Evaluation of the Thermal Inactivation Kinetics of Surrogate Vegetative Bacteria

A Thesis Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Amber Lynne Parham Renix

August 2015

Copyright © 2015 by Amber Renix
All rights reserved.

DEDICATION

In loving memory my beloved brother who pushed me to pursue my Master's Degree,

Dr. Deryk Cooper Jr, father, Milton Earl Parham, and Grandmother, Aletha Parham.

ACKNOWLEDGEMENTS

This process of preparing my thesis was not completed alone and I am appreciative of many people including my faith in God. I would first like to give the most appreciation to my advisor Dr. P. Michael Davidson. Thank you for all of your patience and encouragement and always going above and beyond to mentor me. Thank you for believing in me when I did not believe in myself. I also appreciate my committee members Dr. D'Souza and Dr. Golden for all of their contributions, advice, and encouragement.

I am deeply thankful for Dr. Hayriye Bozkurt and Dr. Emefa Monu. Thank you both for your mentorship. I appreciate all the patience and time you spent training me and providing encouragement. Thank you to all of my lab members Dr. Malcond Valladares, Ellen Simmons, Savannah Hawkins, Stella Chen, Sasha Wilkinson, and Marcel Schmidt for all their encouragement and assistance. I also would like to thank the faculty, staff, and students in the Department of Food Science & Technology for creating a friendly learning and working environment. A special thank you to my friends Marquetta Philips and Jacinda Dunn for all their positivity and prayers.

To all of my sorority sisters, thank you for all your support and love. A special thank you to Kayla Thompson, Ashley Omelogu, Christie Banks, and Rose Charm for your consistent encouragement, prayers, and motivational speeches. I am very appreciative of my friends Treva Miller, Corenthia Starks, and Teven Lee for all their actions of love and support.

Lastly, I would like to thank my entire God family for always being there and for pushing me to purse a Master's Degree. To my God sister, thank you so much for being available at any time to me. Thank you to my biological mother for being there for me throughout my collegiate career. To my brothers Al and Greg, thank you for your protective love and support. I am extremely thankful for my Aunt Mae and Uncle Robert. If God had not blessed me with reuniting us I am not certain I would have made it this far. I appreciate all of the sacrifices, love, and support it took to raise me.

ABSTRACT

Microbial surrogates are defined as non-pathogenic organisms with behavioral and/or survival characteristics correlating to those of specific pathogens. The purpose of this study was to determine the thermal inactivation kinetics (D and z-values) of *Lactobacillus bulgaricus* ATCC 11842*, Lactobacillus fructivorans* ATCC 8288*, Lactobacillus plantarum* ATCC 4008*, Listeria innocua* ATCC 33091, ATCC 51742, and 11/97, and *Staphylococcus carnosus* CS-299 as potential surrogates for hepatitis A virus (HAV; $D_{72°C}$ = 0.9 min). Additionally, the effect of sub-lethal heat on increasing the thermotolerance of *L. bulgaricus* was investigated. All strains were tested in phosphate buffered saline (PBS) as a preliminary step and, contingent upon the survival characteristics, further tested in ultra-high temperature (UHT) 2% fat milk and chopped spinach. Thermal inactivation experiments in PBS, UHT milk, and chopped spinach were performed at a temperature range of 58-70°C for 0-60 min. For sub-lethal heating, *L. bulgaricus* was subjected to 45 or 50°C for 30 min prior to inoculation of milk and subsequent heating at 70°C. *L. fructivorans, L. plantarum,* and all *L. innocua* strains had insufficient thermal resistance to be used for HAV surrogates. In contrast, D-values for *L. bulgaricus* in UHT milk were 9.98, 2.68, and 0.45 min, at 65, 67, and 70˚C, respectively, with a z-value of 3.7°C. When subjected to sub-lethal heating, the $D_{70°C}$ values for the 45 or 50˚C treatment were 0.34 or 0.48 min, respectively. Thus sub-lethal heating did not increase thermal resistance. D-values for *L. bulgaricus* in spinach were 43.29, 10.13, 3.20, 0.91 min for 63, 65, 67, 70°C. *S. carnosus* had D-values in PBS of 3.46, 1.27, and 0.47 min at

65, 67, and 70°C, respectively, and a z-value of 5.84°C. In milk *S. carnosus* D-values were 4.60, 1.94, and 0.46 min at 65, 67, and 70°C, respectively and in spinach D-values of 4.61, 2.42, 0.73 min were found at 65, 67, and 70°C, respectively. While D-values of *L. bulgaricus* and *S. carnosus* were lower than that of HAV, they may have potential value at high cell concentrations in validation studies for inactivation of HAV.

TABLE OF CONTENTS

CHAPTER I Introduction and General Information 1
Thermal Processing 1
CHAPTER II Review of Literature 6
Factors Affecting the Heat Resistance of Microorganisms 6
Thermal Processing of Foods 8
High Temperature Thermal Processing 9
Low Temperature Thermal Processing 10
Validation of Thermal Processes 14
Surrogate Microorganisms 15
CHAPTER III Materials and Methods 21
Thermal Inactivation of Surrogate Bacteria 21
Cultures 21
Growth Conditions and Preparation of Surrogate Bacteria 21
Phosphate Buffered Saline and UHT Milk 22
Chopped Spinach 23
Enumeration of Surviving Bacteria 24
Statistical Analysis 25
CHAPTER IV Results and Conclusions 26
CHAPTER V Conclusions and Recommendations 31
LIST OF REFERENCES 32
APPENDIX 36
VITA 42

LIST OF TABLES

Table 1. Thermal resistance (D- and z-values) of *Lactobacillus bulgaricus* ATCC 11842 and *Staphylococcus carnosus* CS-299 in phosphate buffered saline, UHT milk and chopped spinach. 27
Table 2. Effect of sub-lethal heating on the D-value of *Lactobacillus bulgaricus* ATCC 11842 at 70°C. 29
Table 3. Thermal inactivation projections of HAV and surrogates in spinach 30

LIST OF FIGURES

Figure 1. Survivor Curves for *Staphylococcus carnosus* CS-299 in phosphate buffered saline, 2%UHT milk and chopped spinach at 65, 67, and 70°C..... 37
Figure 2. Survivor curves for *Lactobacillus bulgaricus* ATCC 11842 in 2%UHT Milk and chopped spinach at 65, 67, and 70°C. 38
Figure 3. Thermal Death Time curves for *S. carnosus* CS-299 in phosphate buffered saline, 2% UHT milk and chopped spinach. 39
Figure 4. Thermal Death Time Curve for *L. bulgaricus* ATCC 11842 in 2% UHT milk and chopped spinach. 40
Figure 5. Log D-values of Vegetative Bacterial Surrogates in comparison to HAV..... 41

CHAPTER I

INTRODUCTION AND GENERAL INFORMATION

Thermal Processing

Thermal processing is one of the oldest preservation methods used to ensure the microbial safety of food and is often a critical control point in the HACCP (hazard analysis critical control point) system for food processors. Critical control points are procedures in a food process where a measure of control is applied as a means to prevent, eliminate, or reduce to the acceptable level a specific hazard *(18)*.Thermal processing is the use of heat to reduce the microbial hazards present within food as a measure to protect public health, inactivate spoilage microorganisms to increase shelf life, and to stop unfavorable enzymatic activity that causes spoilage *(29)*.

The penalties of a product failing to undergo the proper preservation method can be detrimental to an operation company resulting in billions of dollars lost, degradation of its reputation and brand, and, most importantly, the potential for illnesses and even deaths. The Centers for Disease Control and Prevention (CDC) reports that about 48 million Americans contract a foodborne illness annually, 128,000 are hospitalized, and 3,000 die. There are 31 known pathogens that cause 20% of these foodborne illnesses and 44% of deaths. Foodborne enteric viruses alone cause over half of these cases resulting in 58% of reports *(10)*. The top five contributors of acquired food-borne illnesses are Norovirus, nontyphoidal *Salmonella*, *Clostridium perfringens*, *Campylobacter* spp., and *Staphylococcus aureus*. *Salmonella*, *Toxoplasma gondii*, *Listeria*

monocytogenes, Norovirus, and *Campylobacter* spp. are the top five pathogens causing to foodborne illness resulting in death *(10)*. Contaminated food sources associated with foodborne illnesses include 46% produce, 22% meat and poultry, 20% dairy and eggs, and 6.1% fish and shellfish *(11)*.

Several methods for heat treating food products have been designed, including high temperature commercial sterilization, pasteurization, blanching, and even cooking. Commercial sterilization and pasteurization are the most efficient processes for ensuring food safety of packaged products from a microbial aspect *(29)*. Blanching and sous-vide (vacuum heating at low temperatures for extended times) are methods primarily used to inactivate enzymes and are capable of inactivating microorganisms at some times and temperatures *(32)*. All of these thermal processing systems include combinations of specified moist heating temperatures and times and cooling regimens that promote microbial death *(41)*.

The concept of thermal processing of food in containers to achieve a good quality product with an extended shelf-life was first accomplished in the early 1800's by Nicholas Appert. By creating the in-container sterilization system, Appert was responsible for inventing the method we call canning. This process is now known as commercial sterilization or appertization *(2)*. Louis Pasteur In 1864, unveiled the microbial science behind the results of thermal processing and packaging to prevent spoilage through development of a heating system to pasteurize wine *(1)*. Later, Bigelow and Ball developed the general method for mathematically analyzing the effects of heat

on the destruction of microorganisms over time (thermal inactivation kinetics), while Stumbo is known for his contributions of expanding these parameters (Featherstone, 2012).

Assessing the resistance of pathogens to heat is an essential component in determining the parameters of a thermal process. Processes are designed based on the thermal inactivation kinetics of the pathogen of most resistance using inactivation testing. To identify the level of inactivation needed, mathematical modeling of the thermal inactivation kinetics of a microorganism in the specific food product is required. Thermal inactivation parameters of particular microorganisms in food products may vary depending on the food matrix and required level of reduction. Death of microorganisms usually follows the mathematical model of a first-order reaction where the logarithmic value of microorganisms surviving a temperature is plotted against time *(1)*. The rate of death is a function of the slope of the death curve which is plotted as the number of survivors at each temperature. This relationship results in a linear decrease and creates what is referred to as a "survivor curve"*(41)*. The D-value or, decimal reduction time, is the time that it takes to achieve a 90% or 1 log decrease for a microorganism at a given temperature. As the temperature increases a D-value should decrease. The equation used to calculate D-value is as follows:

$$D = (t_2-t_1) / (\log N_1 - \log N_2)$$

where $N_1$and N_2 represent the surviving bacteria at times t_1 and t_2 respectively. The reaction rate constant (k) for first order reactions is calculated by k = 2.303/D

The z-value is the temperature increase required to change the log D-value by 90%. A z-value is used to measure the lethality of an organism at varying temperatures*(1)*. The equation used to calculate z-value is as follows:

$$z = (T_2 - T_1)/ (\log D_1 - \log D_2)$$

where D_1 and D_2 represent the D-values at temperatures T_1 and T_2 *(5)*.

To comply with the FDA regulations, validation studies must be conducted to confirm the inactivation of a microbial hazard of a calculated thermal process. However, it is not feasible in food-processing facility settings to validate thermal processes with pathogenic bacteria. Hence, a suitable nonpathogenic microorganism or surrogate is needed for process validation. The resistance of microorganisms used as surrogates must correlate with the resistance of the pathogen of concern in order to confirm the efficacy of the process*(17)*.

Thermal processing of foods to inactivate enteric viruses are not currently required in the U.S. Instead, means to prevent their introduction to food are enforced*(15)*. However there is published information showing that the thermal resistance of potentially the most heat resistant enteric virus (HAV) *(8, 9)* is greater than vegetative bacteria used as targets for thermal processes. In order to validate thermal processing for viruses, non-pathogenic vegetative bacteria or viruses to be used as potential surrogates should be identified. The most common bacterial surrogate used for pasteurization studies, *Listeria innocua*, is not heat resistant enough for validation studies *(24)*. Because viral surrogates would generally be more difficult to culture and handle, a bacterial surrogate

would be preferable. Thus, the objective of this study is (i) to identify potential surrogate bacteria for HAV in peptone buffered saline (PBS), ultra-high temperature (UHT) milk, and chopped spinach, (ii) to assess the effect of sub-lethal heating as an avenue to increase the thermal resistance of a surrogate, and (iii) to compare thermal inactivation kinetics of first-order models as means to provide insight on the validation during thermal processing for industrial applications.

CHAPTER II

REVIEW OF LITERATURE

Factors Affecting the Heat Resistance of Microorganisms

A number of factors affect the measured heat resistance of microorganisms including the microbial type, how the microorganisms are handled prior to heating, the heating system itself and the system used to recover viable cells *(40, 41)*. The type of microorganism is not only related to what the microorganism is, e.g., mold, yeast, bacteria, virus, but its genus, species and even strain. For example, bacteria can be only in the form of vegetative cells or produce endospores. Vegetative cells metabolize and grow in number when the proper nutrients and favorable environments are present *(1)*. Vegetative bacteria have relatively low resistance to thermal inactivation being susceptible to temperatures below 70°C *(13)*. Some gram positive bacteria have the ability to form endospores as a means of survival when unfavorable conditions are presented. All endospores form inside the vegetative cell and are referred to as sporeforming bacteria. Endospores are dormant and form a thick wall which protects them from extreme environments. As a result, they are much more resistant to heat than vegetative cells requiring temperatures above 90°C for inactivation. The two primary endospore forming bacterial genera are *Bacillus* and *Clostridium (1)*. Some viruses are more heat resistant than vegetative bacteria and require temperatures of 70°C and above. *Listeria monocytogenes, Clostridium botulinum,* and hepatitis A virus (HAV) are considered the most heat resistant vegetative, sporulating, and viral

pathogens, respectively. The inherent resistance level of bacteria will vary as different strains of the same species can show different levels of resistance *(41)*.

Prior to exposing cells to heat, how they are handled can influence their apparent heat resistance. For example, incubation temperature can influence heat resistance. Higher incubation temperatures may produce more resistant bacteria by increasing the rigidity of the cell membrane *(40)*. Cells grown to the stationary phase of growth are generally naturally more heat resistant than exponentially growing cells *(1)*. However, when exponentially growing cells are subjected to stressful environmental conditions such as sub-lethal heat, heat shock (subjecting to temperatures above optimum growth temperature), or starvation their stress response mechanisms are initiated and they develop an increased adaptation *(40)*. Heat shock can increase the resistance of a cell by increasing the proteins responsible for stress adaption *(12)*.

During heating, the pH of the food environment has shown to have an effect on the resistance level since high acid environments can enhance inactivation *(40, 41)*. Food with a pH of 4.5 or higher are considered low-acid while foods with a pH of lower than 4.5 are considered high-acid. The consideration of pH with inactivation has shown to be essential as spores of *Clostridium botulinum* vegetative cells are able to grow and produce toxin at pHs above 4.6 and therefore their spores must be inactivated by heat in foods with this pH range *(5, 41)*. Exposure during heating may be affected by the type of food. Heating in the presence of (up to 4%) of salt, and sugar, fat, and reduced water

activity can promote a protective response and increase thermal resistance of microorganisms *(1, 40, 43)*.

Following heating, the recovery media utilized can play a role in the apparent resistance of microorganisms. Microbiological media can be selective or non-selective. The media selected for recovery should possess the ability to support the growth of cells that may be injured as well as healthy or uninjured cells. Non-selective media are always preferred for recovery of stressed cells because they allow for repair and recovery of injured cells. Some selective media contain substances that are far more inhibitory to heat-injured cells than to healthy cells *(41)*. When the true number of surviving bacteria is not represented through the recovery media, the measured heat resistance will be misleading. As an example, when organic acids are present in the recovery media, the apparent resistance level can be decreased *(40)*. In some cases, adding starch, serum albumin, or charcoal to the subculture medium promoted better recovery of spores after heat treatments *(41)*.

Thermal Processing of Foods

Considering public health when manufacturing food products is necessary due to the fact that plant and animal materials are hosts to many microbial pathogens which cause foodborne illnesses *(1)*. There are also many non-pathogenic bacteria that cause spoilage. These bacteria gain access to food through the environment during harvest, storage, or distribution *(26)*. Most food, in its raw state has large amounts of natural microflora present *(1)*. In order to protect public health, the use of thermal energy can

be applied through processing procedures as a means to inactivate or prevent the growth of pathogens. Processes such as commercial sterilization, pasteurization, and blanching, focus on the delivery of heat to preserve products *(38)*. Most thermal processes have a target pathogen of concern or a target enzyme (affecting shelf-life or used as an indicator) to reduce or eliminate. Specificity of a preservation process should be determined by the parameters required to rid the target pathogens or enzymes and are dependent on the specific environment or food matrix *(13, 29)*. Target microorganisms are the pathogens of public health concern associated with a food and the ones identified to be most resistant to a thermal process. A thermal process should result in the practical elimination or reduction of these pathogens to the required levels *(19)*.

High Temperature Thermal Processing

Commercial Sterilization

Commercial sterilization or appertization, refers to a heating process used to produce foods that are “shelf stable”, i.e., able to be stored at ambient temperatures without spoilage by microorganisms. For low acid foods (pH > 4.6), commercial sterilization is designed to ensure the destruction of all pathogenic sporeforming bacteria (i.e., *Clostridium botulinum*) present in a food and any spoilage bacteria that could grow under normal storage conditions *(1)*. The term “commercially” is an indication that the process ensures that the product is free from all microorganisms that could grow under normal storage conditions but not that the product is completely sterile since

thermophilic sporeformers (e.g., *Bacillus stearothermophilus*) can survive but do not grow *(29)*. The regulatory requirement for processing of low-acid canned foods is 121°C for 3 min to inactivate the target pathogen *Clostridium botulinum* spores by 12-logs *(1, 3, 29)*. For high acid foods (pH < 4.6), products may be heated only to 100°C because *C. botulinum* cannot grow at this pH. The goal for these products is to destroy all spoilage microorganisms that could grow at ambient temperatures*(34)*. Steam, steam/air mixtures, and hot water are used as the heating media for commercial sterilization.

After a commercial sterilization treatment, products are hermetically sealed to guarantee protection from recontamination *(38)*. Commercially sterile products generally have a shelf-life of 1-2 years.

Low Temperature Thermal Processing

Blanching

Blanching refers to a heat process applied to fruits and vegetables prior to freezing, drying, or canning. The goal of the process is generally to inactivate unwanted enzymes and secondly to reduce microorganisms. Blanching also can be applied to soften vegetable tissue, enhance the natural color, and remove off flavors and gases *(32)*. Steam, water, or air can be used for heat transfer. The process commonly occurs in batch where products are held at a certain temperature for the specified amount of time needed to reach the reduction of target enzymes and microbes followed immediately by cooling *(26)*. Temperature ranges vary from 75 to 105°C depending on the type of food product and target enzyme or microorganism *(32)*.

Sous Vide

In this method, mild heat (55-60°C) is applied to vacuum-sealed food products for extended periods. It is extensively used in the restaurant industry *(36)*. Sous-vide is usually intended as a method of cooking to hold or chill a product that will retain its original nutritional and quality parameters *(6)*.

A process termed “heatshocking” has been suggested for some products as a way to preserve the quality parameters of a food *(35)* Rico, 2007. It can be used as a washing step and uses a process similar to high-temperature-short-time. Temperature ranges used are from 45-70°C for less than 5 min.

Pasteurization

Pasteurization is considered a mild thermal treatment that is usually applied to foods such as milk and milk products, fruit juices, beer, seafood, and most recently ready-to-eat or heat products *(29)*. The goal of pasteurization is to improve the quality of a product through reducing pathogens and spoilage microorganisms thereby extending shelf life. The process is designed to expose all particles of a food to the required temperature and time required to destroy all target vegetative pathogens, spoilage microorganisms and enzymes *(23)*. Following pasteurization, bacterial endospores and potentially some vegetative cells may not be destroyed, therefore a second preservation process must be used to extend shelf-life to 2-3 weeks such as refrigeration. Low temperature, long time (LTLT), high temperature, short time (HTST), ultrapasteurization, and ultra-high temperature (UHT) are types of processing possible under pasteurization

(29). Products can be introduced to heat in a batch or continuous flow design. Pasteurization of liquids is commonly performed in a continuous flow process using a plate heat exchanger. The time and temperature are controlled by a holding tube and flow diversion valve, respectively. Batch processes are conducted by holding the product in a vessel for a specified time and temperature. The heating media are customarily steam and/or hot water *(38)*.

Pasteurization regulations for food products

Milk pasteurization requirements are governed by the FDA Pasteurized Milk Ordinance (PMO). They state that milk should receive a treatment of 72°C for 15 sec during an HTST process or 63°C for 30 min using a batch process or LTLT process *(22)*. These regulations were developed using the requirements to inactivate the most heat resistant target microorganism, *Coxiella burnetii*, a rickettsia bacteria which causes Q fever in humans *(16)*. For extended shelf-life refrigerated milk products, temperatures above 138°C for at least 2 sec are required for ultrapasteurization. Temperature of 135-150°C for 4-15 sec create ultra-high temperature (UHT) milk product that can be held at room temperature. UHT and ultrapasturized products have an extended shelf life of 6-8 weeks *(29)*.

Regulations for pasteurization of egg and egg products, which are governed by USDA Food Safety Inspection Service regulations and FDA criteria 21 CFR Parts 16 and 118, target the reduction of *Salmonella*. The process requires an 8.75-log reduction of

Salmonella for liquid eggs such as 56.7°C for 3.5 min, and 5-log reduction for whole eggs *(20)*.

The FDA regulates seafood through the Fish and Fisheries Products Hazards and Control Guidance and suggest a 90°C for 10 min to achieve a 6 log reduction of the target pathogen, *Clostridium botulinum* type E and non-proteolytic *C. botulinum* types B and F. However, for crab meat a process achieving a >12 log decrease in *C. botulinum* type E spores is needed requiring a process of 30 min at 85°C *(21)*.

Pasteurization treatments for ready-to-eat products are dependent on the characteristics of the food and the pathogen of concern. The European Chilled Food Federation suggests processes provide a 6 log reduction of the target pathogens *Listeria monocytogenes* and *C. botulinum* in ready-to-eat products. The addition of modified atmosphere or vacuum packaging may also be used for RTE products *(14)*.

Due to the fact that conventional pasteurization methods, especially those with extended times, can result in the degradation of the nutritional and sensory quality of a food, consumers are requesting less processed foods and the use of less harsh technologies such as microwave pasteurization *(29)*. Microwave pasteurization occurs at faster rate resulting in a quicker process and, while still achieving death of microorganisms through heat, less degradation of nutrients and preservation of sensory characteristics. In this technique heat is conducted through a rapidly changing electromagnetic field *(2)*. Applying microwave technology to achieve microbial safety occurs similarly to thermal pasteurization however unpredictable uneven heating

effects occur. This makes it difficult or impossible to predict the slowest heating zone in a package increasing the possibility of microbial survival in treated products *(23)*.The efficacy of inactivation achieved using microwave heating has been evaluated with *Bacillus cereus, Campylobacter jejuni, Clostridium perfringens,* pathogenic *Escherichia coli, Enterococcus* spp.*, L. monocytogenes, S. aureus,* and *Salmonella (2, 23).* These bacteria have all been found to survive various microwave treatments, possibly as a result of uneven heat distribution due to unsystematic cold spots. A microwave pasteurization batch process of milk at 71.1°C for 8, 3, and 10 min was found to cause complete inactivation (8 to 9 log) for *Y. entercolitica, C. jejuni,* and *L. monocytogenes*, respectively *(23)*.

Validation of Thermal Processes

According to the Food Safety Modernization Act, all food companies are required to conduct an analysis of possible hazards that could affect a product, apply the appropriate preventative measures of control, and monitor or validate those means of control *(18)*. The validation of a measure entails the collection and evaluation of scientific and technical data as evidence to determine if a measure of control will effectively and consistently control a hazard. Scientific and technical data can be obtained through inoculated pack challenge studies conducted in facility pilot plants. The processes are determined by the pathogen of highest resistance with its corresponding non-pathogenic or surrogate bacteria. Locations within the process and

food matrix that are expected to have the lowest temperature or cold spot are the primary focus of study.

Surrogate Microorganisms

Within microbiology, surrogates can be any non-pathogenic bacteria or other microorganism used to study the fate of a pathogenic microorganism in a specific environment. Surrogates in history were first used as an indicator of fecal contamination in water *(37)*. Now, the overall goal of using a surrogate microorganism is to validate the effectiveness of sanitization, inactivation or inhibition processes as a means to ensure control of pathogenic microorganisms. When choosing a surrogate for a validation study the physiological and environmental resistance characteristics should mimic that of the target pathogen when exposed to similar conditions. Surrogates grow to high numbers, be simple to cultivate and be easy to detect and recover. Because they are expected to be used with industrial processing equipment, they should not affect the long term use of that equipment when used for a validation study *(37)*. Using non-pathogenic bacteria is ideal because they are biologically safe and possess little risk to public health. As would be expected, introducing a pathogen into a food processing facility for a process validation study is high risk and the consequences of mismanagement could be detrimental *(17)*. The FDA and USDA have deemed the use of surrogate bacteria permissible and the validation of a lethal process is to be part of a company's HACCP plan. In a validation study, products are inoculated with a surrogate bacteria, the product is processed using the lethal process to be validated, and any surviving bacteria

enumerated. When used for thermal process validation, the thermal inactivation kinetics of a selected surrogate should be equal to or higher than that of the target pathogen *(31)*. Thus eliminating the surrogate would indicate that all pathogens of equal or less resistance should also have been killed. Selecting an appropriate surrogate will vary with the food matrix and target pathogen for evaluation *(17)*.

Surrogates have been used in the validation of commercial sterilization and pasteurization processes. For the validation of sterilization, the sporeformers *Clostridium sporogenes* and *Bacillus stearothermophilus* have been used in the place *Clostridium botulinum*. For many food products the targets may be the pathogens *Listeria monocytogenes* or Shiga toxin-producing *Escherichia coli.* The surrogates for these microorganism have been the non-pathogenic *Listeria innocua* and non-pathogenic biotype I *Escherichia coli (19)*.

Surrogates for Thermal Inactivation of Enteric Viruses

HAV is a concern because it can be present on any food that involves human contact. The CDC reports that 45% of foodborne illnesses were caused by human enteric viruses in 1998-2008 *(30)*. These viruses are only able to replicate in live hosts cell and have the ability to cause infections at low doses of 10-10^2. Currently, the primary method to control HAV is to prevent contamination instead of methods of control or reduction *(15, 27)*. Berries, leafy vegetables, and ready-to-eat meats are examples of vehicles of HAV transmission. Recently, Bozkurt et. al found that HAV has D-values at 72°C of 0.88-0.91 min in cell culture media and spinach with z-values of 12.51-13.92°C. The current

guidelines for eradicating the target pathogen of highest resistance have historically been *L. monocytogenes*. However, the heat resistance of *L. monocytogenes* is lower than HAV which calls for a re-examination of the requirements. Problems with using HAV as the target pathogen are the difficulty and complexity of handling and recovering the virus and the fact that it is pathogen. If HAV is accepted as the new target of concern, a new more heat resistant surrogate is needed. It is surprisingly difficult to identify non-pathogenic vegetative bacteria with heat resistances that approach that of HAV. Some potential candidates are *Staphylococcus carnosus,* lactic acid bacteria, including *Lactobacillus bulgaricus*, and specific strains of *L. innocua.*

Staphylococcus carnosus

Staphylococcus carnosus is a gram positive bacterium that can be isolated from processed meats, soy sauce, and fish-based sauces. It is used as a starter culture for fermented meats as it contributes to the flavor and color of the meats due to reduction of nitrate to nitrite and production of other compounds *(4)*. The bacterium was studied as a potential surrogate for *E. coli* and *Salmonella* in frankfurter batter and ground beef of varying fat levels. However, it was reported to have significantly higher thermal resistance compared to the proposed target pathogens so was not recommended as a surrogate. The reported $D_{55°C}$-values for S. carnosus were 152.63, 113.57, 102.94, and 74.35 min at 55°C in ground beef at 7, 15, 27% fat and frankfurter batter, respectively *(43)*.

Lactic acid bacteria

Lactic acid bacteria are gram-positive, non-sporeforming, fermentative rods or cocci capable of producing lactic acid as a major end product of the fermentation of glucose. Lactic acid bacteria are either homofermentative (primary end product of lactic acid) or heterofermentative (end products of lactic acid, carbon dioxide, and ethanol). These bacteria are extensively used in the food industry as starter cultures for many fermented products and are a major part of the human diet. Products produced from lactic acid bacteria include cheese, yogurt, buttermilk, kefir, kumiss, sauerkraut, pickles, kimchi, olives, fermented meats, sourdough breads, soy sauce, and some alcoholic beverages *(28)*.

Lactobacillus bulgaricus is one of the most important lactic acid bacteria. It is used with *Streptococcus thermophilus* in starter cultures for yogurt and "high heat" cheeses such as provolone and Swiss. The bacteria is usually added to yogurt proceeding its treatment by a method of preservation such as spray drying or freeze drying *(39)*. High and low temperatures during culture preservation and process fermentation can cause stress to cells of *L. bulgaricus* resulting in changes in the behavioral characteristics *(25)*. The mechanisms of sub-lethal stress and methods to protect this bacterium are frequently studied because the dairy industry is interested in starter culture bacteria that can tolerate stressful environments. Thus, the effect of lethal processes on *L. bulgaricus* are much less studied.

Lactobacillus fructivorans is a heterofermentative lactic acid bacteria that is widely used in alcohol fermentation. It is important for its contribution to flavor during the ripening process of fermented foods. Some strains of *L. fructivorans* can causes spoilage and have been isolated from spoiled mayonnaise, salad dressing, ketchup, and vinegar. The species can be reported to have a high tolerance with a D-value at 65°C of 1.2 min in ketchup *(7)*.

Lactobacillus plantarum is considered a nonstarter lactic acid bacteria (NSLAB) that assists in ripening and enhanced flavor of cheeses, such as cheddar and Italian. It is also important in vegetable fermentations. The presence of this bacteria post-pasteurization suggests it is relatively thermotolerant. *L. plantarum* DCP 2103 in MRS broth and skim milk demonstrated D-values at 65°C of 0.52 and 0.32 min, respectively. *L. plantarum* DPC2739 had D-values at 60, 72, and 75°C in sterile milk of 32.9, 14.7, and 7.14 s, respectively *(12)*.

Listeria innocua

Listeria innocua is a gram positive, rod shaped organism and typically found in soil and food sources. Due to the behavioral and resistance similarities to *L. monocytogenes* but lack of pathogenicity, *L. innocua* can be used as a surrogate for *L. monocytogenes* one of the most heat resistant vegetative pathogens. In liquid whole eggs, ultrapasteurization at 70°C for 1.5 min would result in 3.5, 5.0, and 6.5 log reductions in *L. innocua* STCC 4030, *L. monocytogenes* 5672, and *L. monocytogenes* 4032, respectively *(33)*. D-values at 70°C in hamburger patties for *L. innocua* M1 and *L. monocytogenes* F4243 were 0.34

and 0.16 min, respectively, making *L. innocua* M1 a suitable surrogate *(24)*. *Listeria innocua* strains have been used extensively in research where a highly heat resistant vegetative bacterial surrogate was needed and it has actually been considered the “go-to” surrogate. However, with new information on the heat resistance of hepatitis A virus, its use may not be the “best” fit.

CHAPTER III

MATERIALS AND METHODS

Thermal Inactivation of Surrogate Bacteria

Cultures

Bacterial cultures were obtained from ATCC (Manassas, VA) or Chr. Hansen (Milwaukee, WI). A total of seven non-pathogenic bacterial strains where evaluated as potential surrogates. *Lactobacillus bulgaricus* ATCC 11842, *Lactobacillus fructivorans* ATCC 8288, *Lactobacillus plantarum* ATCC 4008, *Listeria innocua* ATCC 33091, ATCC 51742, and 11/97, and *Staphylococcus carnosus* CS-299.

Growth Conditions and Preparation of Surrogate Bacteria

Media used for growth included de Man, Rogosa, and Sharpe (MRS; Difco, Becton Dickinson, Franklin Lakes, NJ) broth for *Lactobacillus* species and Brain Heart Infusion (BHI; Difco, Becton Dickinson) broth for *Listeria innocua* strains and *Staphylococcus carnosus*. Stock cultures were created using the appropriate broth medium and 15% (vol/vol) glycerol and stored at -80°C. Fresh cultures for thermal inactivation tests were prepared by transferring 0.20 µl (microliters) of frozen stock to the appropriate broth medium and incubating for 24 h to stationary phase using optimum incubation temperatures. *Lactobacillus bulgaricus* was incubated under anaerobic conditions at 37°C using gas campy container system packs (Becton Dickinson), other *Lactobacillus* and L*. innocua* under aerobic conditions at 37°C, and *S. carnosus* under aerobic

conditions at 30°C. All strains were sub-cultured overnight twice prior to thermal inactivation experiments. Prior to experimentation the cells were plated on MRS or BHI agar to determine the initial number. Next, cells were isolated from the broth medium by centrifuging 1.9 mL for 3 min at 5,000 x g and washed twice with sterile phosphate buffered saline (PBS; Difco, Becton Dickinson). Following the last centrifugation, pellets were resuspended to 10^8 colony forming units/milliliter (CFU/mL) in PBS. When testing *Lactobacillus bulgaricus* in milk, the pellets were resuspended in UHT milk.

Phosphate Buffered Saline and UHT Milk

Thermal inactivation of surrogate bacteria in PBS and 2% fat UHT milk (Hershey's, Hershey, PA) was performed in sterile 2 mL glass screw-capped vials in a circulating water bath (Haake model V26, Karlsruhe, Germany). UHT milk was aliquoted into 50 mL batches in sterile plastic tubes and stored under refrigeration until use. Vials were filled with 2 mL of culture in either PBS or UHT milk from to achieve an initial count of 10^7 CFU/mL. Heat treatments were performed at 65, 67, 70°C for varying times (0-60 min). The come-up time and internal temperature of the vials were measured using a Type T thermocouple (Omega Engineering, Inc., Stamford, CT) placed at the geometric center of the vial through the lid. Thermocouples were connected to MMS3000-T6V4 type portable data recorder (Commtest Inc., New Zealand) to monitor temperature. The temperature of the circulating water bath was monitored using thermocouples and a mercury-in-glass thermometer. The treatment time began when the target temperature was reached in the vials and the come up time was recorded. After treatments, vials

were cooled immediately in an ice bath to stop further heat transfer. The outside of the vial were sterilized with 70% ethanol prior to opening.

Sub-lethal treatments in milk

Cells in UHT milk were subjected to a sub-lethal heat treatment (45 or 50° fro 30 min) using the circulating water bath and 2mL glass vials containing 10^8 CFU/mL of milk. Temperatures were measured as described previously and upon completion of the designed heating process vials were removed and allowed to cool for 30 min at room temperature. This heat-treated culture was then inoculated into fresh UHT milk at 10^7 CFU/mL which was added to 2 mL glass vials. Thermal inactivation was performed as described previously.

Chopped Spinach

Frozen chopped spinach (Kroger; Cincinnati, OH) was purchased from a local grocery store and allowed to thaw completely overnight under refrigeration before use. To create a homogenized sample, thawed chopped spinach was blended (Waring blender, Model 1063, Waring Commercial, USA) and sterilized at 121°C for 15 min, aliquoted in 50 g (gram) batches in sterile plastic tubes and stored under refrigeration until use. For inoculation, a sample of spinach was placed in a sterilized beaker and inoculated gradually with a bacterial culture with constant stirring over a period of 20 min. The target initial count was 10^7 CFU/g. Ten gram samples of inoculated spinach were aseptically weighed into 13 cm x 19 cm polyethylene/nylon bags (Seco Industries, Los Angeles, CA). Two 10 g un-inoculated spinach samples were used as controls. One was

used as a negative microbiological control (i.e., plated to determine presence of contaminants) and the other was used for monitoring the internal temperature oat the geometric center of the spinach in the bags with a Type T thermocouple (Omega). All bags were vacuum-packaged and sealed with a Multivac A300/16 vacuum-packaging unit (Sepp Haggemuller KG, Wolfertschwenden, Germany). Packages were manipulated to achieve approximately uniform thickness to ensure even heat distribution.

Thermal inactivation of bacteria in spinach was performed in the circulating water bath described previously using a thermospacer (plastic holder for multiple bags) filled with the spinach samples. As before, temperatures were monitored using thermocouples and come-up time was measured. After the appropriate treatment, samples were immediately placed in an ice bath. The outer surface of bags were rinsed with 70% ethanol and aseptically opened with sterile scissors. Bags were filled with 90 mL of PBS and stomached (Seward Stomacher 400 Circulator) at 260 rpm for 90 sec.

Enumeration of Surviving Bacteria

Untreated and treated samples were serially diluted in sterile 0.1% peptone or PBS, spread-plated on appropriate agar medium and incubated under optimal conditions for 48 h. Incubation for *L. bulgaricus* was done anaerobically on MRS agar using gas campy container system packs (Becton Dickinson). *S. carnosus* and *L. innocua* were incubated aerobically on BHI at 30 and 37°C, respectively, and other *Lactobacillus* strains were incubated aerobically on MRS at 37°C. Following incubation, colonies were counted and survivor curves plotted as the log CFU/mL or g versus time to determine D-values.

Z-values were determined by plotting the log D-values versus temperature.

Statistical Analysis

The heat resistance data was statistically analyzed using an analysis of variance (ANOVA) with SAS software (version 9.4, SAS Institute, Cary, NC, USA) to determine if there were statistically significant differences in thermal resistance among the surrogates in different media. Mean comparisons were analyzed using a Tukey's with 95% confidence intervals. All experiments were replicated thrice.

CHAPTER IV

RESULTS AND CONCLUSIONS

The thermal inactivation the selected strains of vegetative bacteria was first measured in PBS to determine if they were capable of survival at temperatures as high as 70°C so they might be used as surrogates for HAV ($D_{72°C}$ ~ 0.9 min). *Lactobacillus bulgaricus, L. fructivorans, L. plantarum,* were found to be inactivated during the come-up-time at 65, 67, and 70°C, respectively. For the strains of *Listeria innocua*, either they did not survive come-up-time at 65°C or they had very low D-values of 1.-1.5 min at 58°C (data not shown). In contrast, *S. carnosus* was capable of surviving in PBS for 0.47 min at 70°C (Table 1). The D- and z-values for *S. carnosus* in PBS are shown in Table 1, the survivor curves are shown in Fig. 1, and the z-value curve is shown in Fig. 3. As PBS does not provide any protective elements for bacteria during exposure to heat the D-values would be expected to be lower than in foods. Thus, all except *L. bulgaricus* and *S. carnosus* were not studied further.

L. bulgaricus and *S. carnosus* both survived 65-70°C in 2% fat UHT milk (Table 1). D-values in milk for *L. bulgaricus* were 9.98, 2.68, and 0.45 min and for *S. carnosus* were 3.36, 1.27, and 0.47 min at 65, 67, 70°C, respectively. The z-values of *L. bulgaricus* and *S. carnosus* were 3.73 and 4.98°C, respectively. Survivor curves in UHT milk for *S. carnosus* and *L. bulgaricus* and are shown in Fig. 1 and 2, respectively, and the z-value curves are shown in Fig. 3 and 4, respectively.

Table 1. Thermal resistance (D- and z-values) of *Lactobacillus bulgaricus* ATCC 11842 and *Staphylococcus carnosus* CS-299 in phosphate buffered saline, UHT milk and chopped spinach.

Microorganism	Heating Medium	Temp (°C)	D-value (min)	SD (±)	z-value (°C)
L. bulgaricus ATCC 11842	PBS	65	ND[1]		
	2% Fat UHT Milk	65	9.98 A[2]	1.03	3.73
		67	2.68 A	0.16	
		70	0.45 A	0.08	
	Chopped Spinach	63	43.29	3.03	4.21
		65	10.13 A	1.34	
		67	3.20 A	0.20	
		70	0.91 A	0.13	
S. carnosus CS-299	PBS	65	3.46 B	0.50	5.84
		67	1.27 A	0.26	
		70	0.47 A	0.13	
	2% Fat UHT Milk	65	4.60 B	0.82	4.98
		67	1.94 A	0.26	
		70	0.46 A	0.02	
	Chopped Spinach	65	4.61 B	0.41	6.24
		67	2.42 A	0.41	
		70	0.73 A	0.02	

[1]Not detected after come-up-time.

[2]D-value followed by the same letter within each temperature is not significantly different (P=0.05).

D-values in chopped spinach for *L. bulgaricus* ranged from 43.29, 10.13, 3.20, and 0.91 min at 63, 65, 67, 70°C, respectively and the z-value was 4.21°C. *S. carnosus* had D-values of 4.61, 2.42, 0.73 min at 65, 67, and 70°C with a z-value of 6.23°C. The survivor curves and z-value curves for *S. carnosus* and *L. bulgaricus* in chopped spinach are shown in Fig. 1-2 and Fig. 3-4, respectively.

Using sub-lethal heating as a measure to the increase thermal resistance of *L. bulgaricus* was observed to not have an effect (Table 2). It has been documented that sub-lethal heating can cause increased thermotolerance. Teixeira et al. *(39, 42)* demonstrated that performing sub-lethal heat treatments on exponentially growing cells could result in increased thermal resistance compared to stationary cells. At the stationary phase of growth, a cell has already developed the protective measures it naturally possesses. Thus sub-lethal heating of stationary phase cells may not alter the protection mechanisms. In the present study, sub-lethal heating was carried out on bacteria already in the stationary phase, which could explain why the D-values for *L. bulgaricus* in milk at 70°C were similar with or without a sub-lethal treatment.

It is known that when bacteria are present in food, resistance is influenced by components of the food matrices. Intrinsic properties of foods such as water activity (a_w), pH, and salt content of food have contribute the most to microorganisms ability to withstand a thermal process. However, while the D-values for *L. bulgaricus* and

Table 2. Effect of sub-lethal heating on the D-value of *Lactobacillus bulgaricus* ATCC 11842 at 70°C.

Heating medium	Sub-lethal Heating (°C)	D-value (min)
2% Fat UHT Milk	None	0.45 A[1]
	45	0.34 A
	50	0.48 A

[1] Same letter indicates no significant difference between D-values (P=0.05).

S. carnosus in milk and spinach were different, the food medium did not have a significant effect on the heat resistance of these surrogates. When comparing the D-values of the present study with D-values in the literature, the D_{64} for *L. bulgaricus* in skim milk was 10.87 min and the D_{55} of *S. carnosus* in frankfurter batter of 74.35 min were comparable *(42, 43)*.

The goal of this research was to find potential surrogates that had thermal resistance characteristics similar to that of HAV for the purpose validating inactivation during thermal processing. A suggested thermal process (*19*) for HAV would be a 6 log (6D) reduction in viable cells. HAV in spinach has a D_{72} of 0.91 min and would thus require a 5.4 min process to inactivate 6 logs of the virus. In order to make this comparison the D-values for *L. bulgaricus* and *S. carnosus* were calculated for 72°C using the z-values. The $D_{72°C}$ for *L. bulgaricus* and *S. carnosus* in spinach are 0.30 and 0.35 min (Table 3), respectively. 6D projections for *L. bulgaricus* and *S. carnosus* would be 1.8 min and 2.1

min, respectively. In order to validate a thermal process for HAV, the initial cell numbers for each bacteria would need to be 10^{18} and 10^{15} CFU, respectively. Achieving a cell concentration this high is not practical. However, for validating a 2D process for HAV (1.8 min) using *L. bulgaricus* (0.6 min) and *S. carnosus* (0.7 min) would require an initial cell concentration of 10^{6-8} is achievable. Thus, *L. bulgaricus* and *S. carnosus* could be used as surrogates during validation using a 2D reduction model for HAV.

Table 3. Thermal inactivation projections of HAV and surrogates in spinach

Microorganism	D_{72} (min)	6D at 72°C (min)	Log Reduction After 5.4 min at 72°C	2D at 72°C (min)	Log Reduction After 1.8 min at 72°C
Hepatitis A (HAV)	0.91	5.4	6	1.8	2
***L. bulgaricus* ATCC 11842**	0.30	1.8	18	0.6	6
***S. carnosus* CH-299**	0.35	2.1	15.4	0.7	5.1

CHAPTER V

CONCLUSIONS AND RECOMMENDATIONS

Identifying the appropriate surrogate for HAV is important due to the continuing of outbreaks and recent findings suggesting that it should be the target pathogen of concern instead of *L. monocytogenes*. This research was conducted to identify a vegetative non-pathogenic bacteria that demonstrates similar heat resistance of HAV for the use of easily validating thermal processes designed to inactivate this virus. Of the bacteria tested,

L. bulgaricus and *S. carnosus* demonstrated the closest correlation to HAV but are not heat resistant enough to use in the 6D reduction model required for HAV. Also, using sub-lethal heat as means to increase thermal resistance was not successful. These bacteria are ideal surrogates when using a 2D reduction model. Hence, for the advancement of thermal processes more research must be applied to find a suitable vegetative surrogate for HAV.

LIST OF REFERENCES

1. Adams, M. R., and M. O. Moss. 1995. Food microbiology. The Royal Society of Chemistry, Guildford, UK.
2. Ahmed, J., and H. S. Ramaswamy. 2007. Microwave pasteurization and sterilization of foods. p. 692-711. *In*, Handbook of food preservation Taylor & Francis Group, LLC.
3. Anderson, N. M., J. W. Larkin, M. B. Cole, G. E. Skinner, R. C. Whiting, L. G. Gorris, A. Rodriguez, R. Buchanan, C. M. Stewart, J. H. Hanlin, L. Keener, and P. A. Hall. 2011. Food safety objective approach for controlling Clostridium botulinum growth and toxin production in commercially sterile foods. *Journal of Food Protection*. 74:1956-89.
4. Anonymous. Date, Staphylococcus carnosus. Available at: http://www.tgw1916.net/Staphylococcus/carnosus.html. Accessed April 10,2015.
5. Awuah, G. B., H. S. Ramaswamy, and A. Economides. 2007. Thermal processing and quality: Principles and overview. *Chemical Engineering and Processing: Process Intensification*. 46:584-602.
6. Baldwin, D. E. 2011. Sous vide cooking: a review. *International Journal of Gastronomy and Food Science*.
7. Bjorkroth, K. J., and H. J. Korkeala. 1997. Lactobacillus fructivorans spoilage of tomato ketchup. *Journal of Food Protection*. 60:505-509.
8. Bozkurt, H., D. H. D'Souza, and P. M. Davidson. 2014. A comparison of the thermal inactivation kinetics of human norovirus surrogates and hepatitis A virus in buffered cell culture medium. *Food Microbiol*. 42:212-7.
9. Bozkurt, H., X. Ye, F. Harte, D. H. D'Souza, and P. M. Davidson. 2015. Thermal inactivation kinetics of hepatitis A virus in spinach. *International Journal of Food Microbiology* 193:147-51.
10. CDC. 2011. CDC- estimates of foodborne illness in the united states. p. 2. *In* C.f.D.C.a. Prevention (ed.), Atlanta, GA.
11. CDC. 2011. CDC- estimates of foodborne illness in the united states. *In* C.f.D.C.a. Prevention (ed.), Atlanta, GA.
12. De Angelis, M., R. Di Cagno, C. Huet, C. Crecchio, P. F. Fox, and M. Gobbetti. 2004. Heat shock response in lactobacillus plantarum. *Applied and Environmental Microbiology*. 70:1336-1346.
13. Dooley, J. S. G., and T. A. Roberts. 2000. Control of vegetative micro-organisms in foods. *British Medical Bulletin*. 56:142-157.
14. ECFF. 2006. Recommendations for the production of prepackaged chilled food. *In* E.C.F. Federation (ed.).
15. EFSA. 2011. Scientific opinion on an update on the present knowledge on the occurrence and control of foodborne viruses. *European Food Safety Authority Journal*. 9:2190
16. Enright, J. B. 1961. The pasteurization of cream, chocolate milk and ice cream mixes containing the organism of Q fever. *Journal of Milk and Food Technology* 24:351-355.

17. F.F. Busta, T. V. S., M.E. Parish, L.R. Beuchat, J.N. Farber, E.H. Garrett, L.J. Harris. 2003. The use of indicators and surrogate microorganisms for the evaluation of pathogens in fresh and fresh-cut produce *Comprehensive Reviews in Food Science and Food Safety*. 2:179-185.
18. FDA. Full text of the food safety modernization act. *In* U.S.F.D. Administration (ed.) U.S. Government Printing Office.
19. FDA. Methods to Reduce/Eliminate Pathogens from Produce and Fresh-Cut Produce. *In* U.S.F.D. Administration (ed.).
20. FDA. 2009. Prevention of Salmonella enteritidis in shell eggs during production, storage, and transportation. *In* U.S.F.D. Administration (ed.).
21. FDA. 2011. Fish and fisheries products hazards and control guidance. *In* U.S.F.D. Administration (ed.).
22. FDA. 2011. Grade a pasteurized milk ordinance. p. 1-388. *In* U.S.F.D. Administration (ed.).
23. Foods, N. A. C. o. M. C. f. 2006. Requisite scientific parameters for establishing the equivalence of alternative methods of pasteurization *Journal of Food Protection*. 69:1190-1216.
24. Friedly, E. C., P. G. Crandall, S. Ricke, C. A. O'Bryan, E. M. Martin, and L. M. Boyd. 2008. Identification of Listeria innocua surrogates for Listeria monocytogenes in hamburger patties. *Journal of Food Science*. 73:M174-M178.
25. Gouesbet, G., G. Jan, and P. Boyaval. 2001. Lactobacillus delbrueckii ssp bulgaricus thermotolerance. *Lait*. 81:301-309.
26. Heldman, D. R., and R. W. Hartel. 1997. Principles of food processing. Chapman & Hall, New York.
27. Jaykus, L.-A., D. H. D'Souza, and C. L. Moe. 2012. Food microbiology: fundamentals and frontiers ASM Press, Washington, DC.
28. Konig, H., and J. Frohlich. 2009. Lactic Acid Bacteria. *In*, Biology of Microorganisms on Grapes, in Must and in Wine Springer-Verlag Berlin Heidelberg, Mainz, Germany.
29. Kumar, P., and K. P. Sandeep. 2014. Thermal principles and kinetics. p. 17-31. *In* S. Clark, S. Jung, and B. Lamsal (ed.), Food Processing: Principles and Applications John Wiley & Sons, Ltd, Somerset,NJ.
30. L. Hannah Gould, P., M. Kelly A. Walsh, D. Antonio R. Vieira, M. Karen Herman, P. Ian T. Williams, D. Aron J. Hall, and D. Dana Cole, PhD. 2013. Surveillance for foodborne disease outbreaks- united states, 1998-2008. *In* C.f.D.C.a. Prevention (ed.) Office of Surveillance, Epidemology, and Laboratory Services, Atlanta, GA.
31. LI MA, J. L. K., GUODONG ZHANG, CHIA-MIN LIN, MICHAEL P. DOYLE. 2007. Development of thermal surrogate microorganisms in ground beef for in-plant critical control point validation studies. *Journal of Food Protection*. 70:952-957.
32. Mazzotta, A. S. 2001. Heat resistance of Listeria monocytogenes in vegetables: Evaluation of blanching processes. *Journal of Food Protection*. 64:385-387.

33. Monfort, S., N. Sagarzazu, E. Gayán, J. Raso, and I. Álvarez. 2012. Heat resistance of Listeria species to liquid whole egg ultrapasteurization treatment. *Journal of Food Engineering*. 111:478-481.
34. National Canners Association. Research, L. 1975. Canned foods : principles of thermal process control and container closure evaluation. Berkeley, CA : Food Processors Institute, Berkeley, CA.
35. Rico, D., A. B. Martín-Diana, J. M. Barat, and C. Barry-Ryan. 2007. Extending and measuring the quality of fresh-cut fruit and vegetables: a review. *Trends in Food Science & Technology*. 18:373-386.
36. Schellekens, M. 1996. New research issues in sous-vide cooking. *Trends in Food Science & Technology*. 7:256-262.
37. Sinclair, R. G., J. B. Rose, S. A. Hashsham, C. P. Gerba, and C. N. Haas. 2012. Criteria for selection of surrogates used to study the fate and control of pathogens in the environment. *Appl Environ Microbiol*. 78:1969-77.
38. Singh, R. P., and D. R. Heldman. 2009. Introduction to food engineering. Elsevier/Academic Press, Amsterdam; Boston.
39. Sliva, J., A. S. Carvalho, P. Teixeria, and P. A. Gibbs. 2005. Effect of stress on cells of Lactobacillus delbrueckii sp bulgaricus. *Journal of Food Technology*. 3:479-490.
40. Smelt, J. P., and S. Brul. 2014. Thermal inactivation of microorganisms. *Critical Reviews in Food Science and Nutrition*. 54:1371-85.
41. Stumbo, C. R. 1965. Thermobacteriology in food processing. Academic Press, New York, New York.
42. Teixeira, P., H. Castro, and R. Kirby. 1994. Inducible thermotolerance in Lactobacillus bulgaricus. *Letters in Applied Microbiology*. 18:218-221.
43. Vasan, A., R. Geier, S. C. Ingham, and B. H. Ingham. 2014. Thermal tolerance of O157 and non-O157 Shiga toxigenic strains of Escherichia coli, Salmonella, and potential pathogen surrogates, in frankfurter batter and ground beef of varying fat levels. *Journal of Food Protection*. 77:1501-11.

APPENDIX

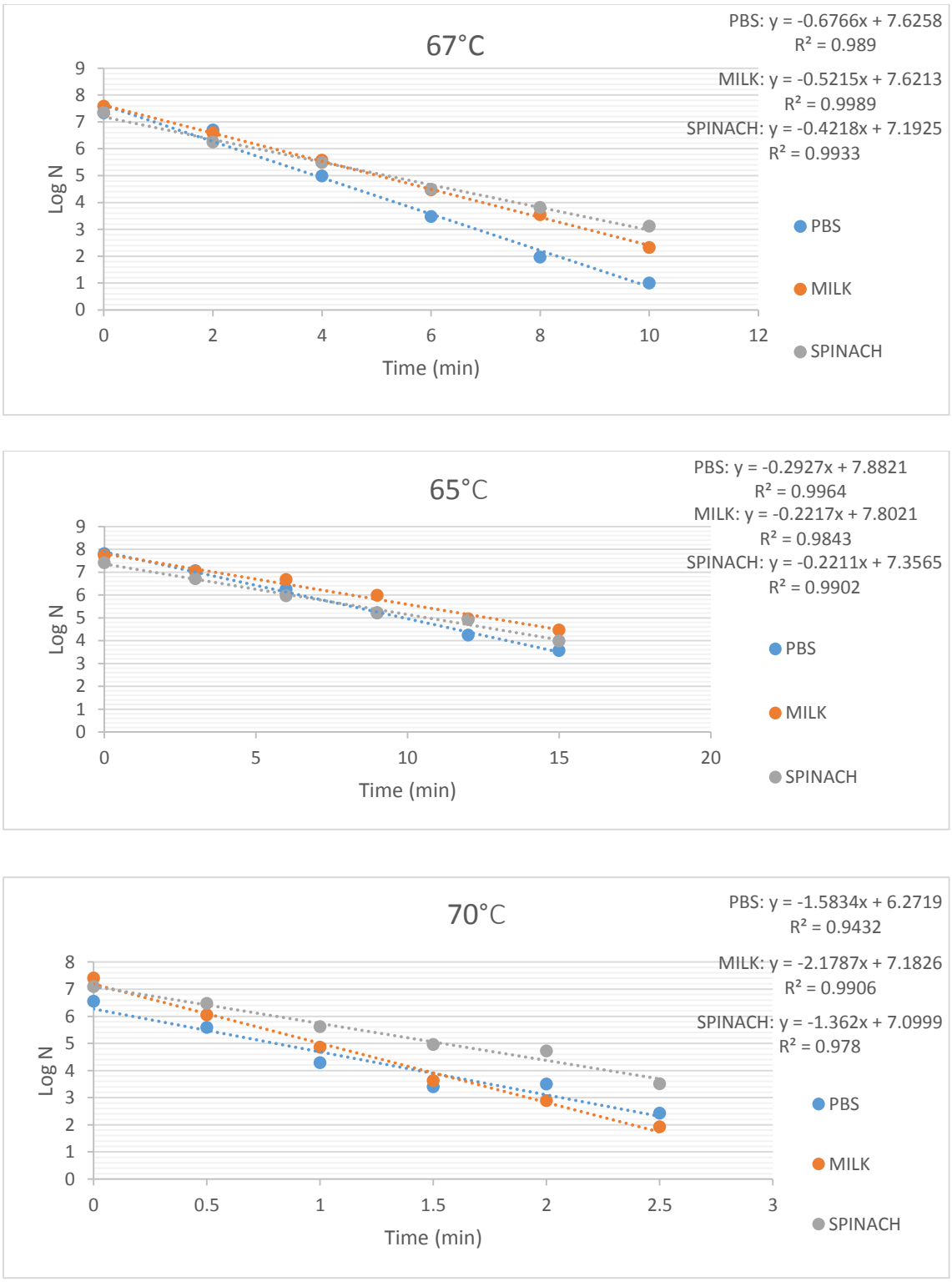
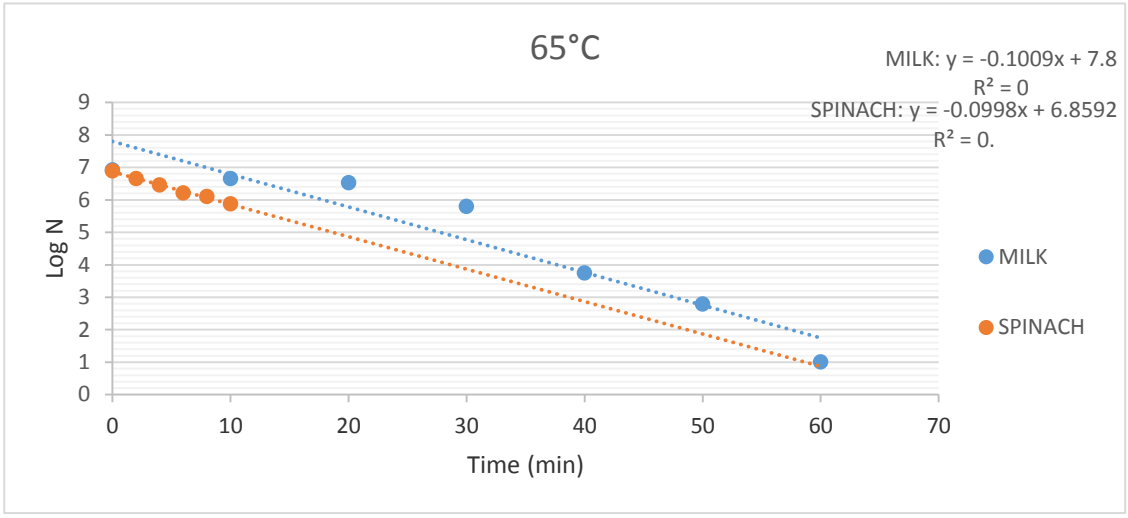
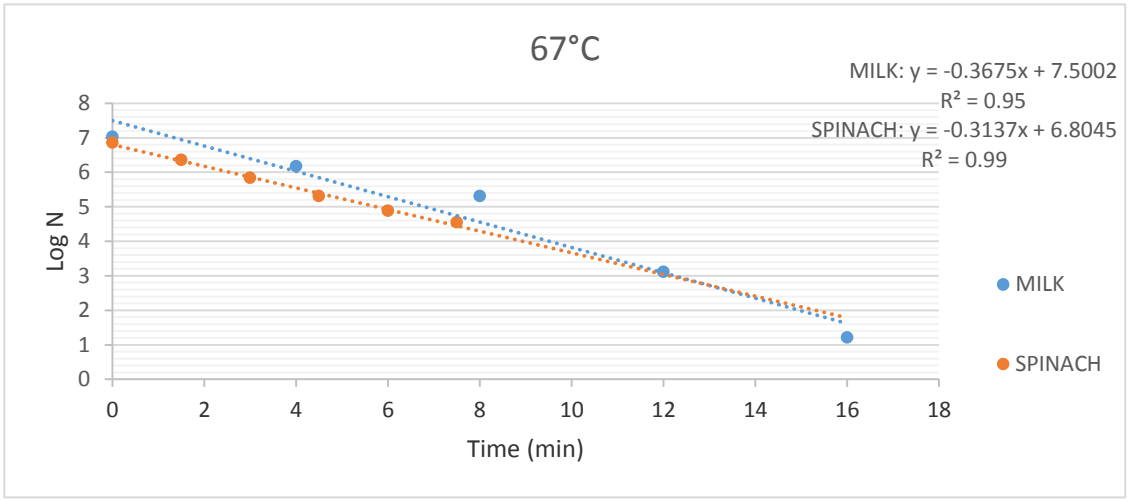


Figure 1. Survivor Curves for *Staphylococcus carnosus* CS-299 in phosphate buffered saline, 2%UHT milk and chopped spinach at 65, 67, and 70°C.





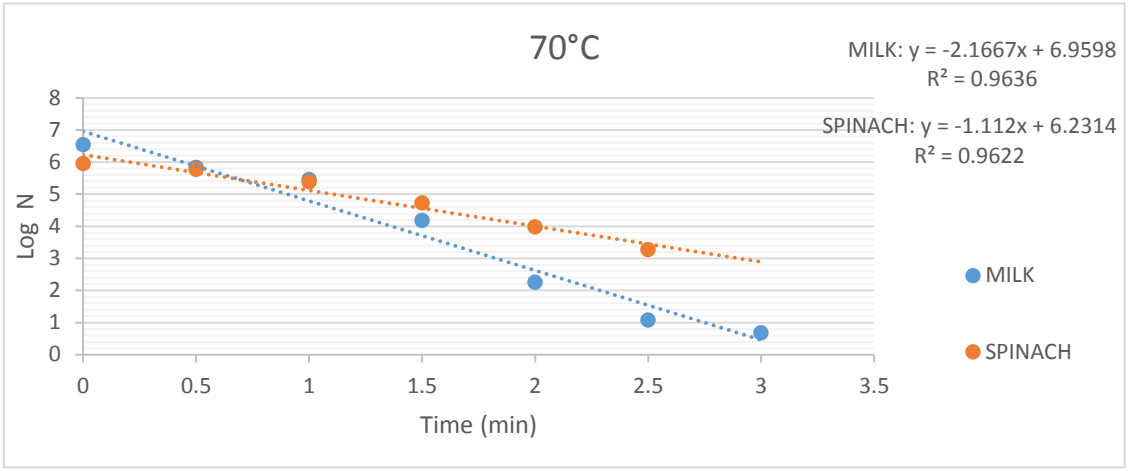
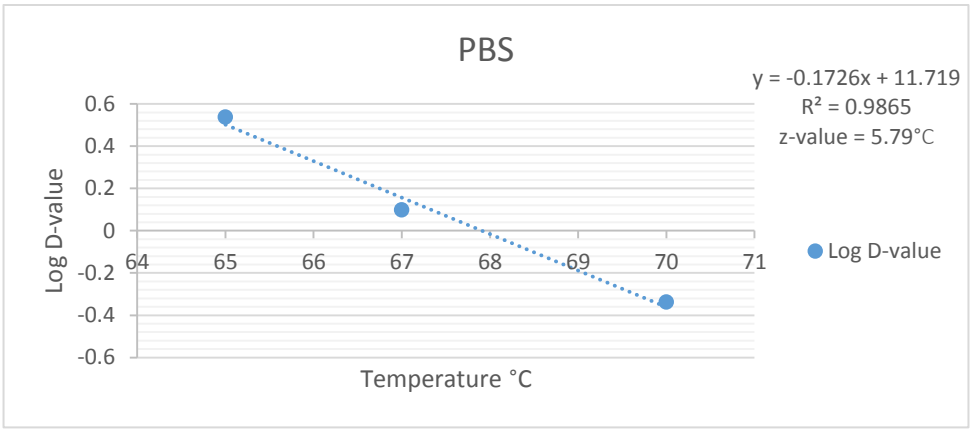
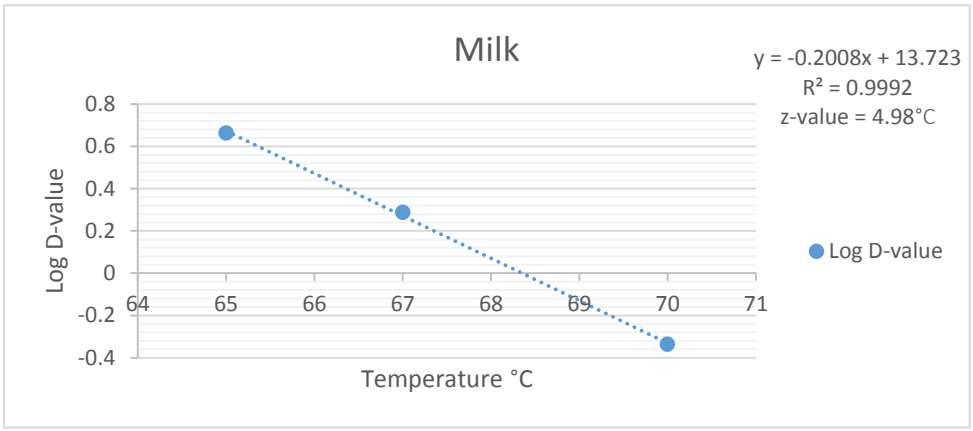


Figure 2. Survivor curves for *Lactobacillus bulgaricus* ATCC 11842 in 2%UHT Milk and chopped spinach at 65, 67, and 70°C.





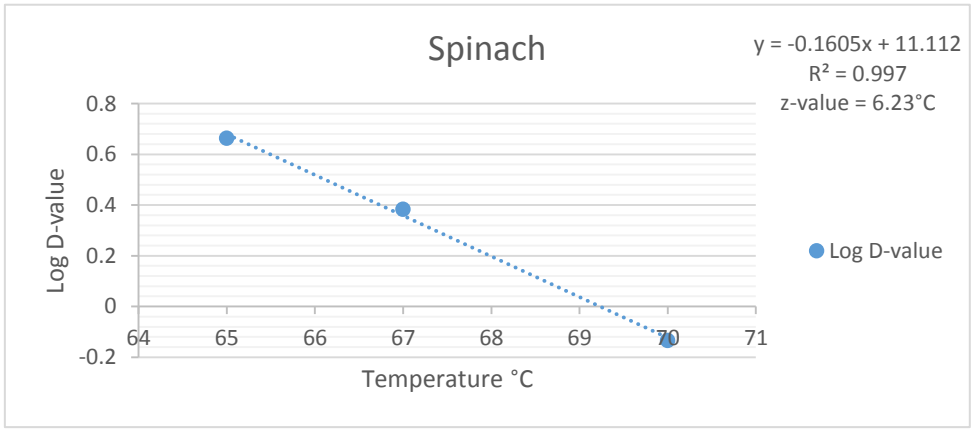
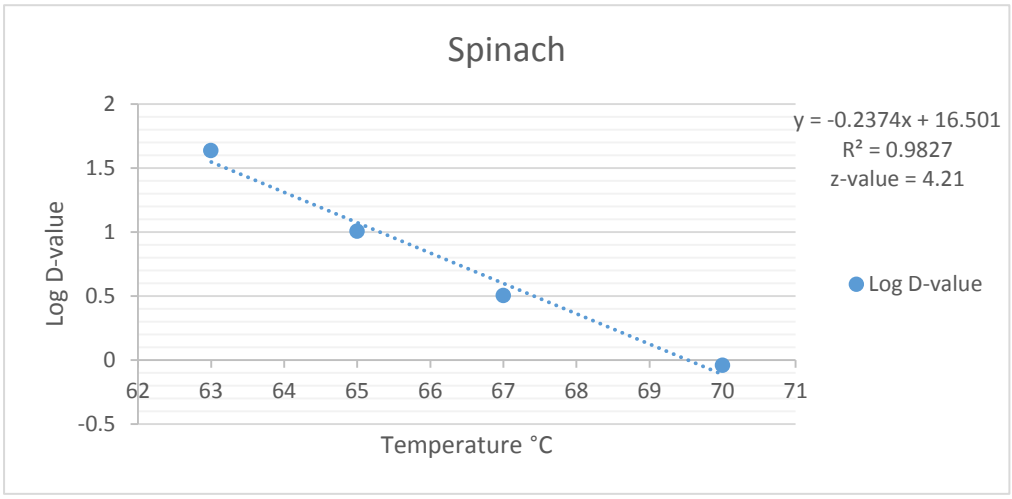


Figure 3. Thermal Death Time curves for *S. carnosus* CS-299 in phosphate buffered saline, 2% UHT milk and chopped spinach.



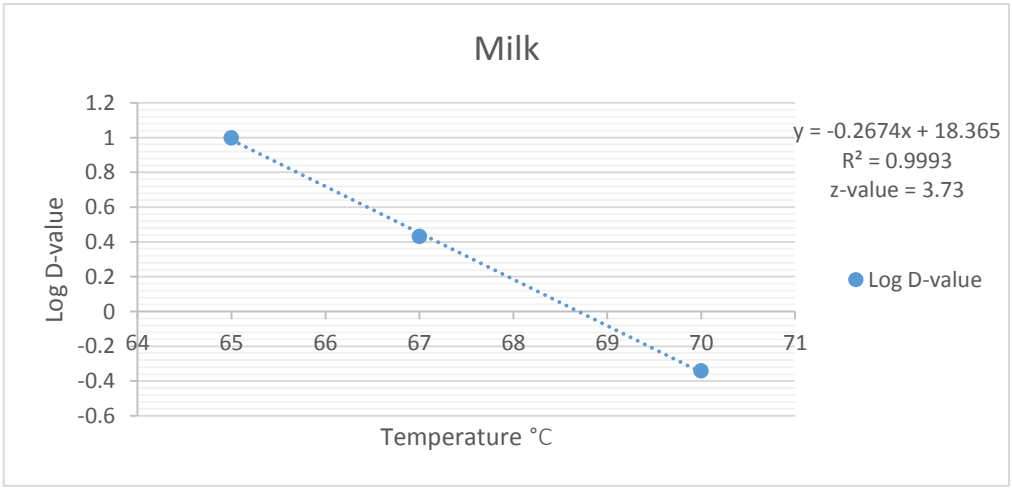


Figure 4. Thermal Death Time Curve for *L. bulgaricus* ATCC 11842 in 2% UHT milk and chopped spinach.

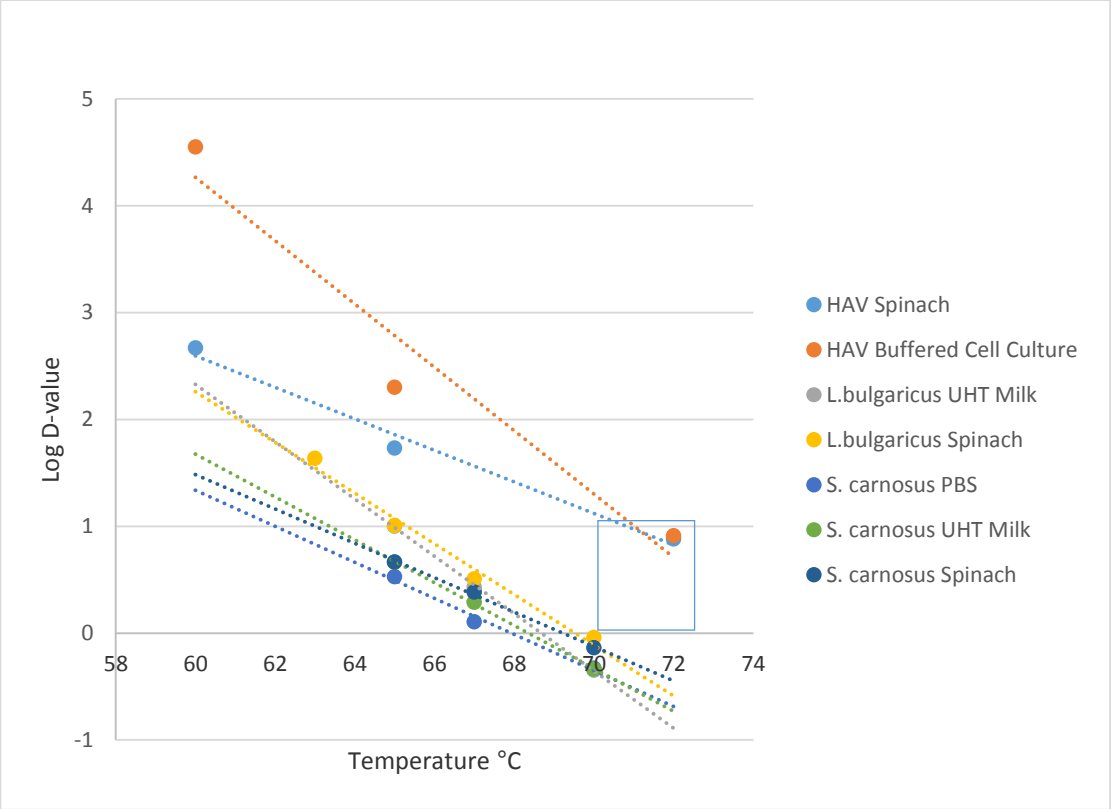


Figure 5. Log D-values of Vegetative Bacterial Surrogates in comparison to HAV.

VITA

Amber Lynne Parham Renix was born in Blytheville, Arkansas on March 27, 1991. She was raised in Memphis, TN where she attended Whitehaven High School. Her Bachelors of Science in Food Science and technology was obtained from the University of Tennessee, Knoxville in 2013. Amber will obtain her Masters of Science in Food Science and Technology, major Food Microbiology in August, 2015.