presence of antimicrobial compounds and changes in environments (Keller et al., 2013; Sun et al., 2014).

During storage of the ground black pepper, from day 3 to day 15 after inoculation, the microbial population of inoculated ground black pepper was also stable (standard deviations <0.3 log CFU/g) with *E. faecium* populations between 7.2 to 7.5 CFU/g and *Salmonella* spp. population between 6.6 to 7.0 CFU/g. The difference in population between day 3 and day 15 for *E. faecium* was less than 0.26 log CFU/g for all three water activities evaluated. Similarly, for *Salmonella* spp. the difference was less than 0.34 log CFU/g. These results confirmed that both organisms were able to survive in low- a_w conditions with *E. faecium* to be slightly more resistant to desiccation than *Salmonella* spp. in ground black pepper when equilibrated to low water activities.

In a previous study, *E. faecium* viable counts were stable over storage (at 16 °C) with a decrease lower than 0.2 log on confectionary, culinary products, pet foods (a_w =

0.565 to 0.655) and chicken meat powder ($a_w = 0.38$) after 21 days (Rachon et al., 2016). In that same study, for *Salmonella* spp. the largest reductions observed were of 0.5 and 0.4 log in confectionary and pet food products, respectively. Differences in viable counts <0.5 log are generally considered non-significant in microbiological analysis (Rachon et al., 2016).

Previous studies have reported that microorganism's adaptation to its environment is important for sample preparation before thermal inactivation studies (Beuchat & Scouten, 2002; Fudge et al., 2016; Jeong et al., 2012). Stable and homogeneous microbial populations are required for inoculated samples used in thermal inactivation experiments to avoid errors in microbial survival counts after treatments.

4.3.2. Influence of water activity on the microbial population before thermal treatments

The microbial population of *Salmonella* spp. and *E. faecium* in ground black pepper before heat treatments were statistically different ($p < 0.05$) between both microorganism and within the three water activities (0.25, 0.45, 0.65 a_w , measured at 25 °C) (Figure 4.2). *Salmonella* spp. counts were significantly lower ($p < 0.05$) than *E. faecium* for the different a_w . In previous studies, *S. Enteritidis* PT 30 and *E. faecium* B-2353 population level in wheat flour at 0.25 a_w were not statistically different ($p > 0.05$) (Villa-Rojas et al., 2017). In the same study, the populations of *S. Enteritidis* PT 30 in wheat flour equilibrated to different a_w (0.25, 0.45, 0.65 a_w) were not significantly different ($p > 0.05$). The discrepancy with the results reported here may be attributed to the differences of response to the equilibration method for a cocktail of *Salmonella* spp. (in this study) and a single strain of *Salmonella* (*S. Enteritidis* PT 30) in the previous

study.

4.3.3 Influence of water activity on the thermal resistance of *Salmonella* spp. and *E. faecium* in ground black pepper

Thermal inactivation experiments were conducted using TDT sandwiches set up to the fastest possible heating rate of 600 °C/min. The CUT at all three a_w and temperatures tested ranged from 41 to 57 seconds (Table 4.1). The achieved CUTs allowed achieving quick isothermal conditions, preventing possible preconditioning of the bacteria due to long CUT, as reported in previous studies (Chung et al., 2008). Short CUT could reduce thermal lag time and increase the accuracy when determining the thermal resistance of microorganisms (Chung et al., 2008).

The a_w of the equilibrated sample influenced the initial survival of the microorganisms during CUT in ground black pepper. Higher log reduction was achieved in ground black pepper with 0.65 a_w (0.27-1.74 log CFU/g) than with 0.25 and 0.45 a_w (Table 4.1). High microbial reduction during CUT reduces the bacterial population leading to levels that may be lower than those required to obtain adequate thermal inactivation curves after the thermal treatment. These results suggested that there could be limitations when using TDT sandwiches to evaluate thermal resistance of *Salmonella* spp. at $a_w \geq 0.65$ if the initial population is not sufficiently high ($>10^8$ CFU/g) to account for potentially high reduction during CUT.

In this study, treatment temperatures were chosen to be close to 75 °C because it is the common pasteurization temperature. However, initial experiments using ground black pepper samples (0.45 and 0.65 a_w) inoculated with *Salmonella* spp. that were treated at temperatures above 75 °C resulted in excessive bacterial reductions before the

target temperatures were reached. Therefore, temperatures ≤ 75 °C (65, 70 and 75 °C) were chosen for black pepper samples inoculated with *Salmonella* spp. conditioned to these a_w . On the contrary, the microbial population in ground black pepper samples inoculated with *E. faecium* and treated at 80 °C did not decrease greatly. Therefore, temperatures of 70, 75, and 80 °C were chosen for thermal inactivation of *E. faecium* in ground black pepper equilibrated to 0.45 and 0.65 a_w . The differences of temperatures used denoted the increased thermal resistance of *E. faecium* compared to *Salmonella* spp. within the range of water activities evaluated (0.45 and 0.65 a_w).

When heating inoculated black pepper samples conditioned to 0.25 a_w , with the same temperatures chosen for 0.45 a_w and 0.65 a_w , the thermal inactivation experiments required longer time to achieve sufficient inactivation for D-value determination. Therefore, the treatment temperatures chosen for black pepper conditioned to 0.25 a_w were 75, 80, and 85 °C for both microorganism to achieve sufficient inactivation in a maximum treatment time of 6 hours. The increased thermal resistance of *Salmonella* spp. and *E. faecium* at 0.25 a_w was evident when compared to 0.45 a_w and 0.65 a_w . In a previous study, it was reported the thermal resistance of *S. Weltevreden* in flour was the highest at a_w between 0.3 and 0.4 (Archer et al., 1998).

Table 4.2 shows the D-values of *Salmonella* spp. and *E. faecium* in ground black pepper at different water activities (0.25, 0.45, 0.65 a_w , measured at 25 °C) and temperatures. As a_w increased, the D-value decreased for both microorganisms. Overall, within each a_w , as the temperature increased the D-value decreased. These results are congruent with previous published data (Archer et al., 1998; Fudge et al., 2016; S. Wang et al., 2013).

4.3.4 Response Surface Model to predict D-value at different water activities

In this study, Response Surface Equations were developed for determination of log D-values of *E. faecium* and *Salmonella* spp. in ground black pepper when equilibrated to 0.25 to 0.45 a_w .

The response surface equations for log D-value determination for *E. faecium* and *Salmonella* spp. under the studied conditions using TDT sandwiches are given in Equations 4.1. and 4.2. The R^2 corresponds to the value resulted from regression statistics using Microsoft Excel.

$$\log D_{\text{value}} = 11.51 - 2.51a + 3.47a^2 - 0.16T + 0.0005T^2 - 0.04aT \quad ; R^2 = 0.925 \quad (4.1)$$

$$\log D_{\text{value}} = 8.90 - 4.79a - 0.08T \quad ; R^2 = 0.956 \quad (4.2)$$

Where:

D_{value} = decimal reduction time (minutes)

a = water activity (decimals)

T = temperature ($^{\circ}\text{C}$)

For *E. faecium*, the obtained model showed that water activity and temperature had a linear and quadratic effect on the log D-value (Eq. 4.1). Additionally, there was a significant interaction between each other for log D-value determination of both microorganisms. Equation 4.1 was used to predict log D-value and then transform these values applying power of 10 to obtained Predicted D-values for *E. faecium* (Table 4.3). The correlation for R^2 when plotting observed D-values against predicted D-values for *E. faecium* was 0.893 (Figure 4.2).

Similarly, the obtained model for *Salmonella* spp. showed that water activity and temperature had linear and quadratic effect on the log D-value for *Salmonella* spp. (Eq. 4.2). Predicted D-values for *Salmonella* spp. were obtained using Eq. 4.2 to predict log D-value and applying power of 10 (Table 4.4). The correlation R^2 of observed D-values against predicted D-values for *Salmonella* spp. was 0.893 (Figure 4.3).

4.3.5. Survival curves obtained after heat treatment using TDT sandwiches

Commercial pasteurization thermal processes are designed based on D-values and on the assumption of a first-order microbial inactivation kinetics (Chung et al., 2008; Humpheson, Adams, Anderson, & Cole, 1998). Other studies have also showed that the shape of the curve is dependent to the strain of *Salmonella* and the food matrix (Fudge et al., 2016). In this study, the survival curves after thermal inactivation treatments for inoculated samples at all three water activities were log linear (Figures 4.5, 4.6 and 4.7). The correlation coefficient (R-square) for each set of experiments was calculated to evaluate the linearity of all curves (Table 4.5). When comparing the R-square of thermal inactivation curves it can be observed that the R-square value for *E. faecium* were higher than those obtained for *Salmonella* spp. R-squares for *E. faecium* curves were between 0.945 to 0.992, indicating better linear-fit. Whereas, for *Salmonella* spp. curves the R-square ranged from 0.90915 to 0.97188. Lower R-square values were achieved with samples inoculated with *Salmonella* spp. equilibrated to a_w of 0.65 and treated at 70 and 75 °C (Table 4.5). By comparison, another study reported linear curves with R-square \geq 0.85 when testing *S. Typhimurium* in flour (0.32 to 0.42 a_w) at 70, 75 and 80 °C (Fudge et al., 2016).

It has been reported that the method used for thermal resistance determination might influence the shape of the survivor curve (Humpheson et al., 1998). Deviations from first-order inactivation include shouldering or tailing. Shouldered death curves are caused by a slower death rate at the beginning of the experiment and tailing are often viewed as consequence of experimental difficulties such as numbering of lower populations (Humpheson et al., 1998). In this study, shouldering was not present but some tailing were observed for *Salmonella* spp. at 0.45 a_w treated at 70 °C and 75 °C and *E. faecium* at 0.65 a_w treated at 70 °C. The tailing affected the R-square obtained for these curves.

Archer and others (1998) reported biphasic curves, with a rapid decrease (> 1 log CFU/g) in population within 5 to 10 minutes of heating, followed by a longer slower, linear decrease. In this study, the D-value was only calculated with the linear portion of the survival curve (Archer et al., 1998). Additionally, other studies have reported non-linear survival curves and have determined the thermal resistance by Weibull model distribution (S. Wang et al., 2013).

4.3.6. z-value of *E. faecium* NRRL-B2354 and *Salmonella* in ground black pepper at 0.25, 0.45 and 0.65 a_w

In this study, the z-values for *E. faecium* and *Salmonella* spp. ranged from 11.87 to 12.82 °C and 9.99 to 13.04 °C, respectively (Table 4.6). The z-values for *Salmonella* spp. in this study were expected to be higher than *Salmonella* spp. in high moisture foods which varied from 3.5 to 8.2 °C, previously reported by Sylvia and Gibbs, 2012. Lower z-values demonstrate smaller temperature changes causing thermal inactivation, therefore, less thermal resistance. Whereas an increase in z-values, there is a higher

requirement for temperature change to reduce the time for destruction, suggesting higher thermal resistance.

In this study, there are no significant difference ($p>0.05$) between z-values of *E. faecium* and *Salmonella* spp. at 0.25 and 0.45 a_w , but there is a significant difference ($p<0.05$) with *E. faecium* (9.9 °C) at 0.65 a_w . The R-square values of thermal death time curves for both microorganisms ranged from 0.96 to 1.00 (Table 4.7.). These results confirmed that reduced a_w increases the thermal resistance of bacteria as reported in previous studies (Archer et al., 1998; S. Wang et al., 2013).

With respect to low- a_w foods, there are limited data of z-values for *Salmonella* spp. The z-value for *Salmonella* Typhimurium and *Salmonella* Senftenberg in whole milk powder was reported as 62.2 °C and 44.3 °C (Lang et al., 2017). In a different study, the z-value for almond kernel flour decreased from 10.4 to 7.2 °C as a_w increased from 0.601 to 0.946 (S. Wang et al., 2013). *Salmonella* Weltevreden in flour with a_w ranging from 0.25 to 0.60 was reported to have z-values between 15.2 and 53.9 °C (Archer et al., 1998).

4.3.7. *E. faecium* NRRL-B2354 as a suitable surrogate for *Salmonella* in ground black pepper

E. faecium NRRL-B2354 has commonly been considered as a suitable surrogate for *Salmonella* spp. (Almond Board of California, 2007). Its safety for use has been evaluated in function of its genomic and functional characteristics (Kopit et al., 2014). However, a surrogate used to predict a target response might work well in a process but not in another (National Advisory Committee on Microbiological Criteria for Foods, 2010). In a previous study, *E. faecium*'s D-value was lower than a cocktail of

Salmonella's in confectionary products stored at 80 °C for up to 21 days (Rachon et al., 2016). In another study, *E. faecium*'s D-value was lower than *Salmonella* Typhimurium TP 42 in flour treated using screw top glass vials in a water bath at 75°C. Therefore, studies using different strains, treatment conditions and type of foods are required when determining a surrogate in process validations.

There are limited data of process validations using *E. faecium* as surrogate for *Salmonella* spp. in black pepper. A recent study evaluated these organisms inoculated in whole and ground black pepper equilibrated to a_w 0.60 and 0.66, respectively, and treated with radiofrequency (Wei, 2017). Other available published data only include inactivation of *Salmonella* spp. in black pepper using different technologies, including radiofrequency (Jeong & Kang, 2014; Kim et al., 2012), gamma irradiation (Song et al., 2014), atmospheric pressure plasma (Hertwig et al., 2015) and vacuum steam pasteurization (Shah et al., 2017) without comparing it to the inactivation of a potential surrogate, such as *E. faecium*.

In this study, the thermal inactivation kinetics of *Salmonella* spp. and *E. faecium* in ground black pepper were compared. Given the differences for treatment temperatures for inoculated black pepper samples conditioned varied depending upon the water activity of the sample it was only possible to compare the thermal resistance of both microorganisms at 75 °C across all three water activities inoculated. The D-value at 75 °C for *E. faecium* in black pepper was higher than for *Salmonella* spp. at all three different water activities (Figure 4.8). Additionally, the higher thermal resistance of *E. faecium* over *Salmonella* spp. was evident when comparing the survival curve of both microorganisms at 75 °C (Figure 4.9). These results confirmed *E. faecium* as an adequate

surrogate for *Salmonella* spp. during thermal inactivation processes at 75 °C for ground black pepper equilibrated to 0.25 to 0.65 a_w . These results are congruent with previous published data evaluating *E. faecium* as a surrogate of *Salmonella* spp. in other low moisture foods and temperatures tested (Bianchini et al., 2014; Ceylan & Bautista, 2015; Jeong et al., 2011; Rachon et al., 2016).

4.4. Conclusions

This study validated that water activity has an effect on the thermal resistance of *E. faecium* and *Salmonella* spp. in ground black pepper equilibrated to 0.25, 0.45 and 0.65 a_w . Thermal resistance of *E. faecium* and *Salmonella* spp. decreased as water activity increased. Furthermore, the thermal resistance of both microorganisms decreased as treatment temperature increased for each water activity. Initial populations of both microorganisms resulted in higher counts for *E. faecium* compared to *Salmonella* spp., indicating higher resistance of *E. faecium* to desiccation. The use of TDT sandwiches provided isothermal conditions during heat treatment, preventing preconditioning of bacteria and increasing accuracy of results obtained. However, this method led to high reductions of *Salmonella* spp. during CUT in black pepper inoculated and conditioned at 0.65 a_w . Additionally, the results from this study reinforced the use of *E. faecium* as a surrogate for *Salmonella* spp. in a low- a_w food, such as black pepper. The Response Surface equations developed in this study can be used to determine log D-value of *E. faecium* and *Salmonella* spp. inoculated in ground black pepper conditioned to water activities between 0.25 to 0.45. The predicted values obtained can be useful tools to establish possible process conditions to decontaminate black pepper at water activity and process temperature different than those evaluated in this study.

4.5 References

1. Almond Board of California. (2007). Guidelines for validation of dry roasting processes. Available from: <http://www.almonds.com/sites/default/files/content/attachments/dry-roast-validation-guidelines.pdf>. Accessed March, 2018
2. Archer, J., Jervis, E. T., Bird, J., & Gaze, J. E. (1998). Heat resistance of *Salmonella* Weltevreden in low-moisture environments. *Journal of Food Protection*, 61(8), 969-973.
3. Beuchat, L., & Scouten, A. (2002). Combined effects of water activity, temperature and chemical treatments on the survival of *Salmonella* and *Escherichia coli* O157: H7 on alfalfa seeds. *Journal of Applied Microbiology*, 92(3), 382-395.
4. Bianchini, A., Stratton, J., Weier, S., Hartter, T., Plattner, B., Rokey, G., . . . Eskridge, K. M. (2014). Use of *Enterococcus faecium* as a surrogate for *Salmonella enterica* during extrusion of a balanced carbohydrate-protein meal. *Journal of Food Protection*, 77(1), 75-82.
5. Blessington, T., Theofel, C. G., & Harris, L. J. (2013). A dry-inoculation method for nut kernels. *Food Microbiology*, 33(2), 292-297.
6. Ceylan, E., & Bautista, D. A. (2015). Evaluating *Pediococcus acidilactici* and *Enterococcus faecium* NRRL B-2354 as thermal surrogate microorganisms for *Salmonella* for in-plant validation studies of low-moisture pet food products. *Journal of Food Protection*, 78(5), 934-939.
7. Chung, H., Birla, S., & Tang, J. (2008). Performance evaluation of aluminum test cell designed for determining the heat resistance of bacterial spores in foods. *LWT-Food Science and Technology*, 41(8), 1351-1359.
8. Emer, Z., Akbas, M. Y., & Ozdemir, M. (2008). Bactericidal activity of ozone against *Escherichia coli* in whole and ground black peppers. *Journal of Food Protection*, 71(5), 914-917.
9. Farakos, S. S., Frank, J., & Schaffner, D. (2013). Modeling the influence of temperature, water activity and water mobility on the persistence of *Salmonella* in low-moisture foods. *International Journal of Food Microbiology*, 166(2), 280-293.
10. Fudge, J., Dunn, M., Pike, O., Robison, R., & Steele, F. (2016). The isolation and identification of *Pantoea dispersa* strain JFS as a non-pathogenic surrogate for *Salmonella* Typhimurium phage type 42 in flour. *International Journal of Food Microbiology*, 219, 1-6.

11. Hertwig, C., Reineke, K., Ehlbeck, J., Knorr, D., & Schlüter, O. (2015). Decontamination of whole black pepper using different cold atmospheric pressure plasma applications. *Food Control*, 55, 221-229.
12. Humpheson, L., Adams, M. R., Anderson, W. A., & Cole, M. B. (1998). Biphasic thermal inactivation kinetics in *Salmonella enteritidis* PT4. *Applied and Environmental Microbiology*, 64(2), 459-464.
13. Jeong, S., Marks, B. P., & Ryser, E. T. (2011). Quantifying the performance of *Pediococcus* spp.(NRRL B-2354: *Enterococcus faecium*) as a nonpathogenic surrogate for *Salmonella enteritidis* PT30 during moist-air convection heating of almonds. *Journal of Food Protection*, 74(4), 603-609.
14. Jeong, S., Marks, B. P., Ryser, E. T., & Harte, J. B. (2012). The effect of X-ray irradiation on *Salmonella* inactivation and sensory quality of almonds and walnuts as a function of water activity. *International Journal of Food Microbiology*, 153(3), 365-371.
15. Jeong, S., & Kang, D. (2014). Influence of moisture content on inactivation of *Escherichia coli* O157: H7 and *Salmonella enterica* serovar Typhimurium in powdered red and black pepper spices by radio-frequency heating. *International Journal of Food Microbiology*, 176, 15-22.
16. Keller, S. E., VanDoren, J. M., Grasso, E. M., & Halik, L. A. (2013). Growth and survival of *Salmonella* in ground black pepper (*Piper nigrum*). *Food Microbiology*, 34(1), 182-188.
17. Kim, S., Sagong, H., Choi, S. H., Ryu, S., & Kang, D. (2012). Radio-frequency heating to inactivate *Salmonella* Typhimurium and *Escherichia coli* O157: H7 on black and red pepper spice. *International Journal of Food Microbiology*, 153(1), 171-175.
18. Kopit, L. M., Kim, E. B., Siezen, R. J., Harris, L. J., & Marco, M. L. (2014). Safety of the surrogate microorganism *Enterococcus faecium* NRRL B-2354 for use in thermal process validation. *Applied and Environmental Microbiology*, 80(6), 1899-1909.
19. Lang, E., Chemlal, L., Molin, P., Guyot, S., Alvarez-Martin, P., Perrier-Cornet, J., . . . Gervais, P. (2017). Modeling the heat inactivation of foodborne pathogens in milk powder: High relevance of the substrate water activity. *Food Research International*, 99, 577-585.
20. National Advisory Committee on Microbiological Criteria for Foods. (2010). Parameters for determining inoculated pack/challenge study protocols. *Journal of Food Protection*, 73(1), 140-202.

21. Rachon, G., Peñaloza, W., & Gibbs, P. A. (2016). Inactivation of *Salmonella*, *Listeria monocytogenes* and *Enterococcus faecium* NRRL B-2354 in a selection of low moisture foods. *International Journal of Food Microbiology*, 231, 16-25.
22. Shah, M. K., Asa, G., Sherwood, J., Graber, K., & Bergholz, T. M. (2017). Efficacy of vacuum steam pasteurization for inactivation of *Salmonella* PT 30, *Escherichia coli* O157: H7 and *Enterococcus faecium* on low moisture foods. *International journal of food microbiology*, 244, 111-118.
23. Silva, F. V., & Gibbs, P. A. (2012). Thermal pasteurization requirements for the inactivation of *Salmonella* in foods. *Food Research International*, 45(2), 695-699.
24. Song, W., Sung, H., Kim, S., Kim, K., Ryu, S., & Kang, D. (2014). Inactivation of *Escherichia coli* O157:H7 and *Salmonella* Typhimurium in black pepper and red pepper by gamma irradiation. *International Journal of Food Microbiology*, 172, 125-129.
25. Sun, S., Anderson, N. M., & Keller, S. (2014). Atmospheric pressure plasma treatment of black peppercorns inoculated with *Salmonella* and held under controlled storage. *Journal of Food Science*, 79(12).
26. Van Doren, J. M., Neil, K. P., Parish, M., Gieraltowski, L., Gould, L. H., & Gombas, K. L. (2013). Foodborne illness outbreaks from microbial contaminants in spices, 1973–2010. *Food Microbiology*, 36(2), 456-464.
27. Vij, V., Ailes, E., Wolyniak, C., Angulo, F. J., & Klontz, K. C. (2006). Recalls of spices due to bacterial contamination monitored by the U.S Food and Drug Administration: The predominance of *Salmonellae*. *Journal of Food Protection®*, 69(1), 233-237.
28. Villa-Rojas, R., Zhu, M., Marks, B. P., & Tang, J. (2017). Radiofrequency inactivation of *Salmonella* enteritidis PT 30 and *Enterococcus faecium* in wheat flour at different water activities. *Biosystems Engineering*, 156, 7-16.
29. Wang, S., Villa-Rojas, R., Tang, J., Gao, M., Kang, D., Mah, J., . . . López-Malo, A. (2013). Thermal inactivation of *Salmonella* enteritidis PT 30 in almond kernels as influenced by water activity. *Journal of Food Protection*, 76(1), 26-32.
30. Wei, X. (2017). *Radiofrequency processing for inactivation of Salmonella spp. and Enterococcus faecium* NRRL B-2354 in whole black peppercorn and ground black pepper (Master's thesis). Available from: Digital Commons@ University of Nebraska-Lincoln. <https://digitalcommons.unl.edu/foodscidiss/88/> Accessed May, 2018
31. Zhang, G., Hu, L., Pouillot, R., Tatavarthy, A., Doren, J. M. V., Kleinmeier, D., . . . Brown, E. W. (2017). Prevalence of *Salmonella* in 11 spices offered for sale

from retail establishments and in imported shipments offered for entry to the United States. *Journal of Food Protection*, 80(11), 1791-1805.

Table 4.1.

Come-up times reductions for *Salmonella* spp. and *E. faecium* NRRL B-2354 populations in ground black pepper with different water activities treated with TDT sandwiches with heating rate of 600 °C/min at different temperatures

Microorganism	Water activity	CUT (s)	CUT reductions (log CFU/g)				
			65°C	70°C	75°C	80°C	85°C
<i>Salmonella</i> spp.	0.25	47-55			-0.04	0.04	-0.07
	0.45	41-44	-0.12	0.11	0.22		
	0.65	49-52	0.48	1.13	1.74		
<i>E. faecium</i>	0.25	47-55			0.11	0.10	-0.01
	0.45	44		0.09	-0.26	0.13	
	0.65	52-57		0.27	0.38	0.96	

Table 4.2.

D-values (minutes + SE) for *Salmonella* spp. and *E. faecium* NRRL B-2354 populations in ground black pepper with different water activities treated with TDT sandwiches with heating rate of 600 °C/min at different temperatures

Temperature (°C)	Microorganism	Water activity (a_w)		
		0.25	0.45	0.65
65	<i>Salmonella</i>		42.21 ± 1.68	10.18 ± 1.68
70	<i>Salmonella</i>		18.07 ± 1.68	2.74 ± 1.68
75	<i>Salmonella</i>	42.86 ± 1.68	6.97 ± 1.68	1.51 ± 1.68
80	<i>Salmonella</i>	17.80 ± 1.68		
85	<i>Salmonella</i>	6.16 ± 1.68		
70	<i>E. faecium</i>		31.98 ± 4.02	17.80 ± 4.02
75	<i>E. faecium</i>	58.35 ± 4.02	12.01 ± 4.02	4.56 ± 4.02
80	<i>E. faecium</i>	22.99 ± 4.02	4.31 ± 4.02	1.63 ± 4.02
85	<i>E. faecium</i>	10.25 ± 4.02		

Table 4.3 Observed and predicted D-values for *E. faecium*

Observation	Water activity (a_w)	Temperature (°C)	Observed D-value (min)	Predicted D-value (min)	Residual
1	0.65	70	30.49	14.80	15.69
2	0.65	70	12.64	14.80	-2.16
3	0.65	70	10.26	14.80	-4.55
4	0.65	75	7.47	4.57	2.90
5	0.65	75	3.15	4.57	-1.42
6	0.65	75	3.07	4.57	-1.51
7	0.65	80	2.30	1.51	0.79
8	0.65	80	1.50	1.51	-0.01
9	0.65	80	1.09	1.51	-0.42
10	0.45	70	28.90	33.76	-4.86
11	0.45	70	35.59	33.76	1.82
12	0.45	70	31.45	33.76	-2.32
13	0.45	75	11.75	11.55	0.20
14	0.45	75	12.50	11.55	0.95
15	0.45	75	11.79	11.55	0.24
16	0.45	80	4.17	4.22	-0.06
17	0.45	80	4.47	4.22	0.25
18	0.45	80	4.28	4.22	0.06
19	0.25	75	63.29	55.28	8.01
20	0.25	75	41.84	55.28	-13.44
21	0.25	75	69.93	55.28	14.65
22	0.25	80	27.62	22.38	5.24
23	0.25	80	13.74	22.38	-8.65
24	0.25	80	27.62	22.38	5.24
25	0.25	85	12.64	9.69	2.95
26	0.25	85	5.62	9.69	-4.06
27	0.25	85	12.48	9.69	2.80

Table 4.3 Observed and predicted D-values for *Salmonella* spp.

Observation	Water activity (a_w)	Temperature (°C)	Observed D-value (min)	Predicted D-value (min)	Residual
1	0.65	65	11.19	8.47	2.72
2	0.65	65	5.60	8.47	-2.87
3	0.65	65	13.76	8.47	5.29
4	0.65	70	2.86	3.30	-0.44
5	0.65	70	1.97	3.30	-1.33
6	0.65	70	3.40	3.30	0.10
7	0.65	75	1.38	1.29	0.09
8	0.65	75	0.91	1.29	-0.38
9	0.65	75	2.25	1.29	0.97
10	0.45	65	34.97	44.27	-9.30
11	0.45	65	44.05	44.27	-0.21
12	0.45	65	47.62	44.27	3.35
13	0.45	70	14.29	17.26	-2.97
14	0.45	70	21.14	17.26	3.88
15	0.45	70	18.80	17.26	1.54
16	0.45	75	5.81	6.73	-0.92
17	0.45	75	8.42	6.73	1.70
18	0.45	75	6.67	6.73	-0.06
19	0.25	75	42.55	42.96	-0.41
20	0.25	75	42.55	42.96	-0.41
21	0.25	75	43.48	42.96	0.52
22	0.25	85	5.95	6.53	-0.58
23	0.25	85	6.14	6.53	-0.39
24	0.25	85	6.40	6.53	-0.13
25	0.25	80	17.21	16.75	0.46
26	0.25	80	18.66	16.75	1.91
27	0.25	80	17.54	16.75	0.79

Table 4.5.

R-square of thermal inactivation curves of *E. faecium* and *Salmonella* spp. in ground black pepper

Microorganism	Water activity (a_w)	Temperature (°C)	Average R-square
<i>E. faecium</i>	0.25	75	0.99
		80	0.99
		85	0.98
	0.45	70	0.99
		75	0.99
		80	0.99
	0.65	70	0.95
		75	0.98
		80	0.95
<i>Salmonella</i> spp.	0.25	75	0.97
		80	0.96
		85	0.97
	0.45	65	0.94
		70	0.93
		75	0.93
	0.65	65	0.95
		70	0.91
		75	0.91

Table 4.6.

z-values (°C) of *E. faecium* and *Salmonella* spp. in ground black pepper equilibrated to different water activities

Microorganism	Water activity (a_w)		
	0.25	0.45	0.65
<i>E. faecium</i>	$13.04 \pm 1.44^{a,x}$	$11.51 \pm 0.40^{a,x}$	$9.99 \pm 0.97^{b,x}$
<i>Salmonella</i> spp.	$11.87 \pm 0.16^{a,x}$	$12.82 \pm 1.11^{a,x}$	$12.13 \pm 0.98^{a,y}$

When comparing between a_w (rows), value followed by different letters (ab) are significantly different ($p < 0.05$).

When comparing between microorganisms (columns), values with different letters (xy) are significantly different ($p < 0.05$).

Table 4.7.

R-square of thermal death curves (z-values) of *E. faecium* and *Salmonella* spp. in ground black pepper equilibrated to different water activities

Microorganism	Water activity (a_w)		
	0.25	0.45	0.65
<i>E. faecium</i>	1.00	1.00	0.99
<i>Salmonella</i>	1.00	1.00	0.96

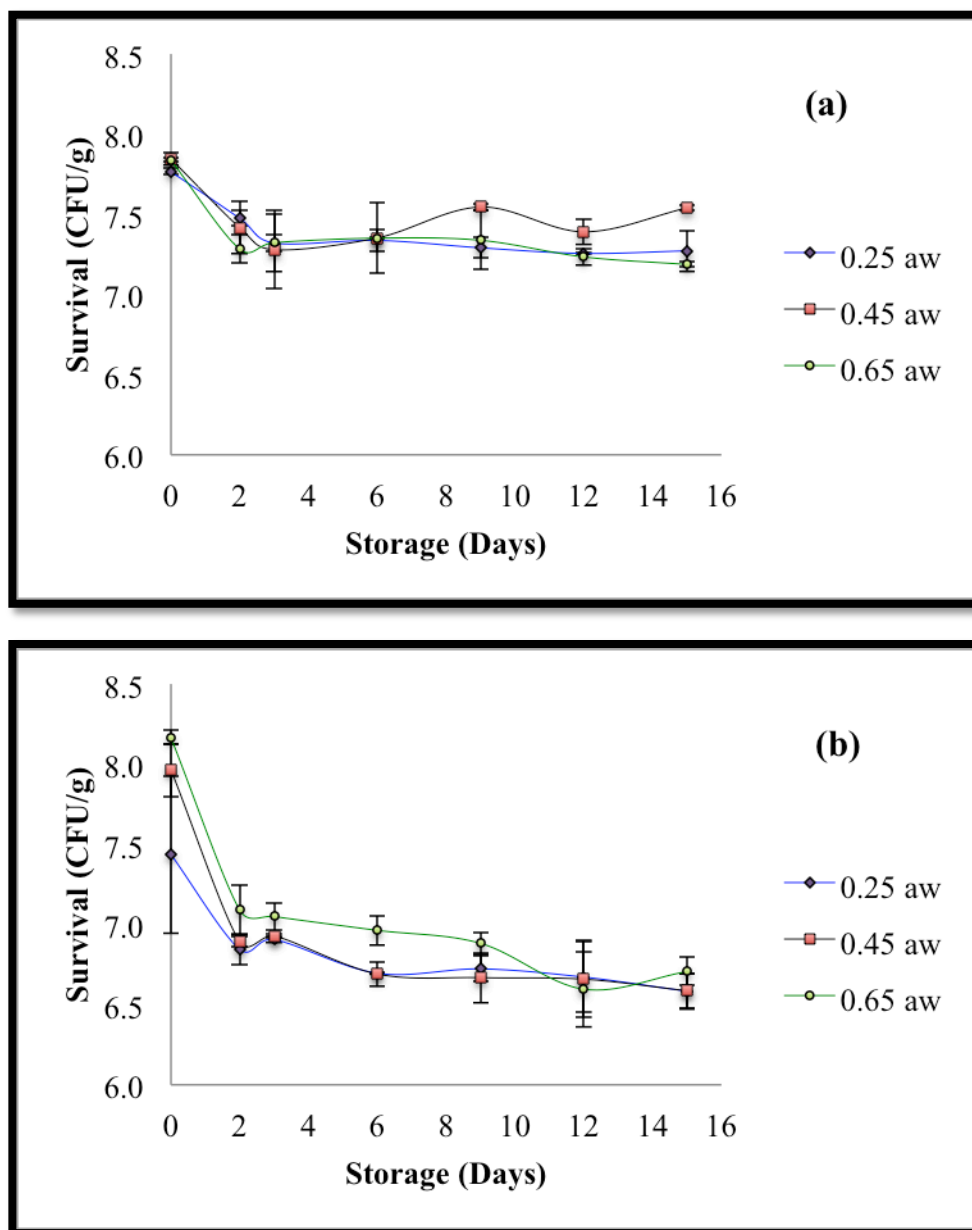


Figure 4.1 Homogeneity and stability of *E. faecium* NRRL B-2354 (a) and *Salmonella* spp. (b) in ground black pepper equilibrated to different water activities (0.25, 0.45, 0.65) and stored until 15 days.

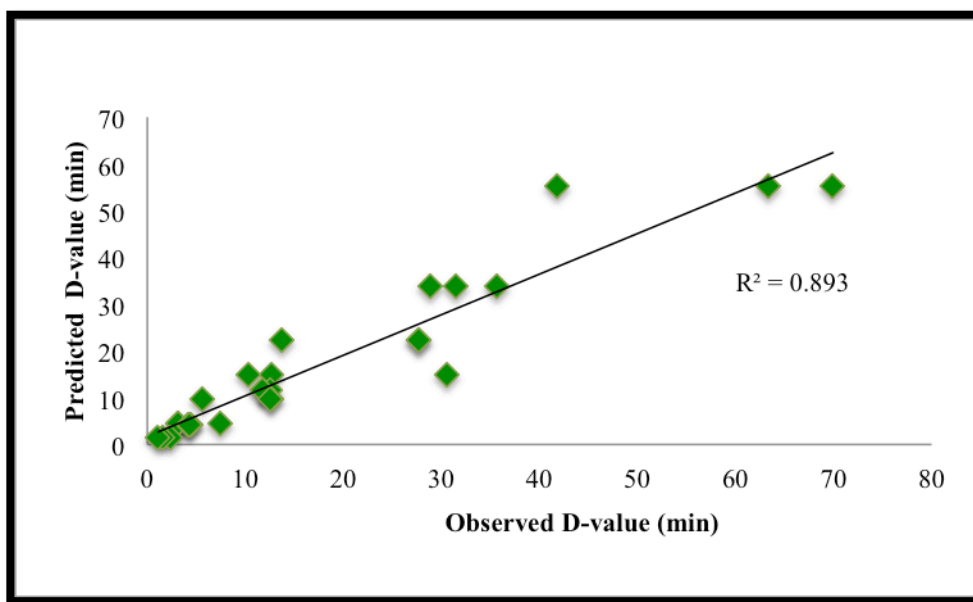


Figure 4.2. Correlation for Observed D-value (min) against Predicted D-value (min) using Response Surface Equation for *E. faecium*.

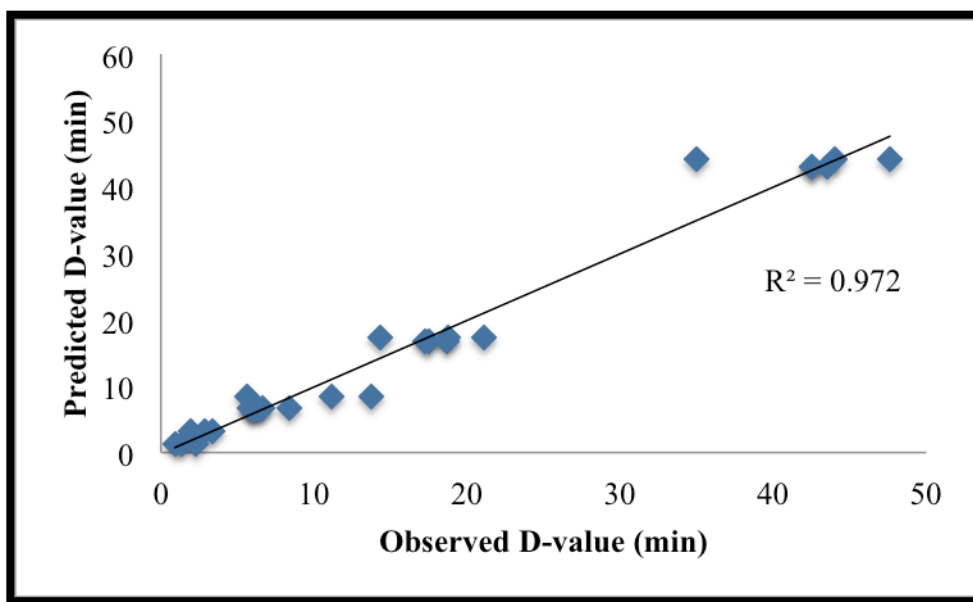


Figure 4.3. Correlation for Observed D-value (min) against Predicted D-value (min) using Response Surface Equation for *Salmonella* spp.

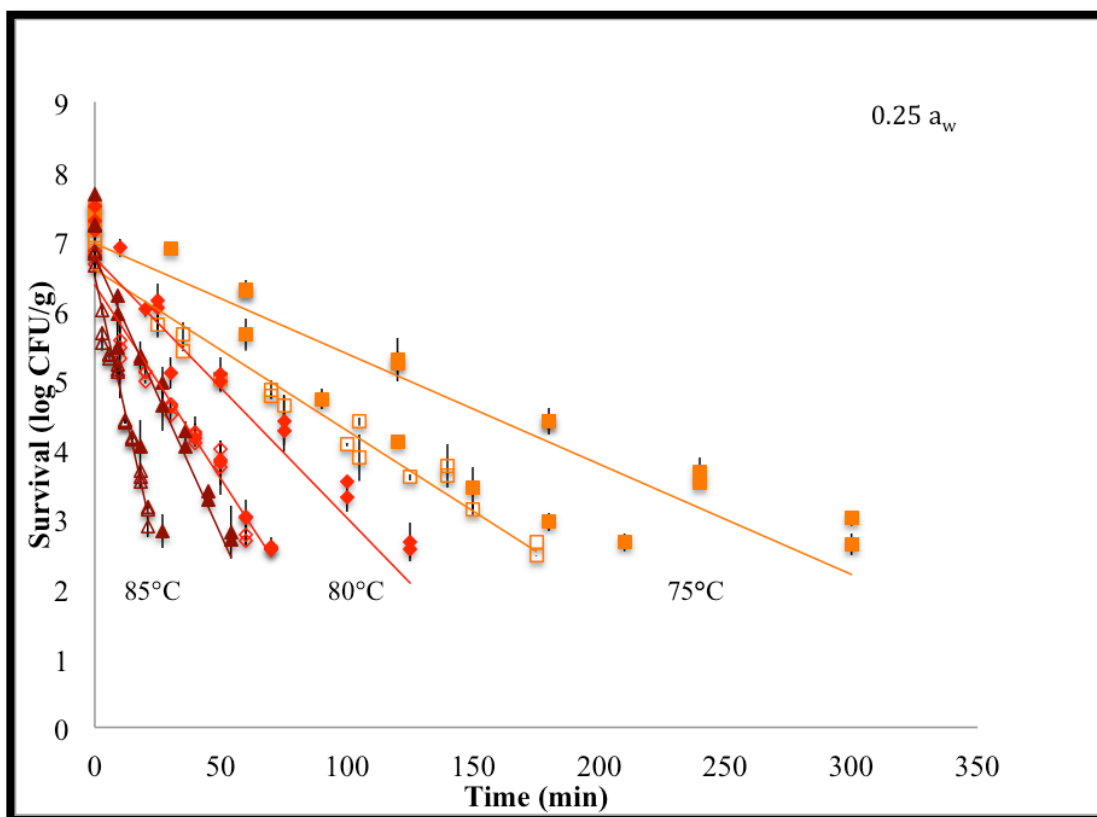


Figure 4.4 Survival curves of *Salmonella* spp. (unfilled shapes) and *E. faecium* NRRL B-2354 (filled shapes) in ground black pepper at 0.25 a_w (measured at 25°C) and different temperatures (■ 75°C, ♦ 80°C and ▲ 85°C) when using TDT sandwiches. Data points were from the average of subsamples (n=2) for all three biological replicates plotted. Error bars indicate standard deviation.

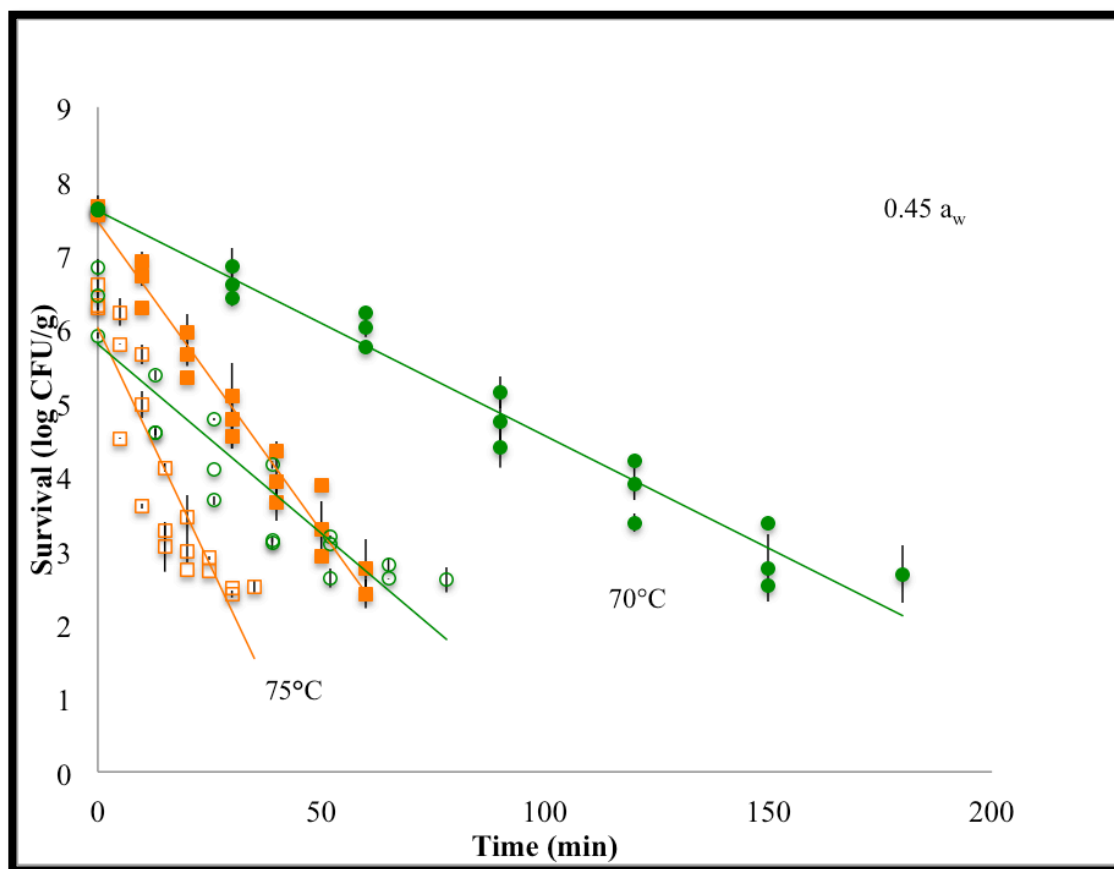


Figure 4.5 Survival curves of *Salmonella* spp. (unfilled shapes) and *E. faecium* NRRL B-2354 (filled shapes) in ground black pepper at 0.45 a_w (measured at 25 °C) and different temperatures (● 70 °C and ■ 75°C) when using TDT sandwiches. Data points were from the average of subsamples (n=2) for all three biological replicates plotted. Error bars indicate standard deviation.

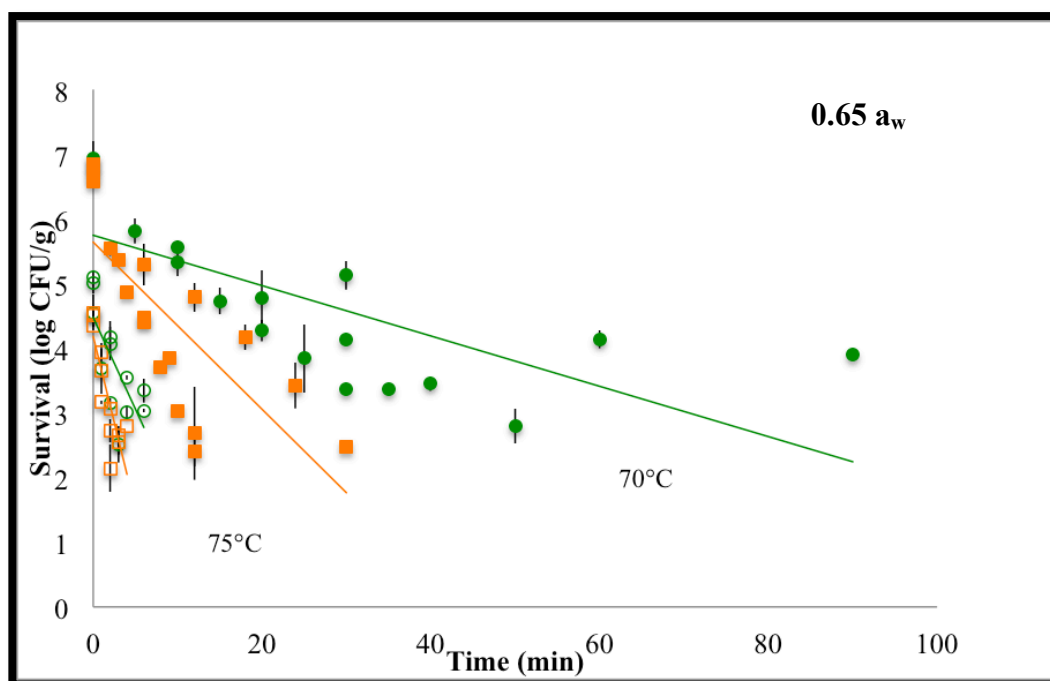


Figure 4.6. Survival curves of *Salmonella* spp. (unfilled shapes) and *E. faecium* (filled shapes) in ground black pepper at 0.65 a_w (measured at 25 °C) and different temperatures (●70°C and ■75°C) using TDT sandwiches. Data points were from the average of subsamples (n=2) for all three biological replicates plotted. Error bars indicate standard deviations.

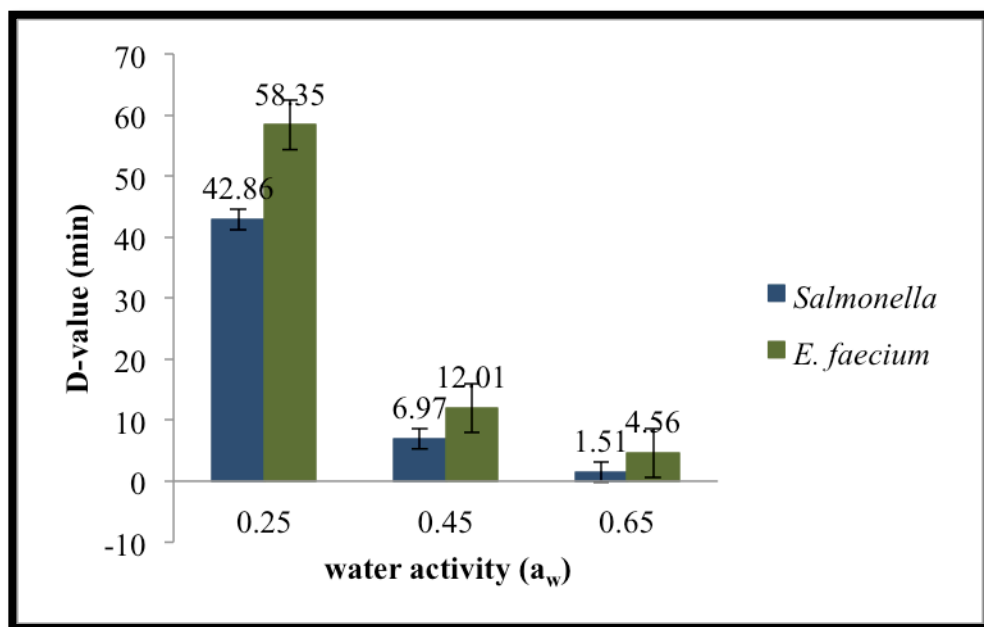


Figure 4.7. Comparison between the D-values (min) at 75 °C of *Salmonella* spp. and *E. faecium* NRRL B-2354 influenced by water activity using TDT sandwiches. Error bars indicate standard error.

Chapter V: Conclusions and Suggestions for Future research

5.1. Conclusions

In this study, TDT sandwiches were validated for thermal inactivation kinetics of *Salmonella* spp. and *E. faecium* NRRL B-2354 in ground black pepper. Additionally, water activity (a_w) was shown to have an effect on the thermal resistance of these microorganisms. Before thermal inactivation, the whole black pepper samples were inoculated with either a 5-strain cocktail of *Salmonella* spp. or *E. faecium* and samples were equilibrated to the desired water activity (0.25, 0.45, 0.65 a_w) and further grinded. Thermal inactivation for 0.45 a_w samples were conducted using (TDT) sandwiches and TDT test cells, a conventional heating method, for comparison between methods. Later, thermal inactivation for 0.25 and 0.65 a_w ground black pepper samples were conducted only with TDT sandwiches using the fastest heating rate (600 °C/min).

The results of thermal inactivation suggested that the heating method applied influenced the determination of thermal resistance of *Salmonella* spp. On contrary, the heating method did not have an effect on *E. faecium*. Moreover, it was proven that heating rate above 6.5 °C/min did not have an effect on the thermal resistance of either microorganism studied. The D-values for *Salmonella* spp. and *E. faecium* increased as water activity decreased. Finally, *E. faecium* was established as a surrogate for thermal process validations of *Salmonella* spp. in ground black pepper for all three water activities and temperatures tested. The response surface equations developed in this study can be use as estimation tools to predict log D-values for *Salmonella* spp. and *E. faecium* in ground black pepper conditioned to 0.25-0.65 a_w and treated under different temperatures.

5.2. Suggestions for Future research

In this study, TDT sandwiches were validated to be an effective dry heating method to determine the thermal inactivation kinetics of *Salmonella* spp. and *E. faecium* in ground black pepper. However, there is more research needed to elucidate the process and sample conditions that are required to be monitored for proper determination of thermal inactivation kinetics of microorganisms in low- a_w foods. For example, the physical properties of samples (density and particle size) should be evaluated if it influences the heating rate and therefore, the thermal resistance of the microorganisms, when using the TDT sandwiches. Additionally, it should be evaluated if the chemical properties and composition of the food sample influences the thermal resistance of microorganisms.

TDT sandwiches were used assuming thermal uniformity of the heating blocks. Current TDT sandwiches were designed and a thermocouple was placed in the middle of each heating element and temperatures were recorded and analyzed. Further studies should validate the heating uniformity profile of the heating blocks by using more precise methods capable of measuring the temperature in all the surface of the heating pads.

Thermal inactivation conditions for each microorganism were determined by trial-and-error method. This was time consuming and many samples and resources were wasted. However, this study attempts to evaluate several parameters including different heating methods, heating rates, temperatures, water activities and microorganisms. Future studies that compare D-value determination using TDT sandwiches against other heating methods could focus on one temperature and heating rate to ease work.

In recent studies, other heating methods including TDT cells and TAC cells have been developed for thermal inactivation determination of microorganisms in low- a_w foods. TAC cells with LiCl solution are reported to be capable of stabilizing the relative humidity inside the cell, and therefore, maintained the a_w constant during heat treatment. With this, it is expected to determine the thermal resistance of microorganisms for samples with equal a_w as during heating treatments using an oil or water bath. In this study, TDT sandwiches, a novel dry method, did not require the use of additional solutions during treatment. Inactivation studies on inoculated samples prepared under the same inoculation and preparation conditions can be conducted using these two methods and evaluate if comparable results can be obtained.

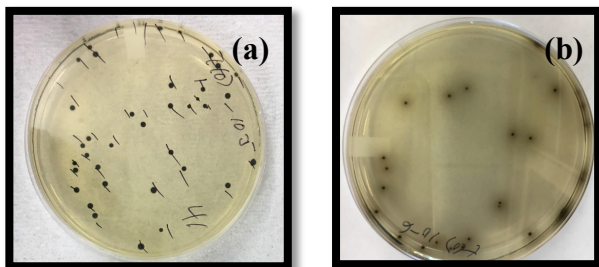
Further studies using TDT sandwiches, can apply slower heating rate to achieve comparable heating rates to those required for thermal decontamination of bulk packages in food processing facilities. Given the low thermal conductivity of low- a_w foods, in thermal processes including steam treatment, bulk packages require long times to achieve isothermal conditions and therefore high come-up-time can result in adaptation of cells and increase thermal resistance. Additionally, thermal process validations using the D-values reported in this study can be conducted for other thermal processes including steam treatment and radiofrequency.

Food processing facilities often need to determine processing conditions based on time, quality and economic evaluations. Thermal treatment might deteriorate the product physical properties; therefore, process conditions should be chosen to warranty food safety while not deteriorating the food product. Therefore, multiple process conditions are required to be tested for quality. Future studies, could conduct quality degradation

kinetics of important bioactive components in food samples. With this, food processors can determine process parameters that are efficient for microbial decontamination while preserving the quality of the product.

Additionally, determination of thermal inactivation kinetics of other pathogens possible surrogates for process validations is necessary to increase the knowledge on thermal resistances of other microorganisms that can be found in low- a_w . A database can be created with the results for several microorganisms in different low- a_w foods.

Finally, a thermal inactivation model for microorganisms can also be developed on different samples and treatment conditions. The model can be developed using the history of D and z values calculated influenced by a_w and other factors that are proven to affect its thermal resistance. The model can become a useful tool for determination of thermal process conditions required to ensure food safety in low- a_w processing facilities.

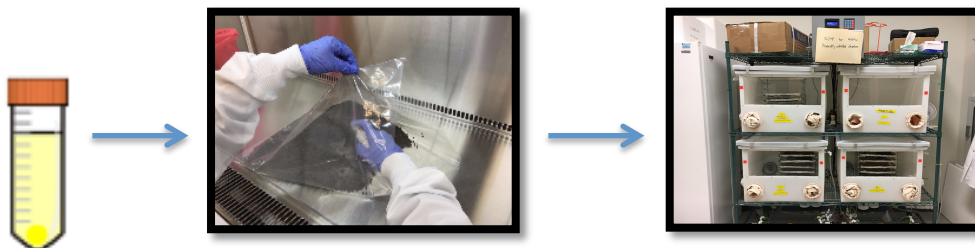
APPENDIX.**Appendix A.** *Salmonella* on mTSAYE an *E. faecium* on eTSAYE plates

(a) *Salmonella* on mTSAYE and (b) *E. faecium* on eTSAYE plates

Appendix B. Sample preparation and inoculation



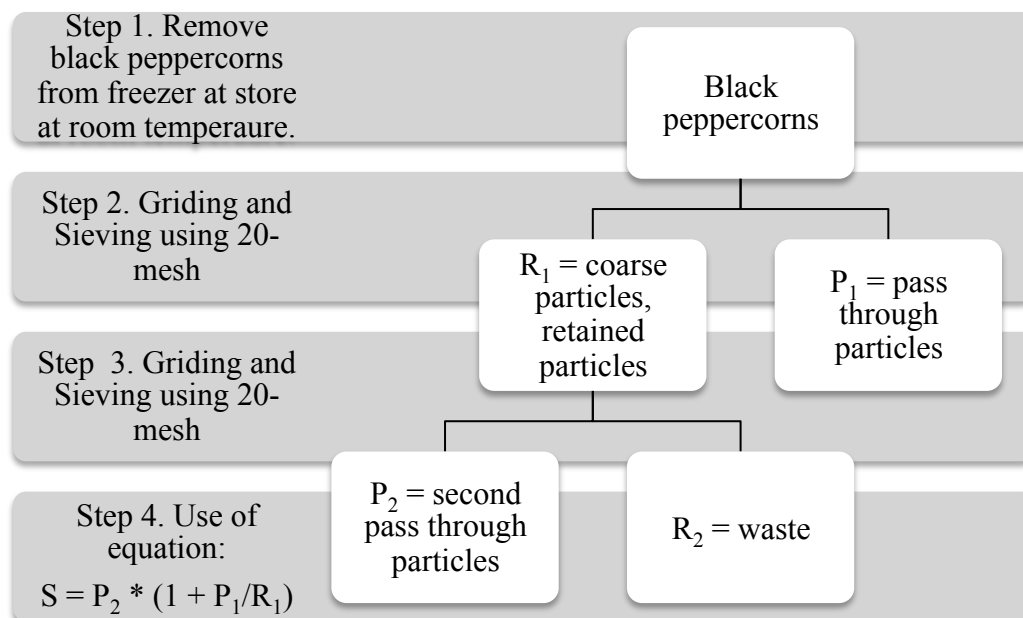
Step 1. Black peppercorns equilibrated to desire a_w (0.25, 0.45, 0.65).

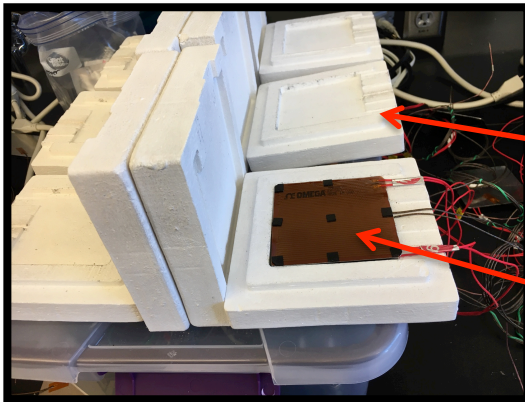


Step 2. Inoculum preparation, spray inoculation and re-equilibration to desire a_w (0.25, 0.45, 0.65).



Step 3. Grinding and re-equilibration to desired a_w (0.25, 0.45, 0.65).

Appendix C. Grinding process

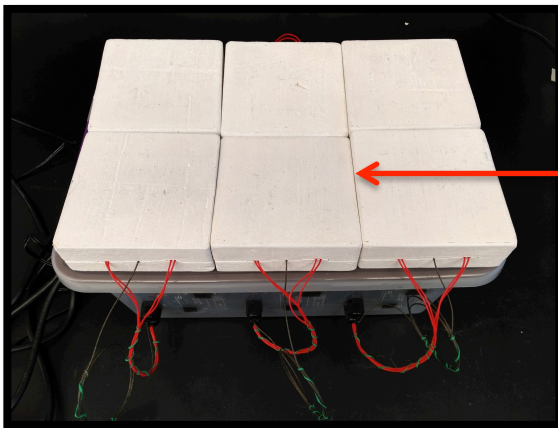
Appendix D. TDT sandwiches components

2 insulating ceramic
fiber blocks

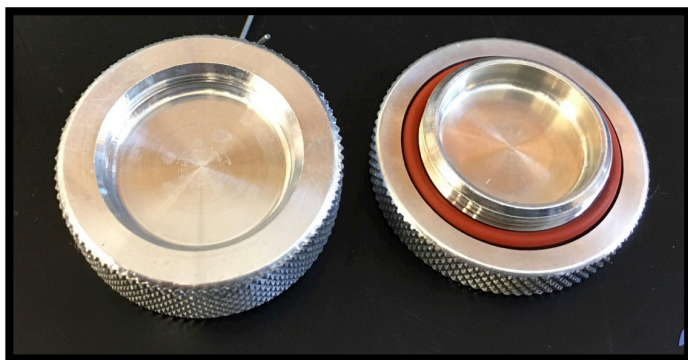
2 flat heating elements



Water-impermeable
aluminized plastic
pouches



18-independent
units of TDT
sandwiches to be
used at a given

Appendix E. TDT test cells

Appendix F. TDT test cells inside water bath during CUT determination

