Identification of potential bacterial surrogates for validation of thermal inactivation processes of hepatitis A virus

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Abstract

Thermal treatment is a method for inactivating pathogens in a wide range of food products. Recent studies have shown that hepatitis A virus (HAV) has a $D_{72°C}$ [D72 degree celcius] of 0.9 min in buffer which is greater than vegetative bacterial pathogens. Common surrogates, such as *Listeria innocua*, are not resistant enough to be used as surrogates for HAV, thus, new surrogates need to be identified. The purpose of this study was to compare the thermal inactivation kinetics (D- and z-values) of *Staphylococcus carnosus* in different foods and different incubation temperatures to identify a potential surrogate for HAV. Thermal inactivation of *S. carnosus* was performed in phosphate buffered saline (PBS), 2% UHT milk, spinach, mussels, and clams following incubation at 32°C or 40°C. Approximately 7 log CFU/ml of *S. carnosus* in PBS was added to 2 ml vials. Thermal inactivation studies were performed at 65, 67, and 70°C and 55, 60, 65, and 70°C when incubated at 32 and 40°C, respectively. Vials were removed at various time points, plated on BHI agar and incubated for 72 h at 32°C or 40°C. Each trial was conducted in duplicate and replicated three times. D- and z-values were determined and compared using a first-order and Weibull model. The fit of the models was investigated comparing regression coefficient (R^2), root-mean-square error (RMSE), and chi-square (χ^2) values. For incubation of 32°C and 40°C and treatment temperatures of 55 to 70°C, D-values ranged from 478.35 ± 78.35 to 0.36 ± 0.07 min and from 1.49×10^6 [1490000] ± 1.19×10^6 to 0.18 ± 0.15 for the first-order and Weibull model, respectively. No significant differences were detected when incubated at 32°C, but for milk and spinach at 65°C when incubated at 40°C, with the first-order

model resulting in significantly higher values. Z-values for both models ranged from 6.17 ± 0.08 to 9.78 ± 7.45°C. Based on inactivation kinetics, ease of incubation, and non-pathogenicity, *S. carnosus* could be used for validation studies of HAV. Additionally, a significant increase in heat resistance could be achieved by increasing the incubation temperature and both models resulted in a good fit of the thermal inactivation kinetics.

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Chapter I

Introduction

In the United States, the Center for Disease Control and Prevention (CDC) reported approximately 48 million Americans are affected by a foodborne illness annually causing 128,000 hospitalizations and 3,000 deaths (*138*). There are 31 known pathogens causing approximately 9.4 million episodes of foodborne illness (20% of annual foodborne illnesses) causing 56,000 hospitalizations and 1,350 deaths each year. Viruses are the leading cause for foodborne illnesses with 5.5 million (59%) cases, followed by 3.6 million (39%) illnesses from bacteria. Norovirus was the most prevalent cause of foodborne illness and among viral foodborne illnesses hepatitis A virus was the second most common one. Both were responsible for most deaths among viruses with 26% and 7%, respectively. Nontyphoidal *Salmonella* spp., *Clostridium perfringens*, *Campylobacter* spp., *Staphylococcus aureus*, non-O157, and O157 Shiga-toxin producing *Escherichia coli* (STEC) were some of the bacterial pathogens with the highest number of foodborne illnesses. The main food sources associated with foodborne illnesses from these pathogens included produce with 46 %, meat and poultry with 22 %, dairy and eggs with 20 %, and fish and shellfish with 6.1 % (*46, 138*).

To eliminate or minimize foodborne outbreaks, the food industry is using various techniques to control pathogens. Thermal processing is one of the oldest and most commonly used methods of preserving foods and plays an important role in developing the unique texture, flavor and color of a product (*8*). This method uses heat to reduce or eliminate the microbial hazards to protect public health, inactivate spoilage microorganisms to increase the shelf-life, and inactivate enzymes that degrade product quality (*112*). Thermal treatment is an important step in the production of a food product and is usually a critical control point (CCP) in the hazard analysis critical control point

(HACCP) system in a food company's food safety plan. A critical control point is defined as a step in a process where control can be applied and it is essential to prevent a food safety hazard (*168*). In the case of thermal treatment, this system would ensure that the temperature and holding time are within the required limits to eliminate the pathogens of concern.

The food industry designed several methods to thermally treat food products to reduce pathogens, including commercial sterilization, pasteurization, and blanching (*112*). A log-linear first order kinetics model is the standard used to design commercial sterilization and pasteurization processes to calculate the efficiency of the process in reducing or eliminating microorganisms on food products. This model assumes that individual microorganisms within a population behave the same and describes the thermal destruction of microorganisms by using the logarithm of the bacterial numbers over time at a given treatment temperature (*153*). However, other models, using non-linear regression, have been used to calculate thermal processes, because a linear regression model is not always the best fit in describing microbial death (*170*). These models have been proposed for their use to describe "shoulder" or "tailing" effects of survivor curves (*102*).

According to FDA regulations thermal processes have to be validated to confirm the inactivation of the pathogens of concern for the product (*41*). However, pathogenic bacteria cannot be used in food processing plants, hence, suitable non-pathogenic microorganisms are needed, which are described by the term "thermal surrogates" (*152*). There are many existing bacterial surrogates for sterilization and pasteurization

processes used to validate for pathogenic bacteria in many different foods. However, the inactivation of enteric viruses is currently not required in the United States; instead, the FDA enforces the prevention of contamination by such microorganisms (*85*). Recently, published data showed that one of the most heat resistant enteric viruses, hepatitis A virus (HAV), had a greater thermal resistance than vegetative bacterial pathogen targets used for designing thermal processes (*32-34*). To validate existing thermal processes for enteric viruses, more resistant bacterial or viral surrogates have to be identified. Viral surrogates exist, but they are difficult to cultivate, thus, bacterial surrogates would be preferred for validation studies (*33*)

Therefore, the objective of this study was to investigate new potential bacterial surrogates for validation of HAV in pasteurization processes. One method for identifying resistant vegetative surrogates was to randomly choose isolates from thermally processed food products using a specific thermal inactivation protocol. The most promising candidate, *Staphylococcus carnosus,* was identified not by the screening studies but rather by literature reports. *S. carnosus* was tested by comparing the thermal inactivation kinetics of the first-order model, and if applicable, of the Weibull model to provide information to validate thermal processes for HAV. Various food model media and methods were used including increased incubation temperature or the addition of salts to determine the behavior of this non-pathogenic bacteria.

Chapter II

Literature Review

Foodborne Outbreaks and illnesses

In the United States, the Center for Disease Control and Prevention (CDC) reported approximately 48 million Americans are affected by a foodborne illness annually causing 128,000 hospitalizations and 3,000 deaths (*138*). There are 31 known pathogens causing approximately 9.4 million episodes of foodborne illness (20% of annual foodborne illnesses) causing 56,000 hospitalizations and 1,350 deaths each year. Nontyphoidal *Salmonella* spp., *Clostridium perfringens*, *Campylobacter* spp., *Staphylococcus aureus*, non-O157, and O157 Shiga-toxin producing *Escherichia coli* (STEC) were some of the bacterial pathogens with the highest number of foodborne illnesses. Viruses are the leading cause for foodborne illnesses with 5.5 million (59%) cases, followed by 3.6 million (39%) illnesses from bacteria. Norovirus was the most prevalent cause of foodborne illness followed by hepatitis A virus. The main food sources associated with foodborne illnesses from these pathogens included produce with 46%, meat and poultry with 22%, dairy and eggs with 20%, and fish and shellfish with 6.1% (*46, 138*).

Scallan et al. reported that nontyphoidal *Salmonella* spp. was the leading cause of hospitalizations among bacteria (*138*). Bacteria of the genus *Salmonella* are Gram-negative bacterial pathogens that cause foodborne gastroenteritis (*93*). In the mid-1900s, several foodborne outbreaks of *Salmonella* Enteritidis caused by contaminated eggs indicated that eggs were a possible source of this bacteria (*114*). Meat, poultry and eggs are the most common food vehicles of salmonellosis to humans (*93*) and *Salmonella* spp. can be found in the intestinal tract, and other parts of the body of

animals. However, control measures successfully reduced human outbreaks of *S.* Enteritidis infections caused by eggs (*35*). Also, the bacterium can be excreted in feces and then be transmitted by other animals or insects, or pollute and contaminate water, which causes contamination of produce (*93*). This shows the need to reduce fecal contamination and cross contamination in pre-harvest, harvesting and storage of produce. Recently, *Salmonella* has been mainly linked to outbreaks with poultry and produce (*14*). In 2015, *S.* Enteritidis infections were linked to raw, frozen, and stuffed chicken entrees by Aspen Foods resulting in 5 cases (*48*). A multistate outbreak of *S.* Enteritidis linked to bean sprouts occurred in 2015 with 115 reported cases (*47*). In March 2016, another multistate outbreak was reported with *S.* Muenchen which was linked to Alfalfa sprouts produced by Sweetwater Farms with 13 cases and 5 hospitalizations (*50*).

One of the most commonly reported cause of bacterial foodborne infection in the United States is the Gram-negative pathogen *Campylobacter jejuni.* This genus was first identified in the 1970s and in the 1980s. It was determined that *Campylobacter* species are one of the most common bacterial causes of diarrhea worldwide (*1*). The food products most associated with sporadic campylobacteriosis are raw poultry through cross-contamination or undercooking (*45*) or outbreaks associated with raw milk consumption (*143*). But there are also reported outbreaks from other sources such as a binational outbreak in Mexico and USA in 2011, which came from inadequately disinfected tap water (*92*). The fraction of outbreak cases is significantly smaller than total cases for *Campylobacter* compared to other foodborne pathogens including *E. coli* O157, *Listeria*, or *Salmonella* (*67*). This difference might be due to the low infectious

dose of *Campylobacter* spp. as well as the bacterium being widespread in the environment and among animals which increases the risk of infections single individuals rather than large groups (*123*).

Escherichia coli is a Gram-negative bacteria and is mostly found in the intestinal tract of humans and warm-blooded animals. However, the genus *E. coli* also contains pathogenic serotypes causing illness such as diarrhea or hemolytic uremic syndrome (*161*). The pathogenic *E. coli* strains are categorized into six pathotypes, with the Shiga-toxin producing *E. coli* (STEC) being the most common cause of foodborne outbreaks linked to *E. coli* (*38, 89, 138*). The toxin of STEC got its name from the Shiga toxin from *Shigella* due to its similarities with Shiga toxins, or Verocytotoxins (*93*). The Shiga-toxin producing *E. coli* O157:H7 can be present in meat, such as beef, pork, poultry, and lamb (*65*). Additional foods linked to foodborne cases with *E. coli* are dairy products and produce (*172*). Other reported transmission routes of *E. coli* are water (*62, 158*) and person-to-person transmission (*133*). One of the most recent Shiga toxin-producing *Escherichia coli* outbreaks was a multistate outbreak of *E. coli* O26 linked to Chipotle Mexican Grill Restaurants with 55 cases and 21 hospitalizations in 11 States. All people fell ill by eating different food items from the restaurants and the investigation did not identify a specific food or ingredient linked to illness (*52*). Most outbreaks with *E. coli* have been linked to undercooked or not adequately pasteurized products such as beef, milk (*126*, *51*), and leafy greens (*60*). Produce outbreaks are mostly linked to raw produce, as seen in an outbreak where 47 airline passengers were ill because of *E. coli* contamination in a garden salad dish (*14*) or in an outbreak linked to cantaloupes (*61*). In 2011, an outbreak of gastroenteritis and the hemolytic-uremic syndrome caused by

Shiga-toxin-producing *E. coli* in Germany was responsible for the second largest *E. coli* outbreak on record. The outbreak was linked to the consumption of sprouts with almost 4000 cases and 50 deaths (*74, 135*). In addition, the USDA Food Safety and Inspection Services declared six non-O157 STEC as adulterants in beef products and included them in the zero tolerance policy (*86*). This declaration was due to a growing concern of a public health risk associated with non-O157 STEC.

Listeria monocytogenes is a psychrotrophic Gram-positive bacterial pathogen, which can be isolated from a large number of environmental sources and it can cause a disease called listeriosis (*73*). The first *L. monocytogenes* outbreaks were mainly reported in dairy products which were either lacking proper pasteurization or may have been cross-contaminated (*73*). *L. monocytogenes* has also been found in soft cheese, fresh and frozen meat, poultry, and on produce. Soft and semi-soft cheeses, such as red smear cheeses are a potential risk product for *L. monocytogenes* contamination (*162*). Soft cheeses provide perfect growth conditions for *Listeria* and have often been linked to outbreaks of listeriosis. A comparison study of hard cheese and soft cheese reported little contamination with *L. monocytogenes* in hard cheeses but contamination in soft cheeses even when pasteurized milk was used (*136*). A study in the U.K. tested retail samples of 100 raw poultry and 222 soft cheeses and found that 60 % of raw poultry and 10 % of soft cheeses were contaminated with *L. monocytogenes* (*131*). Also, the pathogen is widely distributed on plants and can grow on lettuce and endive held at 10°C (*15, 44*), or on asparagus, broccoli and cauliflower at 4°C (*11*) which can cause problems with consumption of ready-to-eat salads. Recently, a multistate foodborne outbreak with *L. monocytogenes* occurred associated with frozen vegetables, indicating

that the blanching process before freezing the vegetables was not sufficient enough or that post-blanch contamination occurred (*49*).

Viruses can be transmitted through the “fecal-oral route” by contamination of food and water, and through person-to person contact (*93*). Food- and waterborne enteric viruses are commonly associated with foodborne illnesses and a cause of acute gastroenteritis in humans. However, they are not tested for in food samples as often as bacterial pathogens (*80*). The properties of viruses are different from those of bacterial pathogens (*58*). Viruses consist of RNA or DNA enclosed in a protein coat (capsid), which functions as the primary protection for the viral particle and supports transfer between host cells. Viruses can be enveloped by an outer lipid membrane, but all human enteric viruses are non-enveloped (*97*). Enteric viruses associated with foodborne illnesses include human noroviruses, hepatitis A virus, rotaviruses, hepatitis E virus, sapoviruses, and other enteroviruses (*138*). Human noroviruses and HAV have very low infectious doses, between 10 to 100 virus particles, to cause a foodborne illness (*163*, *111*). A very common food source for viruses are mollusks, such as clams and mussels. They grow in coastal water, which is also the most common areas for disposal of waste. Due to the fact, that mollusks are filter feeders, bacteria and viruses in the water can get filtered and contaminate the mollusks (*8, 93*).

Human norovirus is the leading cause of foodborne related outbreaks worldwide (*138*, *166*, *23*). Noroviruses belong to the family of *Caliciviridae* and they are non-enveloped RNA viruses. They are approximately 27 to 38 nm in diameter and cannot be cultivated in animal cell-culture systems yet, causing difficulty in detecting the virus in traditional

microbiology laboratories (*80*). Norovirus is transmitted through foodborne, waterborne, or person-to-person contact and typical foods commonly involved in outbreaks are shellfish, fresh fruits, and leafy greens (*166*). Lettuce and leafy greens are the major foods related to norovirus outbreaks (*107*) or the strawberry outbreak in Germany in 2012 (*10*).

Hepatitis A virus (HAV) is the cause of Hepatitis A, a self-limited disease that does not result in chronic infection. However, it is the second largest cause of viral enteric gastroenteritis and has the same transmission routes as human norovirus (*53*). HAV belongs to the *Picornaviridae* family and is a non-enveloped single-stranded RNA virus, with an approximate 27-32 nm in diameter (*93*). The largest viral foodborne outbreak ever documented occurred in clams in China in 1988 and was related to HAV (*81*). In this outbreak, clams were harvested from clam beds in sewage-polluted waters and were consumed uncooked (*81*). Another large outbreak in the United States involved consumption of contaminated green onions served in restaurants with over 600 illnesses and four deaths (*174*).

Control of Microorganisms in Foods

The control of microorganisms in food is essential to reduce or eliminate the risk of foodborne illnesses and spoilage. Due to the large number of foodborne illnesses with a variety of microorganisms in different foods, the food industry is always looking for improvements of traditional methods or creation of new methods to reduce foodborne pathogens and to increase the shelf life and safety of food. General methods used in the food industry to inactive microorganisms are the use of thermal methods such as

pasteurization and sterilization (*8*). Non-thermal methods are another approach to inactivate microorganisms by using techniques such as high pressure or ultraviolet irradiation (*113*). Besides inactivation methods, the food industry uses the inhibitory effects of several methods to reduce or prevent the growth of microorganisms. Common inhibition methods used in the food industry include addition of sugar or salt to reduce the water activity, drying and freezing, or the addition of antimicrobials to inhibit the growth of microorganisms (*165*).

Inactivation methods for controlling microorganisms in foods

Non-thermal inactivation methods for foods

The food industry is constantly searching for new and innovative methods and improve existing methods for inactivating microorganisms in foods while, at the same time, preserving the sensory and nutritional aspects of the food (*113*). While very efficient at inactivating microorganisms, thermal processing causes protein denaturation, non-enzymatic browning and loss of vitamins (*113*). There are several alternative processing techniques which have benefits over thermal processing, such as high pressure processing, ionizing radiation, or ultraviolet radiation (*113*). The importance of using other technologies is that they have to be as efficient as heat in microbial inactivation kinetics and their inactivation of enzymes and toxins, since these are the most important targets while producing food.

High hydrostatic pressure (HHP) is a method where food is pressurized above 100 MPa up to 900 MPA in a pressure vessel (*137*). The advantage is that the pressure is applied isostatically, allowing solid food to keep its original shape. Similar to a thermal treatment,

the pressure is held for a desired treatment time depending on the product and target (*137*). HHP can inactivate vegetative bacteria and enzymes at low temperature without changing the nutritional or sensory characteristics of the product (*175*). To inactivate bacterial spores, a combination of high pressure and heat must be used since pressure alone cannot significantly inactivate spores (*127*). When using pressure on bacterial cells, physical damage to the cell membrane has been demonstrated as well as leakage of ATP (*154*). In a study with *L. monocytogenes,* it was shown that intracellular enzymes, such as 6-phosphogluconate dehydrogenase (6PD), alanine dehydrogenase (ALD), fumarase (FUM), or aconitase (ACO), cannot withstand certain amounts of pressure. ACO was already inactivated between 150 and 200 MPa, whereas ALD was not inactivated when exposed to 550 MPa for 15 min (*151*). An advantage of HHP is that the isostatic compression, compared to thermal treatment, is independent of size and structure of the product, which are important limiting factors in thermal processing (*110*). A major disadvantage of HHP is that spores cannot be inactivated by pressure alone, since they are very pressure resistant (*54, 137*).

Ionizing irradiation is another inactivation method, in which electrons or electromagnetic waves are applied to food using gamma rays, electron beams, and X-rays (*9*). Irradiation is a kind of energy that causes ionization and produces free radicals. It targets the molecular bonds in the DNA, but can also denature enzymes and damage the cell membrane (*4*, *9*). Irradiation can be used with a variety of foods, such as seafood (*84*), poultry (*104*), and chilled ready meals including beef, gravy, and potatoes (*79*). Most pathogens in their vegetative forms are sensitive to irradiation, and the method can be used to eliminate foodborne pathogens and extend the shelf life of food

products (*118*) The advantage of this method is that it offers the possibility of processing packaged products, which is very effective, because there is no chance of recontamination (*26*). It also reduced the use of chemicals as preservatives (*132*). Electron beams are mainly used to decontaminate small items such as grains or to decontaminate surfaces, because they have a poor penetration power (*4, 93*). When using gamma rays, the control of the radiological hazard for the environment is very expensive, since irradiation facilities have to be designed in order to ensure no leakage. In addition, irradiation of foods requires a label indicating that they have received such treatment (*4*).

Pulsed and ultra-violet (UV) light are other methods that involves the use of intense, short-duration pulses of UV light to eliminate microorganisms. This technology has a very poor penetration level but can be used to sterilize or reduce microbial populations on food surfaces, packaging material or work surfaces (*145*). However, new developments have allowed the pasteurization of fruit juices with UV light by using turbulent flow in a chamber allowing a continuously renewing surface to be exposed to UV light (*145*). This can be an advantage over thermal pasteurization of juices, because there is no heating required, which may alter the flavors of the juice. This method is not adequate when trying to reduce microbial contamination in a solid food, since it is only reaching the surface of a product.

Thermal processes for inactivating microorganisms in foods

Thermal inactivation is one of the oldest and still most commonly used preservation treatments in the food industry. It has the benefit of efficient inactivation of foodborne pathogenic and spoilage microorganisms, natural toxins and the prolongation of shelf-life (*169*, *8*). In general, thermal processes can be categorized according to the intensity of heat treatment. The terms pasteurization, blanching, commercial sterilization, and ultra-high-temperature treatment are used for the temperature ranges of 70-80°C, 75-105°C, 110-130°C, and 140-160°C, respectively (*169*, *116*). Depending on the potential risks associated with certain products, the temperature can be adjusted to control any form of pathogen including vegetative cells in pasteurization or spores in sterilization (*105, 153*). Thermal processing is often a part of the hazard critical control point system (*115*). Thermal inactivation uses the property of increasing temperature which results in an increasing speed of reactions. The speed of chemical, biochemical, and microbial reactions increases with higher temperature, and above a certain temperature microorganisms and enzymes become inactivated. The advantage of using thermal inactivation methods is that heating is desired as a process step for many products, it produces flavors and provides a certain texture, which the consumer expects.

Blanching and other low temperature processes

Blanching is a thermal treatment (75°C to 105°C) used for solid food and its main purpose is to inactivate enzymes which may alter the quality of the food during processing or storage (*116, 153*). It is applied to fruits and vegetables prior to freezing, drying or canning to avoid enzymatic browning in these products. Besides this main

effect, blanching is also used to expel gases or to soften the tissues of fruits and vegetables (*116*). With temperatures higher than those of a pasteurization process, blanching can also inactivate most vegetative microorganisms present with sufficient holding time (*8, 116*). The temperature used for blanching are not high enough to eliminate spore forming microorganisms (*37, 153*).

Pasteurization

Pasteurization is a thermal process named after Louis Pasteur who discovered that heating wine to temperatures of 50 to 60°C for a short time inactivated spoilage of the wine without affecting the quality (*8*). Today, pasteurization is used to eliminate most of the vegetative pathogenic and spoilage bacteria from food and is mostly designed for liquid foods such as milk, fruit juices, or beer, but also for seafood and ready-to-eat products (*65, 73, 114*). The advantage of pasteurization is the use of mild heat to improve the quality of a product through reducing microbial load without significantly affecting quality. In pasteurization, food is heated to temperatures between 60°C and 85°C for a few seconds up to an hour. One disadvantage of mild heat treatments is that microbial survivors such as spore formers will likely be present in the food after the process. In addition, a mild heat treatment may even lead to stimulation of spores to germinate, which might result in a food safety or spoilage problems (*8*). For these reasons, pasteurized low-acid (pH > 4.6) foods are stored and transported under refrigerated conditions below 4°C to inhibit the growth of pathogenic and spoilage microorganisms after processing (*150*). For pasteurization processes, heating may take place in continuous flow or in a batch. Liquids are usually pasteurized in a continuous

flow process and the time and temperature are controlled by a holding tube and flow diversion valve. Batch processes are designed to hold the product in a closed vessel for a specific time and temperature with steam or hot water as heating media (*153*). Some of the typical pathogenic bacteria which are targeted during pasteurization include *Listeria monocytogenes*, *Salmonella enterica*, or *Escherichia coli* O157 and non-O157 (*120*). These bacteria have reported D-values at 60°C ($D_{60°C}$) of 1.22 min, 0.47 min, 0.97 min, and 0.93 min, respectively in spinach (*120*). With the temperatures used in pasteurization processes of 70-80°C (*169*), these pathogens are fully inactivated by heat. However, bacterial pathogens are not the only pathogens found on food products such as leafy greens, deli items, or ready-to eat foods. Several studies have shown that viruses, such as human norovirus and hepatitis A virus, are present on many foods, such as spinach, milk, and deli meat (*30-34*, *42*). Human norovirus GII has a reported D-value at 75°C ($D_{75°C}$) of 1.71 min and 1.58 min for basil and mint, respectively (*42*), and a $D_{60°C}$ of 25 min in mussels (*56*). Studies conducted with the human norovirus surrogate murine norovirus showed D-values at 60°C of 0.98 min and 2.7 min in spinach and deli meat, respectively (*30, 32*). HAV was found to be more heat resistant than human norovirus with D-values at 60°C of 4.55 min, 6.13 min, and 5.9 min in spinach, clams, and deli meat, respectively (*31, 32, 34*). Thus, using pasteurization processes that eliminate target bacterial pathogens do not guarantee that human norovirus and hepatitis A virus would be eliminated because of their higher heat resistance (table 2.1).

Commercial sterilization of low-acid foods

Commercial sterilization is defined as the thermal process to achieve a shelf-stable food product, which means the product can be stored at normal non-refrigerated temperature without spoilage caused by microorganisms (*153*). For low-acid foods, it requires an intense heat treatment to inactivate bacterial spores as well as vegetative cells (*8*). Only the most heat resistant thermophilic spore formers have the capability of surviving this process. Vegetative bacteria form spores when stress such as reduced levels of nutrients or other environmental factors such as heat are present to survive under extreme conditions (*130*). Spores are extremely heat resistant and can survive up to 40°C higher temperatures than the growing cells of the same strain which requires sterilization processes to eliminate them (*146, 147*). Typical spore forming pathogenic bacteria, which are targeted during sterilization are *Clostridium botulinum* or *Bacillus cereus*. *C. botulinum* can produce a toxin called “botulinum toxin”. The *C. botulinum* spores cannot germinate and produce the toxin in products with a pH below 4.6, which is the reason why this concept is only used for foods above this pH value (*93*). Another important parameter is that the product must be placed in a hermetically sealed container and handled properly to prevent the product from becoming re-contaminated. The only limitation to shelf-life is some chemical and physical changes of the food can take place during long term storage such as the Maillard Reaction or degradation of nutrients (*8*,*153*).

In commercial sterilization of low acid foods, the target is a process that achieves a minimum 12 log reduction of *Clostridium botulinum* (*2, 164*), since it is the most heat resistant pathogenic spore forming bacterium. This process is based on the 12-D

concept, which was developed for the canning industry. It implies that the minimum heating treatment should reduce the probability of survival of *C. botulinum* spores to 10^{-12} (*93*). The D-value of *C. botulinum* at 121°C is $D_{121°C}$ = 0.21 min which results in a 12D value of 2.52 min, which is the required time to reduce *C. botulinum* spores to one spore in 1 of 1 billion containers (*71, 164*).

Factors affecting heat resistance of microorganisms

The heat resistance of microorganisms can be affected by several different intrinsic and extrinsic factors (*27*). One intrinsic factor is inherent heat resistance of a microorganism, which can differ among different species and strains of this microorganism. For example, different strains of *Listeria monocytogenes*, *Salmonella enterica*, *and E. coli* O157 were compared for their thermal resistance in a study by Monu et al. (*120*). The study showed that two different *Listeria* strains, *L. monocytogenes* Scott A and *L. monocytogenes* LM1 had significantly different D-values at 54°C. More differences were found for other strains among the tested *Salmonella* and *E. coli* strains (*120*).

There are many extrinsic factors which can affect the apparent thermal resistance of a microorganism prior to heating, during heating and during recovery of viable cells. Prior to heating, heat shock, temperature of incubation, presence or absence of certain media components, and water activity can affect increase apparent heat resistance. During heating, the composition and pH of the heating menstruum may increase or decrease apparent heat resistance. Finally, recovery of survivors which will influence number of cells and thus apparent resistance is dependent upon recovery medium.

Factors prior to heating

It has been known for some time, that exposure of organisms to near lethal temperature often lead to an adaption to that temperature (*40*).The cell defense mechanism causes the microorganism to produce proteins called heat-shock proteins, or hsps (*140*). They are also called chaperon proteins and were defined as a group of proteins which are responsible for assembling other polypeptides without being a part of the final functional structure (*69*). These proteins are expressed to prevent aggregation, degradation of misfolded proteins and to refold proteins (*39*, *83*). Depending on the organism, the optimum temperature range of heat-shock protein induction varies (*40*). Neidhardt et al. showed that an increase in growth temperature in *E. coli* caused an immediate accelerate synthesis of 17 polypeptides (*122*). Another study by Knabel et al. compared the effect of different heat-shock times on the heat resistance of *L. monocytogenes* and found that a 5 min exposure to heat shock showed significant effects on the thermal resistance. Also, this study demonstrated that a longer exposure to higher temperatures increases the thermotolerance even more. This effect indicated an increased thermotolerance when increasing time and temperature of the heat shock treatment (*109*). When conducting heat-shock experiments, researchers also analyzed the long term effect of heat stress. It was known that a longer exposure to higher heat created a higher heat resistance in the cells. Therefore, Knabel et al. compared the different heat-shock experiments among each other as well as to an overall increase in incubation temperature to the temperature used for heat shocking the cells. Using a higher incubation temperature yielded in the highest increase in thermal resistance (*109*). A reason for that may be the accumulation of large amounts of heat shock proteins during

the stationary phase of growth (*94*) Besides that, it was shown that heat-shocking *L. monocytogenes* cells had different effects depending on the pH of the broth (*99*). This indicates that the interaction between different parameters plays an important role when analyzing the thermal resistance of microorganisms.

An increase in incubation temperature results in higher heat resistance for many bacteria. A possible reason for an increased apparent resistance is that the microorganism changes during growth by natural selection to more heat adapted cells (*82*). A study with *E. coli* showed that incubation at 38.5°C or 40°C resulted in higher percentage survival than at 28°C or 30°C (*68*). The suggestion in this study was that the incubation temperature may have influenced the physio-chemical properties of the cells (*68*). Studies with different incubation temperatures of *L. monocytogenes* supported the theory that higher incubation temperatures withstand higher heat during a thermal treatment than those grown at lower incubation temperatures (*64*). Juneja et al. incubated *L. monocytogenes* at 10, 19, and 37°C at pH 7, followed by a thermal treatment at 60°C. The results showed a significant increase of the D-value with 0.83, 0.93, and 1.11 min for the incubation temperatures, respectively (Juneja, 1998 #140}). Thermal inactivation of *L. monocytogenes* was conducted in a liver sausage slurry with two different incubation temperatures, 19 and 37°C. This experiment resulted in a $D_{60°C}$ of 0.8 min and a $D_{60°C}$ of 1.6 min for 19 and 37°C, respectively (*16*). Smith et al. conducted experiments incubating *L. monocytogenes* at 37 or 42°C followed by heat treating the washed cell at 52°C for 1 h in phosphate buffer. This experiment resulted in a 10^3 to 10^4 fold higher survival rate than incubation at 28, 19, 10 or 5°C followed by heat treating at 52°C (*156*). Those results indicated that bacteria are more susceptible

to heat induced death when growing at low temperatures. The exact mechanism behind the higher heat resistance when incubating at higher temperature is still unclear (*93*). Reasons may be that the growth temperature affects the protein and lipid biosynthesis and the composition of cell membranes, which influences the ability of cells to withstand thermal inactivation (*100*).

Composition of growth medium

All microorganisms require certain amounts of nutrients in order to survive and reproduce. The amount and type of nutrients required are very depending on the microorganism. The most important nutrients are water, any source of carbon, nitrogen, minerals, and vitamins (*8, 101*). Depending on the composition of the growth medium, varying amount of nutrients are available. Meat products have high contents of protein, lipids, minerals, and vitamins, whereas muscle food lack high amounts of carbohydrates. Plant foods, such as leafy greens, have high concentrations of carbohydrates, but different levels of proteins, minerals, and vitamins (*93*). Water is essential for microorganisms to grow, with most spoilage bacteria not being able to grow below a_w = 0.91 (*93*). Microorganisms have several ways to generate energy, such as utilizing alcohols, amino acids, fats, and carbohydrates. The main nitrogen source usually are amino acids, and sometimes peptides. *E. coli* can also utilize nitrogen from nitrates and ammonia to generate amino acids (*8, 93*). Some organisms are in need of more specific growth factors, such as purines, pyrimidines, or vitamins, which can be added to the growth medium.

Effect of salts in the growth and heating medium

Salts can be added either during cell conformation state or to the cell suspension during the thermal treatment. In 1954, Jensen reported that increasing salt levels show a protective action against heat in microorganisms (*95*). In addition, studies showed that the effect depends on what kind of salt, what concentration, and what media was used on the test organism (*82*). One effect of salts on the cells can be the change of transport of acid through the osmotic barrier, which would affect the pH of the cells. They also can decrease the differences in osmotic pressure between the exterior and interior of the cells, resulting in less leakage of essential components during heating (*82*). Salts such as potassium or sodium chloride have a strong effect on the hydration of proteins which can influence the stability of enzymes or other proteins such as the ribosomal complex. Calcium- or magnesium ions are divalent cations which may increase the heat resistance of cells by linking proteins to stronger complexes (*82, 173*). Besides that, the addition of high concentrations of soluble salts decreases the water activity, which results in an increase of the resistance of the bacterial cells (*93*).

Effects of water activity and compatible solutes

“The water activity is an index of the availability of water for chemical reactions and microbial growth” (*8*). Water activity, a_w, is defined as the partial pressure of water vapor (p_w) above the surface of a food or material divided by the vapor pressure of pure water (p'_w) at the same temperature as the sample, as shown in equation (2.1) (*93*)

$$a_w = \frac{p_w}{p'_w} \qquad (2.1)$$

An increase or decrease in water activity is well known to have a tremendous effect on the heat resistance of microbial cells. This effect was observed in a study with

superheated steam, which was used as dry air. The dry air steam had less killing effect at 140-150°C than wet steam at 100°C due to the change in a_w (*173*). The denaturation of nucleic acids, enzymes or in general proteins is very likely to be the main reason for the heat destruction of microbial cells. It is also known, that proteins are more stable in a dry state, which makes them more resistant to heat when the water activity in the cells is low (*173*). This suggests that the effect of hydration on thermostability is directly related to protein dynamics (*72*). This effect helps to stabilize essential proteins and ribosomal units against irreversible damage (*160*). Water is always present in protein structures and can be bound osmotically (Donnan effect) or can be present as inclusion water in the protein structure (*173*). It can also be bound to protein molecules having free charges or to dipole groups such as carbonyl- and amino groups (*173*).

The more water present in proteins the more swollen they are, which results in an increase of chain mobility and flexibility (*72*). The dynamic, swollen, and more flexible structure of the proteins allows the water to gain greater access to salt bridges and peptide hydrogen bonds when heat treating microbial cells in a medium with high water activity. This effect results in a lower denaturation temperature compared to cells, which are in a drier state (*72*).

The increased thermal resistance due to low water activity played an important role in many outbreaks with low-moisture foods such as *Salmonella* in spices and peanut butter, *Clostridium botulinum* in honey or *Staphylococcus aureus* in salami (*13*). It is very difficult to adequately design safe thermal processes in such low-moisture foods. Recently, it was reported that elevated temperatures during thermal processing can change the characteristics of a food matrix and the microorganisms (*160*).

Due to the relationship between the ambient pressure $p_{ambient}$ and the saturated vapor pressure at product temperature, as seen in equation (2.2)

$$a_w = \frac{p_{ambient}}{p_{vs}} \quad (2.2)$$

there is a maximum water activity of a food system when using higher temperatures in an open system (*160*). Syamaladevi et al. described this relationship using an example of 120°C as treatment temperature. They found that when increasing the treatment temperature of a food product with high a_w, the food will actually lose water and reduce the a_w to get to the equilibrium a_w (*160*).

Protein- and carbohydrate-rich food are known to show increasing a_w, when increasing the treatment temperature due to their loss in the ability of binding water (*90*). Fats and oils, on the other hand, are hydrophobic and an increase in temperature may result in a decrease in a_w. (*144*). This effect may be explained by an increase in the solubility of nonpolar solids, such as oils and fats in water at elevated temperatures (*108*). That behavior was tested and confirmed in a study with peanut butter. The water activity of peanut butter was measured while increasing the temperature at fixed water contents showing a decreasing a_w with increasing temperature (*160*). The described water activity changed with different food components demonstrated that the water activity changes of different foods at elevated temperatures are unique depending on the composition.

Another study by Syamaladevi et al. tested the water activity changes of all-purpose wheat flour and peanut butter with increasing temperatures and how it affected *Salmonella*. Both foods had a reported a_w of 0.45 at 20°C, however, the $D_{80°C}$-value for all-purpose flour was 6.9 min and for peanut butter 17.0 min (*159*). In the past, this

effect was described by the protective attributes of a higher protein content in peanut butter (*12, 27*). With this new methodology, the researchers were able to measure the actual water activity in the heating chamber at 80°C and found water activities of 0.8 and 0.04 for all-purpose flour and peanut butter, respectively (*159*). This finding explained the higher heat resistance of *Salmonella* in peanut butter when trying to thermally inactive the cells at 80°C. In addition, it showed the importance of determining the water activity at the actual treatment temperature in order to design safe treatments.

For many years, it has been known that certain nutrients such as low molecular weight solutes improve the adaption to high osmolarity conditions (*117*). Those solutes have obtained the term “compatible” solutes (*36*), because high levels of these solutes do not interfere with the cellular metabolism within the cytoplasm. Several different nutrients such as amino acids, their derivatives, carbohydrates, and dipeptides have all been shown to function as compatible solutes for a variety of bacteria. The positive effect can be explained by the fact that accumulation of compatible solutes within the cell allows the bacteria to grow at high osmolarity, because of the positive turgor pressure in the bacterial cytoplasmic membrane caused by the compatible solutes (*25, 57*). Compatible solutes found in bacteria are K^+ ions, glutamate, proline, alanine, or quaternary amines such as glycine betaine (*91*). These compatible solutes might have different effects on the osmotic stress tolerance of cells. Some can dramatically stimulate the growth rate of cells in media of high osmolarity, whereas others have no detectable effects on the growth. The effect seen with these stimulating compatible solutes might be based on interactions with cellular macromolecules resulting in higher stability of these molecules in the cell (*141*). A study reported, that intracellular proline and glycine betaine

accumulated to high concentrations when *Staphylococcus aureus* was grown in a medium with a high NaCl content (*117*). This study also found that the addition of exogenous proline or glycine betaine as compatible solutes significantly stimulated the growth rate of *S. aureus* in a medium of high osmolarity. The addition of 5 mM of the compatible solutes allowed growth in the presence of 15 % NaCl whereas the control without the addition of the compatible solutes did not show growth (*117*). The results of this study indicated that a controlled addition of selective compatible solutes can enhance the growth of microorganisms in low a_w foods.

Another study analyzed the effect of four different compatible solutes, choline, glycine betaine, proline, and taurine, on the osmotic stress response of *S. aureus* (*78*). Defined concentrations of 1 mM of the compatible solutes were added to the growth media and the effect was analyzed by addition of different concentrations of NaCl. Again, proline and glycine betaine showed the best enhancing effect on growth of *S. aureus* when adding NaCl. The authors of this study suggested that the uptake of these compatible solutes upon osmotic stress may have been due to activation of preexisting transport systems (*78*). A study by Smiley et al. analyzed the uptake of the compatible solute taurine in *S. aureus* by using radioactive labeled taurine. They found that several metabolic pathways may have been responsible for the uptake of taurine, since it was found in the nucleic acid, capsular polysaccharide, protein, and peptidoglycan fractions of the cells (*155*). This shows that the uptake of compatible solutes is complex and it is not fully understood what exactly enhances the resistance against osmotic stress situations. Bae et al. identified two transport systems for proline and one transport system for glycine betaine in *Staphylococcus carnosus* (*5, 6*). For glycine betaine a

high-affinity transport system was reported with K_m = 45 µM (*5*) and for proline a high- and low affinity transport system was reported with K_m = 7 µM and 420 µM, respectively (*6*).

Composition of the heating menstruum

The composition of the heating menstruum is important when determining thermal inactivation kinetics of microorganisms. Researchers suggested that food components may have protective effects on the cells of microorganisms (*59, 103*). Deboosere et al. found that sugar components of a fruit-based product had a protective effect on heat inactivation of hepatitis A virus (*59*). Another study on different bacteria also reported a thermoprotectant effect of carbohydrates, lipids, proteins, salts (*120*). However, most of those thermoprotectant effects seem to be explained by the change in a_w of the products and while heating. Sugiyama et al. showed the protective effect of long-chain fatty acids on *C. botulinum* by increasing the concentration of oleic acid in the heating menstruum, which resulted in more survivors at the same time (*157*). The different composition of foods was also reported to result in different changes of the a_w while applying heat (*90, 144*). This change in a_w may be a main reason why foods with a high protein content result in a different heat resistance of the microorganism than foods with a high fat content.

Effects of the pH value

It is known that every microorganisms has a pH optimum and that a change in the pH in both, acid and alkaline, directions causes a decrease in the heat resistance of bacteria due to increased heat denaturation of proteins (*82, 100*). In 1948, Jordan et al. already found abnormal sensitivity of *E. coli* cultures when exposing them to 51°C at pH 6.4 and

7.0 (*98*). It was reported that a cell might have to maintain a pH close to neutral to have the maximum heat resistance (*87*). Proteins, in general, are more heat resistant at their isoelectric point, which is close to neutral pH. At neutral pH, most proteins are negatively charged, but their net electrostatic repulsive energy is small compared to other beneficial interactions at this pH (*72*). Besides, the pH in a cell may differ from that of the surrounding media, which is why a change in the pH of the heating menstruum may give different results depending on the substance used to change the pH (*82, 87*). When adding sodium chlorite to the heating media an effect was reported on the pH because sodium ions can replace hydrogen ions from the negatively charged ampholytes (*82*). However, bacteria can survive at low pH and even have the ability to adapt to low pH over time (*87*). This effect is similar to the adaption to increased incubation temperature. In a case study, it was reported that *Listeria monocytogenes* showed increased resistance to a pH of 3.5 when exposed to a pH of 5.5 prior to the treatment (*125*). The bacteria cells were more resistant to a low pH, but also to thermal stress, osmotic stress, crystal violet, and ethanol. This demonstrated another possibility for cells to adapt to a new environment.

Measuring the effect of heat on microorganisms

Thermal processing as a method to increase the shelf life of food started in the late 1800s with producing canned foods and pasteurizing young wine. However, the temperature and time during the processes depended on the experience of the workers (*19*). There were standards for temperature or holding time and researchers realized that those new processes were not always good in preventing spoilage. In the early

1900s, Schorer and Rosenau established the first standards for the pasteurization of milk with 20 min at 60°C (*142*) and Bigelow et al. focused on determining suitable process conditions for the production of canned foods (*17*). These studies were one of the first attempts to set parameters for time and temperature when thermally processing food products to reduce or eliminate foodborne microorganisms. Temperature and time in a thermal process are still the most important factors when measuring the effectivity of a thermal process to eliminate microorganisms. Today, the food industry is using special parameters, such as time, temperature, or even pH and food composition, to describe and characterize thermal processes. These parameters are important to design and ensure sufficient heating time and adequate heating temperature of the product to inactivate the pathogens of concern.

First-order model

The D-value, the thermal decimal reduction time, is the time necessary to reduce the microbial population present in a medium by 90 %. The z-value is a reference to the thermal resistance of a microorganism at different process temperatures and is the change in the temperature required to change the D-value by 90 % (*88*). The D-value can be calculated using the following equation (2.3)

$$D = \frac{t_2 - t_1}{logN_1 - logN_2} \qquad (2.3)$$

with N being the number of viable cells present at time t.

The calculation of the D-value at different temperatures results in the z-value, which allows to integrate the lethal effect of different temperatures within a thermal process (*22*). The z-value is obtained by constructing a thermal death time (TDT) curve by

plotting the logarithm of the D-value over temperature and represents the number of degrees to cause 90 % change in log D. The z-value can be calculated using following equation (2.4)

$$z = \frac{T_1 - T_2}{logD_2 - logD_1} \quad (2.4)$$

with the D-values D_1 and D_2 corresponding to the temperatures T_1 and T_2, respectively (*18, 70*).

The F-value is another parameter, which can help to better describe a thermal process. It is the time required to inactivate a certain known population of microorganisms in a given menstruum under specific conditions. The most known F-value is the 12D for sterilization, which is equivalent to a 12 log_{10} reduction of the most heat resistant species of spores in canned food. This reduction is needed to achieve commercial sterility (*128*). The F-value is calculated by multiplying the D-value by the number of log reductions desired, given a $D_{121°C}$ of 1 min a 12D will be of 12 min. This mathematical term expresses that there is a chance of getting one contaminated can out of 1 trillion cans when heating the cans 12 times as long as the D-value.

Weibull-Model

Most published studies describe survivor curves by using first order models to generate D- and z-values (*43, 75, 77, 96*). The first order models are based on mathematical models with temperature being the essential parameter for the inactivation of the microorganisms. The general assumption is that the mortality of cells are fully described by first order kinetics. This model is based on the approach that all cells in a population have identical sensitivity to heat. Over the year, safety records of this principal have

served the food industry as evidence to not consider other methods to calculate the thermal parameters (*128*). However, there has been proof that the first-order model does not always fully describe the microbial inactivation kinetics during thermal processing (*32, 128*). A widely used and discussed non-linear model is the Weibull distribution. It takes into account that individual organisms behave different from the population and that various mechanisms are responsible for a cell to be thermal inactivated (*128, 129*). These different behaviors can be seen in an upward or downward concavity when plotting survival curves (*129*). The Weibull mode uses the two parameters α (time) and the dimensionless shape parameter β. In this approach, the classic first-order model is only a special case with $\beta = 1$, which represents a linear survival curve. The terms $\beta < 1$ and $\beta > 1$ stand for upward or downward concavity, respectively. Upward concavity indicates that remaining cells might adapt to the stress resulting of the thermal treatment, whereas downward concavity means that remaining cells increasingly are getting damaged. In addition, $\beta < 1$ and $\beta > 1$ can also be expressed as tailing and shouldering effect, respectively (*170*). In a study of Martinus van Boekel, fifty-five case studies were analyzed to find out that the first-order model is more the exception rather than the rule and that the Weibull model better describes most studies (*170*).

Surrogates

Food processing companies are required to conduct certain steps to assure that the production of a product is safe and that the chance of any pathogenic microorganisms or other hazards is minimized (*167*). Traditionally, for the thermal inactivation of foodborne pathogens during sterilization and pasteurization, surrogate microorganisms are used as a substitute for a pathogenic microorganism to validate the efficacy of a food process (*152*). Because industrial food processing operations cannot introduce a pathogenic microorganisms into their environments, target pathogens cannot be used to validate or verify a new or existing processing method. Thus, surrogate microorganisms are of great importance to ensure microbiological safety of the facility and of the process (*41*). Surrogates may be microorganisms, particles, or substances used to analyze the behavior (growth or inactivation) of a pathogen in a specific environment (*152*). *Escherichia coli* was one of the first bacteria used as a surrogate to indicate if water was contaminated with feces and thus *Salmonella*. *E. coli* was used as a surrogate or indicator because it has similar resistance and growth characteristics as *Salmonella.* Thus, *E. coli* could be used to develop treatment processes and validate them without using *Salmonella*. Pathogenic as well as non-pathogenic organisms have been used as surrogates for different purposes, such as fecal indicators, tracers, or process indicators (*7*, *41*, *106*, *152*). However, safety is most important in the food industry and therefore, non-pathogenic surrogate organisms are used to validate processes. Selection of a surrogate is extremely important since under- or overestimating of the resistance of a pathogen may negatively impact the risk assessment and therefore the public health (*152*).

When validating a thermal process, the first step is to identify the most resistant pathogen of concern and fully characterize the process and survival characteristics (*24, 152*). Ease of use is another attribute every surrogate should have. The microorganism has to be easy to grow to high-densities, be easily detectable and enumerated using fast, sensitive, and cheap detection methods (*152*). A surrogate should also be similar to a target pathogen in its shape, size, and in an ideal case in its surface properties and life cycle. Microorganisms that are categorized as spoilage organisms on equipment or in the production area generally should not be selected as possible candidates due to the risk of cross-contamination into food products (*41*). But most importantly, a surrogate must have the ability to survive and grow in the same engineered or natural system as the pathogen of concern. Conditions of concerns are pH, temperature, relative humidity, organic matter, and other nutrients (*152*). Surrogate candidates for thermal process validation can be divided into three groups: non-ideal, semi-ideal, and ideal (figure 2.1-2.3). Non-ideal conditions occur, when the surrogate and the pathogen have identical z-values, but the heat resistance (D-value) of the surrogate is lower at a given treatment temperature. Semi-ideal conditions occur, when the z-value of the surrogate is lower than the one of the pathogen, resulting in a higher slope for the surrogate. In this case, the surrogate can only be used for validation in the temperature range where the D-values are higher than the pathogen. Ideal conditions occur, when the z-values are identical, and the heat resistance of the surrogate is slightly higher at any given treatment temperature.

Types of surrogates

Over the years, the industry has discovered many suitable surrogate microorganisms which can be used for in-plant critical control point validation to ensure the microbiological safety of the product. Different pathogens and processes are associated with certain products. Therefore, surrogates have to be identified for the pathogens of concern in every process. Sometimes, different surrogates are used for the same pathogen but in combination with another food product or process. Validation of thermal processes can be divided into the two main methods, sterilization and pasteurization, with both inactivation methods targeting different temperature ranges, 110-120°C and 70-80°C, respectively (*2, 8, 65*,*73*).

For sterilization processes, surrogates are generally spores which are the target during sterilization (*2*). The nonpathogenic spore forming bacteria *Clostridium sporogenes* and *Geobacillus stearothermophilus* (formerly *Bacillus stearothermophilus*) have been used for many years to establish and validate the inactivation of *Clostridium botulinum* in the low-acid canning industry (*41*). A study by Gaze et al. showed that *C. sporogenes* would be a suitable candidate for validation of processes up to 120°C and *G. stearothermophilus* for temperatures above 130°C (*76*). Brown et al. summarized many studies to calculate mean $D_{121°C}$-values, which resulted in D-values of 0.21 min and 0.2 min for *C. sporogenes* PA3679 and *C. botulinum*, respectively, in phosphate buffer (*37*). Another study confirmed these results by collecting datasets from 38 research studies to evaluate *C. sporogenes* PA3679 as a possible surrogate for *C. botulinum.* Overall, it

showed that the heat resistance of *C. botulinum* was significantly lower than the one of *C. sporogenes* PA3679 (*63*).

Bacillus anthracis is another pathogenic spore forming bacteria causing anthrax which can result in anorexia, nausea or vomiting (*119*). A study by Montville et al. analyzed potential thermal surrogates in different food media. The surrogate candidates *Bacillus cereus* ATCC 9818 and *B. cereus* ATCC 4342 had D-values of 22 min and 4.8 min at 90°C, respectively and *Bacillus subtilis* ATCC 6633 had a D-value of 6.0 min at 90°C. The three *B. anthracis* strains Sterne, Pasteur, and Vollum had D-values of 2.9 min, 0.86 min, and 4.9 min, respectively. These results showed, that not every surrogate can be used for every strain of a pathogen and that it is very challenging to find the best surrogates. *B. cereus* ATCC 9818, for example, was too heat resistant, which could result in unnecessary overheating of the product (*119*).

For pasteurization processes, spore forming bacteria surrogates, such as *C. sporogenes* or *B. subtilis*, cannot be used as surrogates because their heat resistance is too great. For target pathogens, such as *Listeria monocytogenes*, *Escherichia coli*, *Salmonella* Enteritidis, or even viral pathogens such as norovirus and hepatitis A virus, vegetative bacterial surrogates are most often used. There have been many studies done to characterize non-pathogenic vegetative bacteria as potential surrogate candidates to validate pasteurization processes. For example, the thermal inactivation kinetics of *Enterococcus faceium* B2345 were compared with *L. monocytogenes* and *Salmonella* Senftenberg. The purpose was to determine if *E. faecium* was a suitable thermal surrogate for in-plant critical control point validation in ground beef. The D-value

of *E. faecium* at 68°C was reported to be 16.5 min in ground beef with a fat content of 12 % compared to D-values of 4.2 min and 4.5 min for *Listeria monocytogenes* and *Salmonella* Senftenberg. These results indicated that *E. faecium* could be used as a surrogate in validation studies for the two pathogens (*115*).

Listeria innocua is a well-studied non-pathogenic surrogate candidate which has been often used as surrogate for *L. monocytogenes* as the target pathogen (*41*). A study by Friedly et al. analyzed the thermal inactivation of *L. innocua* strains and one *L. monocytogenes* strain in hamburger beef patties to validate *L. innocua* as a possible ideal surrogate. *L. monocytogenes* had D-values ranging from 1.17 min to 0.17 min from 62.5°C to 70°C, respectively. Three different *L. innocua* strains were compared to these kinetics. *L. innocua* M1 with D-values ranging from 3.17 min to 0.34 min from 62.5°C to 70°C was the most resistant strain and should serve as nonpathogenic surrogate guaranteeing the best safety in validation processes for *L. monocytogenes*. Other *L. innocua* strains were also more resistant than *L. monocytogenes* and the perfect surrogate should be evaluated from case to case (*75*). *L. innocua* is not only used as a surrogate for *L. monocytogenes*, even though those two are closely related to each other. *L. innocua* has relatively heat resistant which makes it a good possible surrogate for other target pathogens in thermal processes. For example, *L. innocua* was evaluated as surrogate for different serovars of *Salmonella* at 55 to 70°C in chicken breast meat. The reported D-values at 55°C were 24.7 min and 56.2 min and at 70°C 0.09 min and 0.13 min for *Salmonella* and *L. innocua*, respectively. This showed that *L. innocua* was more heat resistant over the whole temperature range which would allow it to be used in the verification of new thermal processes for *Salmonella* (*121*).

Inactivation of *L. innocua* would ensure inactivation of *Salmonella* under the same conditions, since *Salmonella* has a lower heat resistance.

Another commonly used thermal surrogate for pasteurization processes is the non-pathogenic *Escherichia coli* K12. In a study in liquid egg, *E. coli* K12 was evaluated as a surrogate for *Salmonella* Enteritidis, a pathogen sometimes found in eggs (*148*). The pathogen as well as the surrogate candidate were heated in liquid egg white and reported D-values were tested from 52°C to 68°C with D-values ranging from 6.12 min to 0.28 min for *S.* Enteritidis and from 10.18 min to 0.28 min for *E. coli* K12. The results of this study showed that *E. coli* K12 had a slightly higher thermal resistance than *S.* Enteritidis at each tested temperature, making it an acceptable surrogate (*96*).

Recently, it was reported that viruses are another foodborne pathogen of concern in pasteurized foods (*32, 34*). Surrogates for human norovirus or hepatitis A virus are already in use (*21*), but, for a very different reason. Attempts to grow human norovirus and wild-type strains of HAV in normal cell or primary tissue culture have not been successful yet (*66, 134*). Therefore, viral surrogates have been used as indicators to understand inactivation of foodborne enteric viruses to validate and design food processing systems (*21*). However, to date, the primary method to control HAV is to prevent contamination of food instead of using methods of control or reduction (*85*). Viral surrogates would be the best fit to validate thermal processes, but are generally more difficult to culture and handle than bacterial surrogates (*33*). Bozkurt et al. showed that HAV had D-values at 72°C of 0.88 min, 0.91 min, 1.55 min, 1.07 min, in buffered cell media, spinach, clams, and mussels, respectively (*28, 29, 31, 34*). These D-values

are higher than those of current target pathogens in pasteurization, as shown in table 2.1. Historically, *L. monocytogenes* was thought to be the most resistant vegetative bacterial pathogen. However, the finding of a higher heat resistance of HAV indicated that the guidelines for thermal processes should be reconsidered in order to reduce the risk of more viral foodborne outbreaks. Therefore existing and new surrogate candidates have to be evaluated for validation.

Lactobacilli are one genera of bacteria which may potential to be used as surrogates in thermal validation studies. The advantage of using lactic acid bacteria is that many of them are extensively used in the food industry as starter cultures for fermentation processes, making ideal candidates as surrogates. *L. fructivorans,* a heterofermentative bacteria used in alcohol fermentations was reported to have a D-value of 1.2 min at 65°C in ketchup (*20*). The reported heat resistance of *L. fructivorans* is too low to consider it as a surrogate for HAV. Renix et al. analyzed another *Lactobacillus* strain, *L. bulgaricus* ATCC 11842, with D-values ranging from 9.98 min to 0.45 min at 65 to 70°C. *L. bulgaricus* showed a slightly higher heat resistance than *L. innocua*. However due to the fact that it is an anaerobic microorganism (*149*), it does not perfectly fit the criteria of a surrogate being easily cultivated and enumerated (*152*).

Staphylococcus carnosus is a Gram positive, non-motile, non-spore forming, facultative anaerobic bacteria which was first isolated from dry sausage. The bacteria grows well at temperatures ranging from 15 to 45°C and shows some potential for a higher heat resistance (*139*). In one study *S. carnosus* was tested along with other potential surrogates in frankfurter batter and ground beef (*171*). Among the tested bacteria, *S.*

carnosus had the highest reported D-value at 55°C with 152.63 min and 74.35 min in ground beef with 7 % fat and frankfurter batter, respectively. Several factors make this microorganism to an interesting candidate for further investigations at higher treatment temperatures. It is non-pathogenic, has wide range of growth temperatures, and it is easy to recover and grow.

Chapter III

Methods

Bacterial cultures

The bacterial isolate used in this research was the Gram-positive bacteria *Staphylococcus carnosus* CS-299. The culture was donated by Chr. Hansen (Milwaukee, WI).

Growth Conditions and Preparation of Surrogate Bacteria

The growth medium used for *Staphylococcus carnosus* was Brain Heart Infusion (BHI; BBL™ Brain Heart Infusion, Becton Dickinson and Company, Sparks, MD, USA) broth. For preparation of stock cultures BHI with 80 % glycerol was used followed by storage at -18°C. 0.20 µl of the frozen stock was resuscitated by transferring to BHI, followed by incubating aerobically for 24 h to stationary phase using 32°C or 40°C, depending upon the experiment, as incubation temperatures. Prior to thermal inactivation experiments, frozen stock cultures were transferred twice in BHI. In addition, 100 µl of the overnight culture was plated onto BHI agar (Fisher Scientific, Fair Lawn, NJ, USA) to determine the initial cell count. Cells were collected from the overnight stock by centrifuging 2.0 ml for 3 min at 5,000 x g at room temperature and washed twice with sterile phosphate buffered saline (PBS; Fisher Scientific). Following the last centrifugation, the supernatant was discarded and pellets were suspended in 1 ml of PBS to approximately 8.0 log CFU/mL in PBS. When conducting an experiment with milk, PBS was replaced with milk in the last step of the preparation.

Thermal Resistance of *S. carnosus* in Phosphate Buffered Saline or Milk

Thermal inactivation experiments with PBS and 2 % fat UHT milk (Hershey's, Hershey, PA) as heating media were performed in sterile 2 ml glass screw-capped vials in a circulating water bath (Lauda Eco Silver, Type E 40S, Karlsruhe, Germany). PBS was prepared as above and UHT milk was purchased from a grocery store, both were stored under refrigeration until use. 2.0 ml of washed cell suspension was added to 18 ml of PBS or UHT milk to get a 1:10 solution of the culture which resulted in an initial count of approximately 7.0 log CFU/ml. For cells incubated at 32°C, heat treatments were performed at 65, 67 and 70°C with varying time points (0-10 min). For cells incubated at 48°C, heat treatments were performed at 55, 60, 65, and 70°C for varying times (0-180 min). To record the come-up time as well as internal temperature of the vials, a Type T thermocouple (Omega Engineering, Inc., Stamford, CT) was placed at the geometric center of one vial through the lid. The thermocouple was 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 a thermocouple as well as a mercury-in-glass thermometer as a second control. The treatment time began when the target temperature was reached in the vials and the come up time was recorded. After treatments, the vials were immediately cooled down by placing them into an ice bath. The outside of the vials was sterilized with 70 % ethanol prior to opening.

Thermal Resistance of *S. carnosus* in Spinach

Frozen chopped spinach (Kroger, Cincinnati, OH) was purchased from a local grocery store. The spinach was allowed to thaw completely overnight under refrigeration and a sterilized blender (Waring blender, Model 1063, Waring Commercial, USA) was then used to homogenize the spinach. 50 g of homogenized spinach were added to sterile plastic tubes and stored in a -18°C. To inoculate the spinach, 75 g of spinach and 5 ml of culture were gradually added to a sterilized beaker followed by continuously hand-stirring over a period of 10 min. The final mass of the sample was 80 g. The target initial count of *S. carnosus* was approximately 8.0 log CFU/g. For each experiment, seven 10 g samples of inoculated spinach and one 10 g sample of un-inoculated food sample were aseptically weighed into 13 cm x 19 cm polyethylene/nylon bags (Seco Industries, Los Angeles, CA). All but one inoculated bag were vacuum-sealed with a Multivac A300/16 vacuum-packaging unit (Sepp Haggenmüller KG, Wolfertschwenden, Germany). After vacuum-sealing the bags, the food sample was spread to achieve a uniform thickness of about 1 to 3 mm and guarantee heat distribution as evenly as possible. The un-inoculated unsealed food sample was used for monitoring the internal temperature at the geometric center of the spinach with a Type T thermocouple (Omega Engineering, Inc., Stamford, CT). A thermal spacer was used to prevent bags from touching each other. Bags were spaced 2 cm from each other and it was a very open design to allow a good water exchange. Thermal inactivation of in spinach was performed as above. After cooling, 90 ml of PBS was added to the bags to obtain a 1:10 (w/v) suspension and stomached (Stomacher® 400 Circulator, Seward, UK) at 260 rpm for 90 sec.

Thermal Resistance of *S. carnosus* in Clams and Blue Mussel

Fresh clams (*Mercenaria mercenaria*) and fresh blue mussels (*Mytilus edulis*) were purchased from a local seafood market, stored on ice during the transport, followed by immediately placing them into the refrigerator. The flesh was removed from shell of the shellfish and was then homogenized in a blender (Waring) which had been sterilized using 70 % ethanol. Then, 50 g portions were added to sterile plastic tubes and stored in a -18°C until use. The day before conducting an experiment, the samples were removed from the freezer and allowed to thaw completely overnight under refrigeration. For preparation of the inoculated samples for both types of shellfish, 40 g were placed in a sterilized beaker and inoculated with the bacterial culture followed by continuous stirring over a period of 30 min. A total of 10 ml of the washed bacterial culture was added to the seafood samples to achieve a final weight of 50 g in the sterilized beaker. For each experiment, seven 6 g samples of inoculated clams or mussel and one 6 g sample of un-inoculated sample were aseptically weighed into 13 cm x 19 cm polyethylene/nylon bags (Seco Industries). As above, samples were vacuum sealed and heat treated as described for spinach. Bags were removed at 6 time points and immediately placed in an ice bath. 54 ml of PBS were added to the bags to obtain a 1:10 (w/v) suspension and the bags were stomached (Stomacher® 400 Circulator, Seward, UK) at 260 rpm for 90 sec.

Enumeration of Bacterial survivors and construction of survivor curves

Heat-treated samples as well as untreated samples were serially diluted in sterile 0.1 % peptone (Becton, Dickinsons and Company) and spread-plated on BHI agar. Plates were incubated aerobically for a total of 72 h at 32 and 40°C depending on the experiment. After incubation, colonies were enumerated and survivor curves plotted with log CFU/ml versus time to obtain D-values, or the time in minutes for the survivor curve to be reduced by 1 log or 90%. Z-values were determined by plotting the logarithm of the D-values over the temperatures used in the experiments. The z-value gives the changes in degrees (°C) to change the D-value by 90%.

Inactivation kinetic models

First-order model

Counts of surviving bacterial counts (log CFU/ml) were plotted against the heating time at each temperature to obtain the survivor curves. To describe the survivor curve quantitatively, the first-order model (equation 3.1) was used

$$log_{10}\frac{N(t)}{N_0} = -\frac{t}{D} \qquad (3.1)$$

N(t) is representing the number of survivors after an exposure time (t) in CFU/ml, N_0 is the initial bacterial population and the D-value is the decimal reduction time in minutes, which means the time required to kill 90% of the population.

The z-value describes the change in temperature required to change the D-value by 90% or 1 log. The z-values were calculated using the negative reciprocal of the slope of

the line obtained after plotting temperature versus log D-value and is shown in the following equation (3.2)

$$z = \frac{T_2 - T_1}{logD_1 - logD_2} \quad (3.2)$$

The numerator represents the difference between two temperatures T and the denominator the difference between the equivalent D-values on a logarithm basis.

"6D" projections at 72°C were obtained by calculating the $D_{72°C}$ for *S. carnosus* using equation (2.2) for the D-value, which was then multiplied with the factor 6. This indicates how long a sample needed to be heated to inactivate 6 logs of *S. carnosus* at 72°C. This methods is widely used in the industry to validate thermal inactivation processes

Weibull model

Another method for quantitatively describing the survivor curve that takes into account non-linear survival is the Weibull method (*55, 129*). It can be calculated using the following equation (3.3)

$$S(t) = \exp(-(\frac{t}{\propto})^{\beta} \quad (3.3)$$

Which is equivalent to equation (3.4)

$$S(t) = -\frac{1}{2.303}(\frac{t}{\propto})^{\beta} \quad (3.4)$$

Where *α* and *β* are the scale and shape parameters, respectively.

This equation can also be transformed into equation (3.5) by using the logarithm

$$logS(t) = -bt^{\beta} \quad (3.5)$$

With *b* being a variable for equation (3.6)

$$b = (\frac{1}{2.303}) \propto^{-\beta} \quad (3.6)$$

The time required to achieve a certain logarithmic reduction using this approach can be calculated by using equation (3.7) where *d* is the number of decimal reductions desired.

$$t_d = \propto (-\ln(10^{-d})^{\frac{1}{\beta}} \quad (3.7)$$

For this study, t_d was calculated using $d = 1$ and $d = 6$.

Statistical Analysis

The data obtained while conducting the experiments to heat-inactivate *S. carnosus* was statistically analyzed using an analysis of variance (ANOVA) and mean comparisons were analyzed using Tukey's test with a 95% confidence interval using SAS (SAS 9.4, Cary, NC). The goal was to determine if there were statistically significant differences in thermal resistance of *S. carnosus* at different incubation temperatures within different treatment temperatures and with different food models. All experiments were conducted in duplicate and repeated three times. For non-linear regression analysis of the Weibull model SPSS 23 statistical package (SPSS, IBM Armonk, NY) was used. The statistical parameters used to evaluate goodness of the fit of the models to the experimental data were higher R^2 (regression coefficient), lower chi-square (χ^2), and lower root mean square error (RMSE). For both models, RMSE and χ^2 were predicted for each replicate

of each treatment temperature using values of experimental and predicted survival ratios for each time (3.8, 3.9)

$$RMSE = [\frac{1}{N}\sum_{i=1}^{N}(S_{exp,i} - S_{pred,i})^2]^{\frac{1}{2}} \quad (3.8)$$

$$\chi^2 = \frac{\sum_{i=1}^{N}(S_{exp,i} - S_{pred,i})^2}{N-n} \quad (3.9)$$

The parameters $S_{exp,I}$ and $S_{pred,i}$ were the *i*th experimentally observed survival ratio and the *i*th predicted survival ratio, respectively. *N* was the number of obtained observations and *n* the number of constants.

Chapter IV

Results and Discussion

The objective of this study was to find a potential vegetative bacterial surrogate which could be used in validation of thermal pasteurization processes for elimination of hepatitis A virus. The thermal inactivation kinetics of the bacterium selected as a potential surrogate, *Staphylococcus carnosus* CS Chr-299, were determined under varying conditions. The influence of different media and incubation conditions on the thermal inactivation kinetics of *S. carnosus* was analyzed.

Thermal resistance of *S. carnosus* at different incubation temperatures and in various foods

The purpose of this study was to determine the thermal inactivation kinetics of *S. carnosus* and use several techniques in an attempt to increase the native thermal resistance of *S. carnosus.* The native heat resistance of *S. carnosus* was determined using a moderate incubation temperature of 32°C and PBS as a heating menstruum. Then, the thermal inactivation kinetics of *S. carnosus* were measured in milk, spinach, mussels, and clams. The objective of using different foods was to determine the influence of different compositions of various foods on heat resistance of *S. carnosus*. It has been shown previously with several microorganisms that heating in foods changes the thermal kinetics of the microorganisms (*59, 90, 120, 144*). Additionally, the foods selected were those used in determining the thermal inactivation kinetics of HAV and comparisons could be made between the two microorganisms (*28, 29, 31, 34*). In addition to different heating media, a second incubation temperature of 40°C was chosen as a comparison to the incubation temperature of 32°C to analyze the effect of a higher incubation temperature on the thermal resistance of *S. carnosus.* In previous

studies, increasing the incubation temperature was shown to increase the thermal resistance of microorganisms (*64, 68*).

The thermal inactivation kinetics of *S. carnosus* in different foods and under different incubation conditions were determined by enumerating survivors in CFU per ml over time at a specific temperature. Then, a first-order approach was used to obtain parameters to describe the thermal inactivation behavior of *S. carnosus.* First, the thermal decimal reduction time, or D-value, was determined. Using several treatment temperatures and the resulting D-values allowed for determination of the z-value for *S. carnosus*. The calculation of those two parameters allowed for comparison of the thermal inactivation kinetics of *S. carnosus* among different foods and different incubation temperatures.

In addition the linear model, a non-linear regression model, the Weibull model, was used as a comparison to the first-order model. This model considers that individual organisms behave different from the population and that several mechanisms are responsible for the inactivation of a cell (*129, 170*). It is more sensitive to changes in the thermal inactivation kinetics and can be used as an additional tool to interpret the behavior of *S. carnosus* when conducting thermal inactivation studies.

One other measurement calculated was the time to reduce a population of the test bacterium by 6 logs or the “6D” value. For the log-linear model it is simply 6 times the D-value at a specific temperature. For the Weibull model, the parameter t_d was used as a comparison to the D-value with $d = 1$ (*170*).

In experiments using the incubation temperature of 32°C, treatment temperatures of 65, 67, and 70°C were used. For incubation at 40°C, treatment temperatures were 55, 60, 65, and 70°C. As expected with increasing time, *S. carnosus* survivor counts were reduced for all tested combinations of food and incubation temperature. Also, with increasing treatment temperatures D-values for all combinations decreased significantly ($p < 0.05$) (table 4.2, 4.3). For incubation at 32°C, the D-values in PBS, milk, spinach, mussels, and clams at temperatures from 65 to 70°C calculated using the first-order model were in the range of 1.59 ± 0.20 to 0.36 ± 0.07 min, 2.57 ± 0.08 to 0.43 ± 0.08 min, 3.13 ± 0.59 to 0.46 ± 0.14 min, 2.83 ± 0.48 to 0.46 ± 0.04 min, and 3.02 ± 0.09 to 0.49 ± 0.04 min, respectively (table 4.2). Z-values in PBS, milk, spinach, mussels, and clams were found to be 7.65 ± 0.38, 6.45 ± 0.77, 5.97 ± 0.67, 6.36 ± 0.73, and 6.35 ± 0.18°C, respectively (table 4.6). For each food, the higher the treatment temperature the lower the D-value ($p < 0.05$), but for PBS when incubated at 32°C. Treatment temperature of 65°C was significantly different from 70°C, but not from 67°C and 67°C was not significantly different from 65 and 70°C. When comparing the different heating media, it was found that the $D_{65°C}$ of *S. carnosus* in PBS, which was considered the base D-value, was lower than the D-values of *S. carnosus* in all the foods. This observation confirmed the protective effects of foods on the thermal inactivation of microorganisms observed by other researchers (*59, 120*). However, while there was a significant difference at 65°C, there were no observed differences in the heat resistances between PBS and the foods at 70°C. This may have been a result of the rapid inactivation of *S. carnosus* at such high temperatures and weaker protective effects of the food components at this temperature.

Among the different foods, there were no significant differences in thermal resistance of *S. carnosus* observed. One possible reason may have been that all foods had similar high water activities, which resulted in very similar thermal resistance of *S. carnosus.* In addition, the pH of all foods was close to neutrality, which is another explanation for similar thermal resistances. All foods had similar fat content and protein content were slightly higher for mussels and clams compared to milk and spinach (tables 2.8). However, those differences did not seem to be significant enough to result in protective effects. It is difficult to compare results of the thermal inactivation behavior of *S carnosus* to the behavior of other bacterial surrogates in previously published studies due to the differences in used food matrices, or techniques. However, comparison of D-values among different surrogate microorganisms can be used as indication for the differences in resistance. D-values of *S. carnosus* at 70°C were slightly higher than reported D-values for *Listeria innocua* M1, one of the most heat resistant surrogate in use for pasteurization processes. The reported D-values for *L. innocua* M1 at 70°C were 0.21 min in raw chicken patties, 0.13 min in cooked chicken breast, 0.29 min in raw chicken tenders (*124*), and 0.34 min in hamburger meat patties (*75*). The lowest observed $D_{70°C}$ for *S. carnosus* in the present experiment was a $D_{70°C}$ = 0.36 min in PBS which was higher than any of the reported D-values for *L. innocua* M1. This indicates the potential of *S. carnosus* being a surrogate for microorganisms with relatively high heat resistance. However, comparing the observed D-values of *S. carnosus* incubated at 32°C to the D-values of HAV at 70°C showed that *S. carnosus* was not heat resistant enough to be used as a surrogate for HAV. Calculated $D_{70°C}$ of HAV was 1.27 min, 1.26 min, 1.51 min, and 2.11 min for cell buffered medium, spinach, mussels, and clams,

respectively (table 4.7) (*28, 29, 31, 34*). These D-values were two to three times higher, than the observed D-values of *S. carnosus* at 70°C (table 4.2).

In literature, it has been reported that an increased incubation temperature may lead to an increased thermal resistance of microorganisms (*68, 82*). Based on these studies, additional experiments with *S. carnosus* in PBS were conducted at an incubation temperature of 40°C. The D-values at 65 to 70°C ranged from 3.13 ± 0.48 min to 0.50 ± 0.03 min for *S. carnosus* in PBS. In comparison to the D-values for *S. carnosus* incubated at 32°C and heated in PBS, a significantly higher D-value was found at 65°C (table 4.1). These results suggested that an increase in incubation temperature increased the thermal resistance of cells. Based on these findings, thermal inactivation studies for all five media were conducted with cells incubated at 40°C. D-values in PBS, milk, spinach, mussels, and clams heated from 55 to 70°C ranged from 12.69 ± 0.83 to 0.50 ± 0.03 min, 478.35 ± 78.35 to 0.44 ± 0.09 min, 77.77 ± 13.68 to 1.24 ± 0.04 min, 58.39 ± 9.80 to 0.31 ± 0.06 min, and 165.04 ± 10.45 to 0.82 ± 0.02 min, respectively (table 4.3). Z-values in PBS, milk, spinach, mussels, and clams were found to be 9.54 ± 2.08, 5.59 ± 1.22, 9.11 ± 0.38, 6.65 ± 0.49, and 6.17 ± 0.08°C, respectively (table 2.6). Results from the two incubation temperatures, 32 and 40°C, were analyzed using a factorial randomized block design (RBD factorial) on the interaction of incubation temperature and food media (table 4.1). At 65 and 70°C, the D-values in PBS at 65°C, spinach at 65 and 70°C, and clams at 70°C were significant higher ($p < 0.05$) for the 40°C incubation than the 32°C incubation. Thus, a higher incubation temperature can result in a significantly greater thermal resistance of a microorganism. Besides comparing the D-values of the two incubation temperatures, the calculated D-values

were logarithmically plotted over temperature to obtain the z-value curves (figure 4.1 - 4.5 [B]). This was done to get a better impression of the general behavior at the two incubation temperatures over a wide treatment temperature range. The curves for the two incubation temperatures had similar slopes for each medium with spinach being the only food medium with a significantly higher z-value at 40°C. In literature, it was also shown that growing microorganism in an environment with a higher temperature resulted in a higher percentage of survivors when applying a thermal treatment (*64, 68*). A reason for the observed increase in heat resistance may be an enhanced expression of heat-shock proteins which can help to refold denatured proteins or other proteins which can increase the resistance of the cell membrane (*100*).

Different heat resistance of microorganisms in food media may be explained by the influence of food components on survival. A higher protein, fat or carbohydrate content might function as a protective barrier against heat (*59, 120*). Milk has a relatively high protein content which may have been a reason for the high D-value at 55°C. At 70°C, the D-value of *S. carnosus* in milk was significantly lower than in spinach, which may be explained by the denaturation of protective proteins at temperatures of about 70°C. Milk can be treated with high temperatures and pressure or even be dried to powder without significant damage to the casein micelle system. However, temperatures above about 70°C can cause denaturation of major whey proteins in milk (*3*). Another reason for differences among the foods may be based on the difference in the water activity (a_w) of the foods. Many studies have shown that a lower a_w of a media results in a higher heat resistance of a microorganism (*173*). In this study, the a_w of the used heating models was not very different, due to a high water content in all samples. However, an

explanation might be that foods with similar a_w showed different changes in a_w when applying heat (*90, 144*). Syamaladevi et al. showed this effect by heating all-purpose flour and peanut butter from 20°C to 80°C. At 20°C, both foods had an a_w of 0.45, but when increasing the temperature to 80°C, the a_w of all-purpose flour changed to 0.80 and the a_w of peanut butter to 0.04 (*159*). This effect might explain the different behavior of *S. carnosus* among foods even if the initial a_w was about the same.

Comparing the thermal inactivation kinetics of *S. carnosus* incubated at 40°C to those of HAV at 70°C, reported by Bozkurt et al. (*28, 29, 31, 34*) showed that the D-values of *S. carnosus* were generally lower than those of HAV, with one exception. *S.* carnosus had a $D_{70°C}$ of with 1.24 ± 0.04 min in spinach, which was close to the calculated $D_{70°C}$ of 1.26 min of HAV. This indicated that *S. carnosus* may be used in pasteurization as surrogate for thermal inactivation of HAV in spinach. The $D_{70°C}$ of the other foods were two to three times lower when comparing to the $D_{70°C}$, indicating that the thermal resistance of *S. carnosus* in these foods may not be sufficient for validation of HAV (table 4.7).

In the food industry, a six log reduction is often used to design pasteurization processes. The D-values of the experiments with an incubation temperature of 40°C were chosen for the 6D model. To compare the 6D values of *S. carnosus* with HAV, the $D_{72°C}$ values had to be calculated by using the z-value (table 4.6). D-values of *S. carnosus* were compared to HAV within the same food sample and under the same heating conditions. The comparison of different food matrices may result in differences in inactivation due to compositional differences of food samples. Calculated process times to achieve a 6 log

reduction of HAV at 72°C for the first-order model were 5.3, 5.5, 9.3, and 6.4 min for PBS, spinach, mussels, and clams, respectively (table 4.7). When processing *S. carnosus* for the required times to achieve a 6 log reduction of HAV, log reductions for *S. carnosus* of 17.1, 7.3, 40.1, 23.8 log reductions for PBS, spinach, mussels and clams, respectively would be needed (table 4.7). Based on those results, only the combination of *S. carnosus* and spinach showed a heat resistance high enough to achieve a realistic log reduction. For PBS and clams, a 2D log reduction could be used, resulting in approximately 5.5 and 8 log reductions needed, respectively. For mussels, the obtained heat resistance was not strong enough to use *S. carnosus* as a surrogate in validation studies.

The Weibull model was also utilized as a nonlinear regression approach to describe the inactivation of *S. carnosus* during thermal inactivation. The Weibull model has a shape factor (β) and a time factor (α) integrated in the equation to better fit a curve to the data. The shape factor indicated that *S. carnosus* had monotonic upward concave (tailing) curve behavior ($\beta < 1$) and monotonic downward concave (shoulder) behavior ($\beta > 1$) depending on the temperature for both incubation temperatures (table 4.2 and 4.3). Tailing behavior indicates that some cells of a population are more sensitive to heat and are destroyed more quickly while others show an ability of surviving longer when applying heat. Shoulder behavior indicates that survivors were more resistant initially but become increasingly damaged over time (*170*, *129*).

To identify differences between the first-order and Weibull model, a completely randomized design (CRD) factorial was used to analyze for each food and treatment

temperature. Tukey's test was run to check for differences between the first-order and Weibull model per food and treatment temperature for both incubation temperatures, 32 and 40°C. Both models gave a good fit to the experimental data for all tested treatment temperatures at 32°C incubation since no significant differences were obtained between the two models. This shows that the Weibull model can be used as a non-linear regression approach to describe the thermal inactivation kinetics of *S. carnosus* incubated at 32°C. When incubated at 40°C, the Weibull model did not result in useful values for spinach at 65°C as well as when calculating the $t_{d=6}$ for PBS at 60°C (table 4.2 and 4.3). This was due to the distribution of the experimental data, which the Weibull model was not able to explain. The first-order model had a very poor fit to the observed data of *S. carnosus* in milk at 55°C (R^2 = 0.468 (table 4.5)) compared to the Weibull-model (R^2 = 0.998 (table 4.5), this can also be seen when comparing the D-value and the t_d-value. The number of survivors of *S. carnosus* was barely decreased when heat treating it at 55°C in milk. However, there was a slight initial drop in survivors, followed by consistent numbers of survivors for the rest of the treatment time. This effect can also be described as a tailing effect by the Weibull model ($\beta < 1$). The first-order model is calculated by using a trend line through the experimental data points, which results in a poorer fit, when having a strong tailing or shoulder effect in the data. Therefore the D-value calculated by the first-order model did not represent the actual time required to achieve a 90 % log reduction. The Weibull model, as a non-linear regression approach, has a shape (β) and time (α) factor, which help to get a better fit to the observed data points, resulting in a better representation of the real D-value. In this case, the shape parameter was extremely low with β = 0.06, which resulted in a strong

tailing effect. Since the first-order model lacks the ability to observe these effects, the Weibull model is doing a better job explaining data with very strong tailing or shouldering effects.

At an incubation of 32°C, there was no significant difference between the first-order and Weibull model for all tested combinations with the exception of milk at 67°C (table 4.2). At 40°C incubation temperature, there was no significant difference between the first-order and Weibull model for all tested combinations except for milk and spinach at 65°C (table 4.3). However, the $t_{d=1}$ of *S. carnosus* in spinach at 65°C with 0.11 was lower than the $t_{d=1}$ at 70°C. The D-values usually decrease with increasing treatment temperature indicating false observations at one of the two treatment temperatures when the experiments were conducted. Differences between the two models were not significantly different, which may have been due to low numbers of observation. However, the nonlinear regression approach demonstrated that it is more flexible and may result in a significantly better fit when having enough numbers of observation. Further analysis was carried out by calculating the regression coefficient (R^2), the root mean square error (RMSE), and chi-square (χ^2) for the first-order and Weibull model (table 4.4 and 4.5). There were few cases where the first-order model was having a higher R^2-value than the Weibull model, such as for spinach incubated at 32°C at 67°C or incubated at 40°C at 70°C (table 4.4 and 4.5). This may be explained by a shoulder and tailing effect. In this case, the Weibull model can be worse than the first-order model, because it can only explain one or the other. However for most cases, the regression coefficient was comparatively higher and the RMSE and χ^2 values were comparatively lower for the Weibull model.

Also, the z-value curves were plotted for the Weibull model approach (figure 4.1 – 4.5 [A]) to see the behavior of the survivors over a wide range of treatment temperatures when using a nonlinear approach. In comparison to the figures representing the first-order model, no significant differences were obtained between the z-values of the two models within one food medium and one incubation temperature.

To compare the 6D and $t_{d=6}$ values of *S. carnosus* to those of HAV. The 6D and $t_{d=6}$ values were calculated for the first-order and Weibull model, respectively, and statistically compared. The only statistical differences were obtained at incubation temperature of 40°C for the treatment temperature of 70°C for all foods (table 4.2 and 4.3). When calculating the time required to obtain a 6 log reduction, the shape factor (β) played an important role. This can be seen at the incubation temperature of 40°C with clams at a treatment temperature of 70°C. For one log reduction, D-values were 0.79 and 0.82 min for the Weibull and first-order model, respectively. However, due to a low shape factor $\beta = 0.44$, the order changed when calculating a 6 log reduction. Reported values were 5.96 and 4.90 min for the Weibull and first-order model, respectively (table 4.3). This showed the better fit of the Weibull model when a tailing effect occurs.

Chapter V

Conclusion

The identification of potential bacterial surrogates for validation of thermal inactivation processes of HAV is important due to continue outbreaks and a new concern that it should be the target pathogen in pasteurization processes instead of *L. monocytogenes*. This study was conducted to analyze several methods to increase the heat resistance of *S. carnosus*. A higher incubation temperature demonstrated the closest heat resistance to HAV and the bacteria could be used in the 6D model in combination with the food model spinach. A 2D model would be appropriate in buffer and clams, showing that *S. carnosus* has the potential to be used in validating thermal processes for HAV. Hence, the results of this study provide useful information that can help to design appropriate thermal processes to ensure food safety. Further studies are needed to investigate and describe the thermal inactivation kinetics of *S. carnosus* while altering the growth media during incubation.

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Appendix

Tables

Table 2.1 D-values of the first-order model for different bacterial and viral pathogens during thermal inactivation

Pathogens	Medium	D-values (min) at 60°C (D_{60})	D-values (min) at 72°C (D_{72})
L. monocytogenes	PBS	0.66 ± 0.16	NC
	Spinach	1.22 ± 0.12	NC
S. enterica	PBS	0.20 ± 0.02	NC
	Spinach	0.47 ± 0.06	NC
E. coli O157	PBS	NC*	NC
	Spinach	0.97 ± 0.33	NC
E. coli non-O157	PBS	NC	NC
	Spinach	0.93 ± 0.07	NC
HAV	Buffered Cell Culture Medium	2.67 ± 0.42	0.88 ± 0.11
	Spinach	4.55 ± 0.82	0.91 ± 0.14

*NC = not calculated, because organism was inactivated too fast

Table 4.1 D-values for the first-order model of *S. carnosus* in food media during thermal treatment at 65 and 70°C

Heating medium	Incubation Temperature	Treatment Temperature	D-value
PBS	32	65	$1.59 \pm 0.20^{A*}$
	40		3.13 ± 0.48^{B}
	32	70	0.36 ± 0.07^{A}
	40		0.5 ± 0.03^{A}
Milk	32	65	2.57 ± 0.08^{A}
	40		2.46 ± 0.06^{A}
	32	70	0.43 ± 0.08^{A}
	40		0.44 ± 0.09^{A}
Spinach	32	65	3.13 ± 0.59^{A}
	40		5.59 ± 0.95^{B}
	32	70	0.46 ± 0.14^{A}
	40		1.24 ± 0.04^{B}
Clams	32	65	3.02 ± 0.09^{A}
	40		2.45 ± 0.02^{A}
	32	70	0.49 ± 0.04^{A}
	40		0.82 ± 0.02^{B}
Mussels	32	65	2.83 ± 0.48^{A}
	40		1.93 ± 0.29^{A}
	32	70	0.46 ± 0.04^{A}
	40		0.31 ± 0.06^{A}

*Values with different capital letters within one food media and one treatment temperature are significantly different ($p < 0.05$)

Table 4.2 Coefficients of the Weibull and first-order models for the survivor curves of *Staphylococcus carnosus* Chr Cs-299 in PBS, Milk, Spinach, Mussels, and Clams incubated at 32°C

Heating medium	T (°C)	Weibull distribution				First-order kinetics	
		β	α	$t_{D=1}$ (min)	$t_{D=6}$ (min)	D (min)	6D (min)
PBS	65	0.9 ± 0.15	0.54 ± 0.16	1.36 ± 0.25	10.32 ± 2.16	1.59 ± 0.20	9.53 ± 1.19
	67	0.92 ± 0.17	0.34 ± 0.16	0.82 ± 0.24	5.84 ± 0.76	0.91 ± 0.10	5.48 ± 0.63
	70	0.9 ± 0.10	0.13 ± 0.05	0.32 ± 0.10	2.3 ± 0.37	0.36 ± 0.07	2.14 ± 0.40
Milk	65	0.78 ± 0.10	0.6 ± 0.24	1.72 ± 0.51	17.07 ± 0.11	2.57 ± 0.08	15.4 ± 0.51
	67	0.66 ± 0.11	0.19 ± 0.08	0.66 ± 0.16	10.42 ± 2.58	1.38 ± 0.14	8.26 ± 0.84
	70	0.74 ± 0.41	0.08 ± 0.10	0.25 ± 0.17	4.2 ± 2.11	0.43 ± 0.08	2.6 ± 0.49
Spinach	65	0.74 ± 0.03	0.68 ± 0.19	2.07 ± 0.54	22.98 ± 5.86	3.13 ± 0.59	18.8 ± 3.55
	67	1.16 ± 0.42	0.75 ± 0.35	1.57 ± 0.40	8.73 ± 4.13	1.53 ± 0.40	9.21 ± 2.43
	70	1.06 ± 0.22	0.21 ± 0.05	0.47 ± 0.08	2.8 ± 1.15	0.46 ± 0.14	2.74 ± 0.81
Mussels	65	1.1 ± 0.33	1.31 ± 0.39	2.87 ± 0.13	18.87 ± 2.12	2.83 ± 0.48	16.95 ± 2.87
	67	0.88 ± 0.17	0.57 ± 0.20	1.45 ± 0.30	11.6 ± 2.26	1.57 ± 0.15	9.43 ± 0.87
	70	1.18 ± 0.21	0.25 ± 0.06	0.5 ± 0.06	2.35 ± 0.32	0.46 ± 0.04	2.76 ± 0.22
Clams	65	0.74 ± 0.08	0.80 ± 0.19	2.45 ± 0.32	27.68 ± 3.32	3.02 ± 0.09	18.13 ± 0.51
	67	1.01 ± 0.01	0.59 ± 0.07	1.34 ± 0.17	7.94 ± 1.13	1.32 ± 0.19	7.93 ± 1.16
	70	1.42 ± 0.56	0.31 ± 0.14	0.56 ± 0.14	2.30 ± 0.75	0.49 ± 0.04	2.93 ± 0.27

Table 4.3 Coefficients of the Weibull and first-order models for the survivor curves of *Staphylococcus carnosus* Chr Cs-299 in PBS, Milk, Spinach, Mussels, and Clams incubated at 40°C

Heating medium	T (°C)	Weibull distribution				First-order kinetics	
		β	α	$t_{D=1}$ (min)	$t_{D=6}$ (min)	D (min)	6D (min)
PBS	55	0.54 ± 0.04	0.74 ± 0.14	3.47 ± 0.27	97.32 ± 14.74	12.69 ± 0.83	76.11 ± 4.98
	60	0.36 ± 0.01	0.35 ± 0.45	1.64 ± 0.09	150.27 ± 37.34	8.91 ± 0.71	53.45 ± 4.25
	65	0.55 ± 0.10	0.43 ± 0.33	2.26 ± 0.89	53.59 ± 18.60	3.13 ± 0.48	18.77 ± 2.86
	70	1.00 ± 0.30	0.21 ± 0.11	0.48 ± 0.16	3.22 ± 0.97	0.5 ± 0.03	2.98 ± 0.20
Milk	55	0.06 ± 0.01	3.00 ± 4.20	1.49E+06	1.20E+21	478.35 ± 78.35	2870.12 ± 470.07
	60	0.77 ± 0.05	4.86 ± 1.75	14.33 ± 5.07	115.97 ± 53.05	17.73 ± 4.82	106.39 ± 28.94
	65	0.56 ± 0.01	0.16 ± 0.01	0.72 ± 0.04	6.93 ± 0.75	2.46 ± 0.06	14.74 ± 0.39
	70	0.55 ± 0.13	0.05 ± 0.05	0.18 ± 0.15	4.06 ± 0.55	0.44 ± 0.09	2.64 ± 0.55
Spinach	55	1.08 ± 0.29	39.47 ± 11.28	86.02 ± 6.49	495.28 ± 185.94	77.77 ± 13.68	466.6 ± 82.07
	60	0.4 ± 0.10	0.32 ± 0.37	2.13 ± 1.48	211.19 ± 73.56	12.67 ± 0.48	75.99 ± 2.89
	65	0.18 ± 0.05	0.00 ± 0.00	0.11 ± 0.05	12080.66 ± 14809.08	5.59 ± 0.95	33.51 ± 5.70
	70	1.08 ± 1.28	0.72 ± 0.42	1.25 ± 0.25	5.55 ± 3.65	1.24 ± 0.04	7.44 ± 0.21
Mussels	55	0.99 ± 0.24	23.62 ± 0.23	57.48 ± 12.87	429.56 ± 289.84	58.39 ± 9.80	350.33 ± 58.80
	60	0.68 ± 0.02	2.72 ± 0.08	9.30 ± 0.11	89.67 ± 12.24	13.25 ± 0.46	79.5 ± 2.74
	65	0.88 ± 0.35	0.64 ± 0.46	1.58 ± 0.60	10.69 ± 10.29	1.93 ± 0.29	11.58 ± 1.72
	70	1.35 ± 0.93	0.19 ± 0.17	0.32 ± 0.23	2.09 ± 1.39	0.31 ± 0.06	1.88 ± 0.37
Clams	55	0.92 ± 0.19	65.48 ± 20.99	161.58 ± 22.78	1217.05 ± 316.93	165.04 ± 10.45	990.21 ± 62.72
	60	0.72 ± 0.18	11.77 ± 5.86	36.84 ± 7.93	518.5 ± 212.08	38.21 ± 4.80	229.23 ± 28.82
	65	0.65 ± 0.04	0.44 ± 0.07	1.57 ± 0.13	24.53 ± 2.53	2.45 ± 0.02	14.68 ± 0.11
	70	0.44 ± 0.40	0.12 ± 0.16	0.79 ± 0.39	5.96 ± 0.00	0.82 ± 0.02	4.9±0.12

Table 4.4 Statistical comparison of the Weibull and first-order models for the survivors of *Staphylococcus carnosus* Chr Cs-299 in PBS, Milk, Spinach, Mussels, and Clams incubated at 32°C

Heating medium	T (°C)	Weibull distribution			First-order kinetics		
		R^2	RMSE	χ^2	R^2	RMSE	χ^2
PBS	65	0.993	0.073	0.012	0.995	0.115	0.021
	67	0.991	0.082	0.011	0.995	0.112	0.020
	70	0.995	0.074	0.012	0.994	0.102	0.025
Milk	65	0.990	0.121	0.026	0.977	0.870	1.414
	67	0.986	0.150	0.035	0.959	0.813	1.286
	70	0.987	0.121	0.028	0.974	0.295	0.166
Spinach	65	0.959	0.218	0.072	0.962	0.821	1.271
	67	0.647	0.289	0.150	0.995	0.868	1.513
	70	0.975	0.237	0.109	0.987	0.253	0.125
Mussels	65	0.867	0.255	0.115	0.894	0.016	0.114
	67	0.992	0.133	0.039	0.991	0.012	0.008
	70	0.900	0.075	0.013	0.951	0.008	0.070
Clams	65	0.995	0.046	0.003	0.981	0.105	0.017
	67	0.989	0.071	0.013	0.992	0.099	0.016
	70	0.966	0.118	0.021	0.970	0.145	0.033

Table 4.5 Statistical comparison of the Weibull and first-order models for the survivors of *Staphylococcus carnosus* Chr Cs-299 in PBS, Milk, Spinach, Mussels, and Clams incubated at 40°C

Heating medium	T (°C)	Weibull distribution			First-order kinetics		
		R^2	RMSE	χ^2	R^2	RMSE	χ^2
PBS	55	0.982	0.171	0.049	0.895	0.382	0.191
	60	0.990	0.080	0.010	0.822	0.451	0.306
	65	0.993	0.046	0.004	0.925	0.323	0.206
	70	0.985	0.105	0.024	0.989	0.656	1.443
Milk	55	0.995	0.042	0.003	0.468	0.139	0.031
	60	0.985	0.086	0.013	0.973	0.840	2.481
	65	0.989	0.132	0.036	0.926	0.404	0.250
	70	0.997	0.062	0.006	0.929	0.310	0.184
Spinach	55	0.945	0.182	0.050	0.947	0.923	1.296
	60	0.987	0.114	0.021	0.855	0.407	0.252
	65	0.793	0.463	0.347	0.543	0.612	0.582
	70	0.620	0.398	0.243	0.770	0.510	0.403
Mussels	55	0.900	0.102	0.022	0.908	0.106	0.023
	60	0.995	0.043	0.003	0.968	0.121	0.022
	65	0.961	0.148	0.034	0.967	0.224	0.085
	70	0.956	0.197	0.094	0.913	0.351	0.229
Clams	55	0.994	0.032	0.001	0.993	0.045	0.004
	60	0.978	0.051	0.004	0.967	0.078	0.010
	65	0.997	0.044	0.003	0.963	0.142	0.031
	70	0.864	0.170	0.036	0.743	0.271	0.116

Table 4.6 Z-values of *S. carnosus* Chr CS-299 in food media incubated at 32 and 40°C for the first-order [A] and Weibull model [B]

A

Heating medium	Incubation Temperature (°C)	z-value (°C)
PBS	32	$7.65 \pm 0.38^{A*}$
	40	9.54 ± 2.08^{A}
Milk	32	6.45 ± 0.77^{A}
	40	5.86 ± 1.85^{A}
Spinach	32	5.97 ± 0.67^{A}
	40	9.11 ± 0.38^{B}
Mussels	32	6.36 ± 0.73^{A}
	40	6.65 ± 0.49^{A}
Clams	32	6.35 ± 0.18^{A}
	40	6.17 ± 0.08^{A}

B

Heating medium	Incubation Temperature (°C)	z-value (°C)
PBS	32	8.07 ± 1.88^{A}
	40	19.55 ± 3.85^{A}
Milk	32	5.59 ± 1.22^{A}
	40	5.25 ± 1.40^{A}
Spinach	32	7.87 ± 1.66^{A}
	40	9.78 ± 7.45^{A}
Mussels	32	6.56 ± 0.37^{A}
	40	6.67 ± 1.53^{A}
Clams	32	7.72 ± 0.66^{A}
	40	5.55 ± 0.27^{A}

*Values with different capital letters within one food media are significantly different ($p < 0.05$)

Table 4.7 Thermal inactivation projections of HAV and *S. carnosus* in PBS, spinach, mussels, and clams using the first-order model

Microorganism	Model	$D_{70°C}$	$D_{72°C}$	6D at 72°C (min)	log reduction using 6D at 72°C for HAV
HAV in buffered cell medium	First-order model	1.27	0.88	5.3	6.0
S. carnosus in PBS		0.50	0.31	1.9	17.1
HAV in spinach	First-order model	1.26	0.91	5.5	6.0
S. carnosus in Spinach		1.24	0.75	4.5	7.3
HAV in clams	First-order model	2.11	1.55	9.3	6.0
S. carnosus in clams		0.82	0.39	2.3	23.8
HAV in mussels	First-order model	1.51	1.07	6.4	6.0
S. carnosus in mussels		0.31	0.16	1.0	40.1

Table 4.8 Composition of 2 % UHT milk, spinach, mussels, and clams per 100g

	2% UHT Milk	Spinach	Mussels	Clams
	Nutrients in g per 100 g food			
Fat	2.12	0.00	2.27	0.97
Carbohydrate	5.08	3.70	3.67	2.56
Protein	3.39	2.47	11.93	12.78
Sodium	0.05	0.15	0.29	0.06
Potassium	0.14	0.00	0.32	0.14

Figures

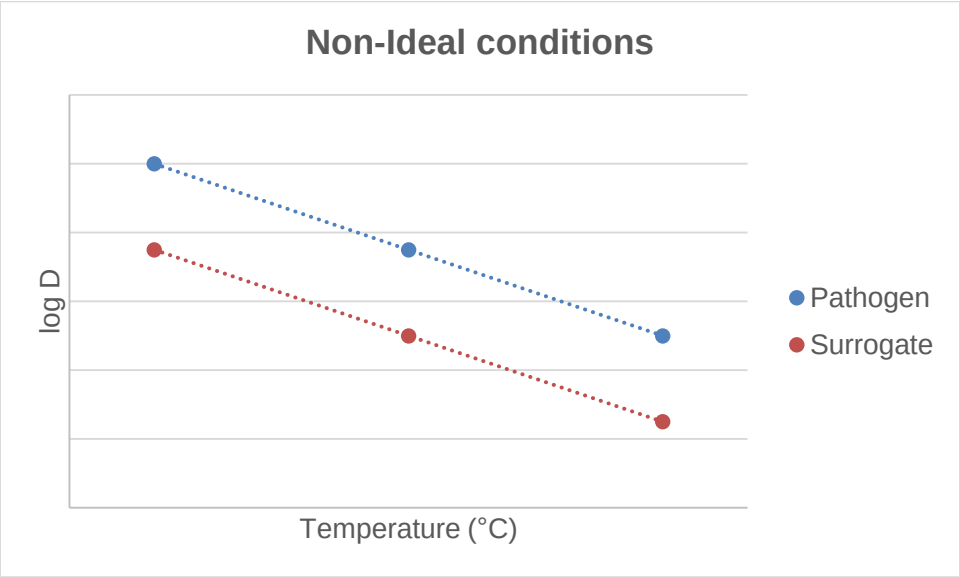


Figure 2.1 Non-ideal conditions of a surrogate microorganism

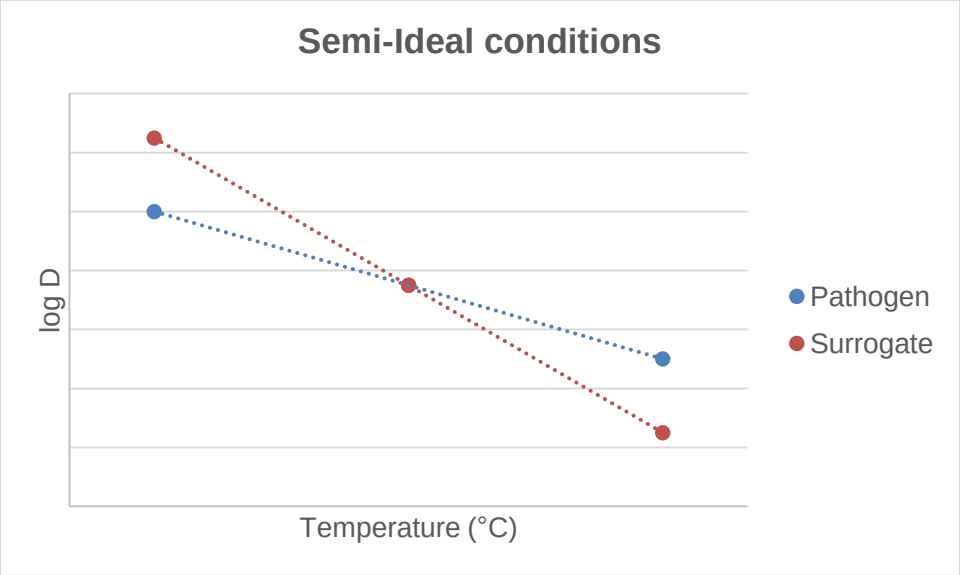


Figure 2.2 Semi-Ideal conditions of a surrogate microorganism

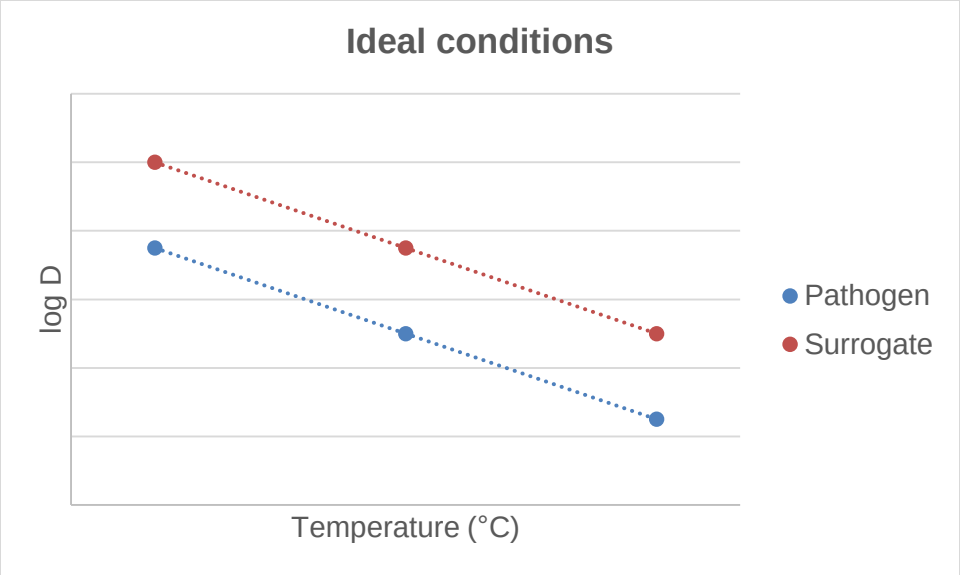
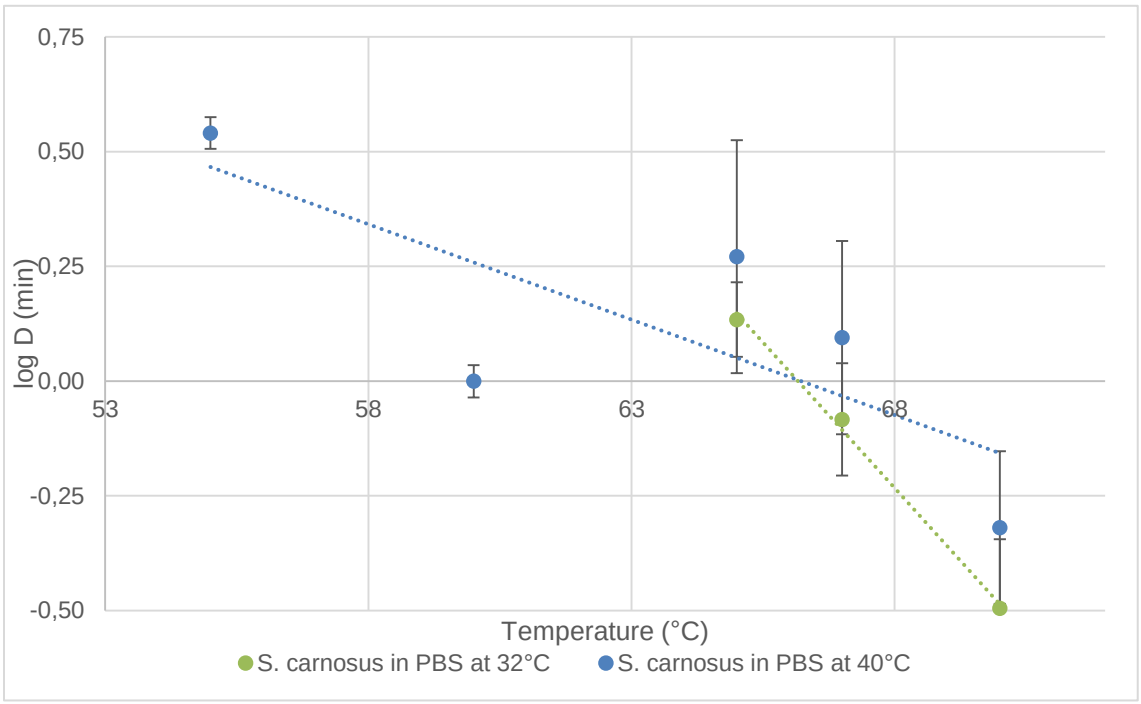


Figure 2.3 Ideal-conditions of a surrogate microorganism

A



B

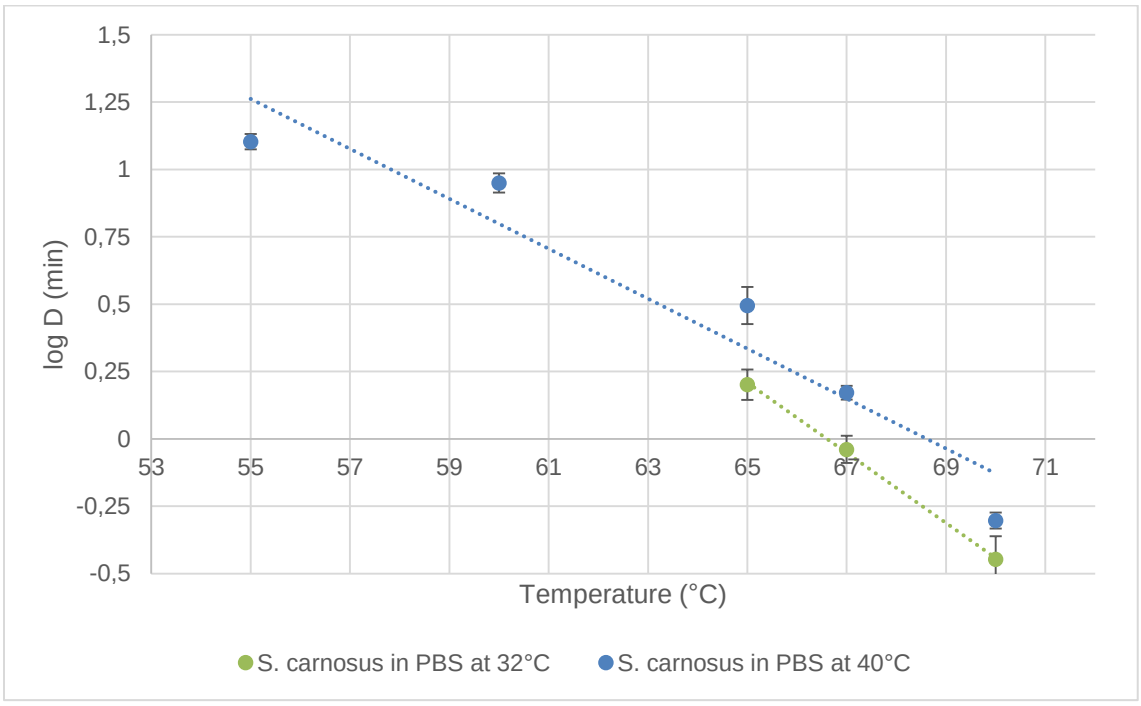
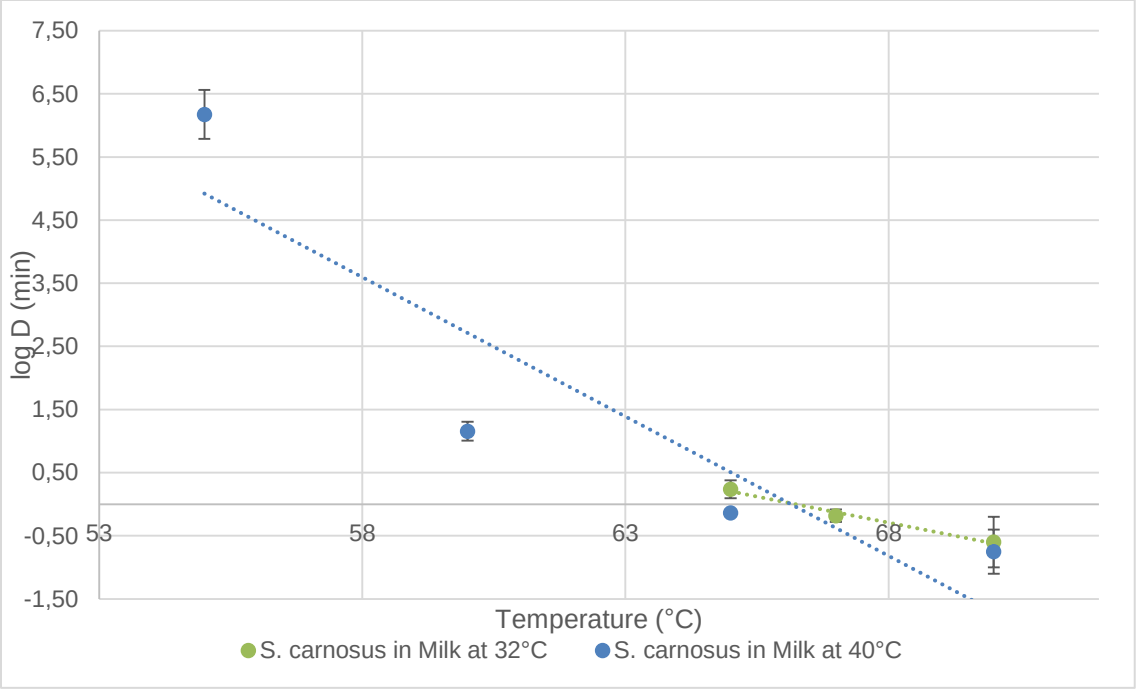


Figure 4.1 Thermal inactivation curves of *Staphylococcus carnosus* for the Weibull model [A] and first-order model [B] incubated at 32°C and 40°C in phosphate buffered saline (PBS)

A



B

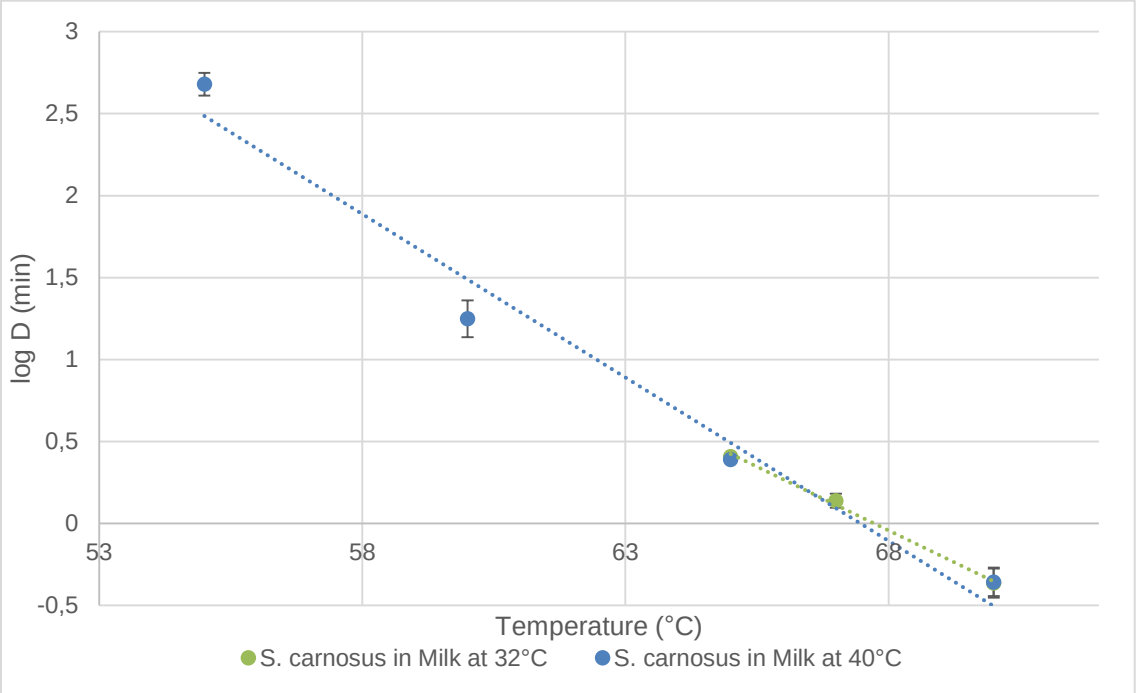
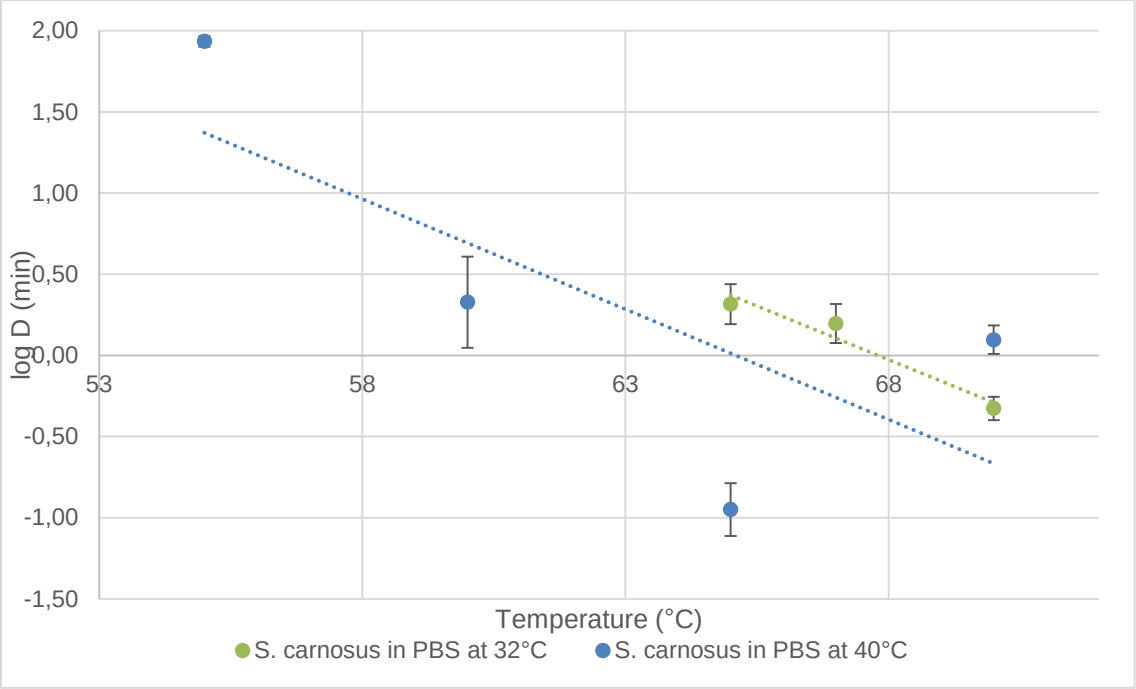


Figure 4.2 Thermal inactivation curves of *Staphylococcus carnosus* for the Weibull model [A] and first-order model [B] incubated at 32°C and 40°C in 2% UHT milk

A



B

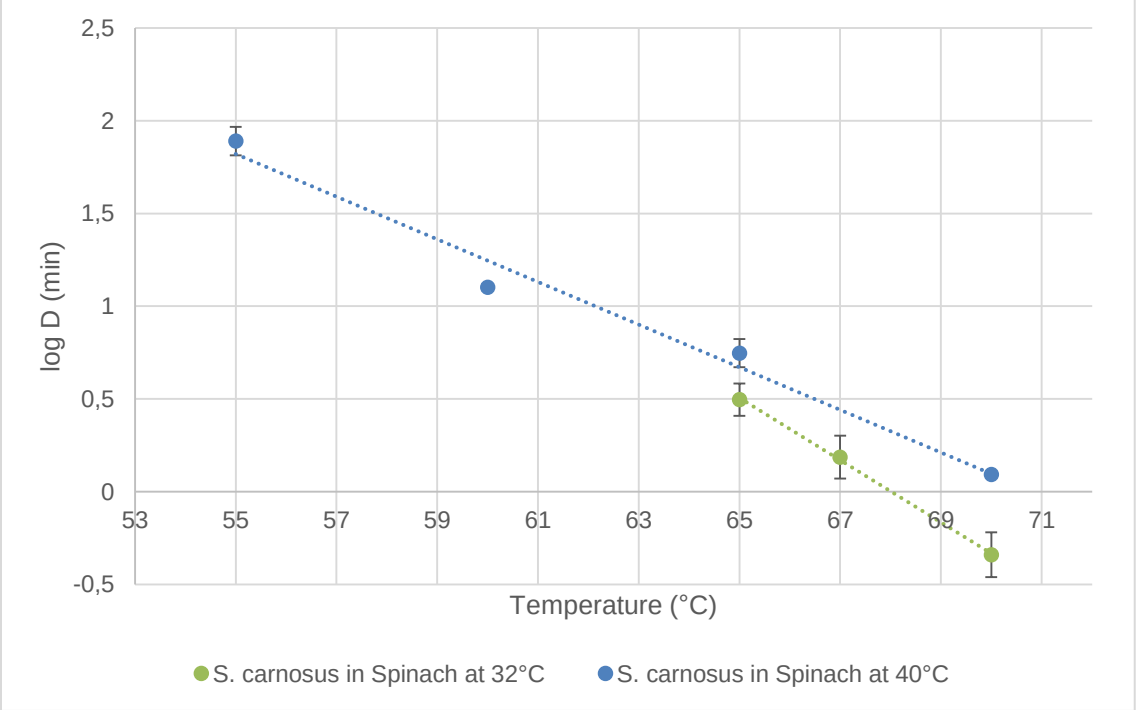
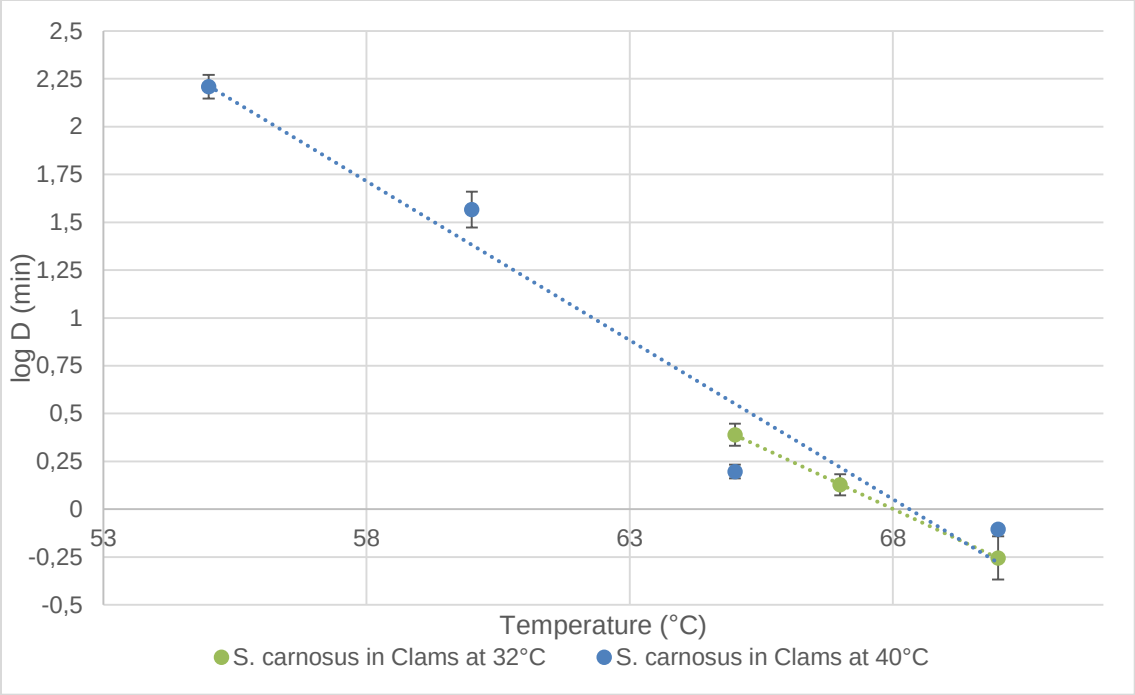


Figure 4.3 Thermal inactivation curves of Stapyhlococcus carnosus for the Weibull model [A] and first-order model [B] incubated at 32°C and 40°C in spinach

A



B

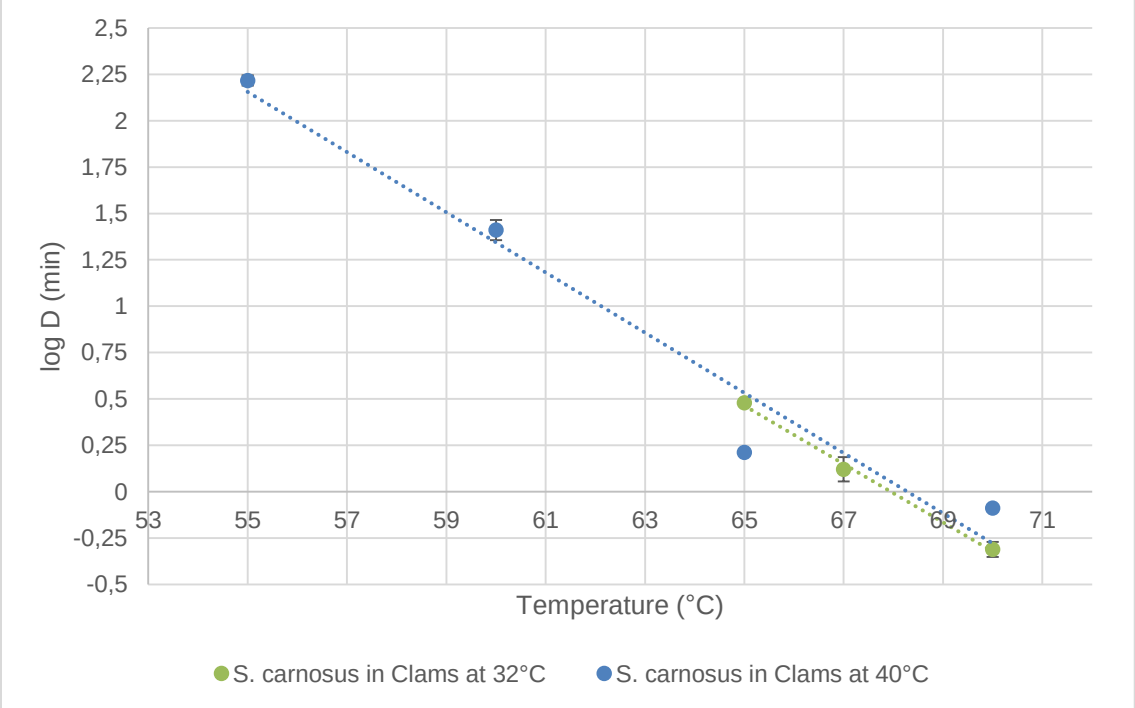
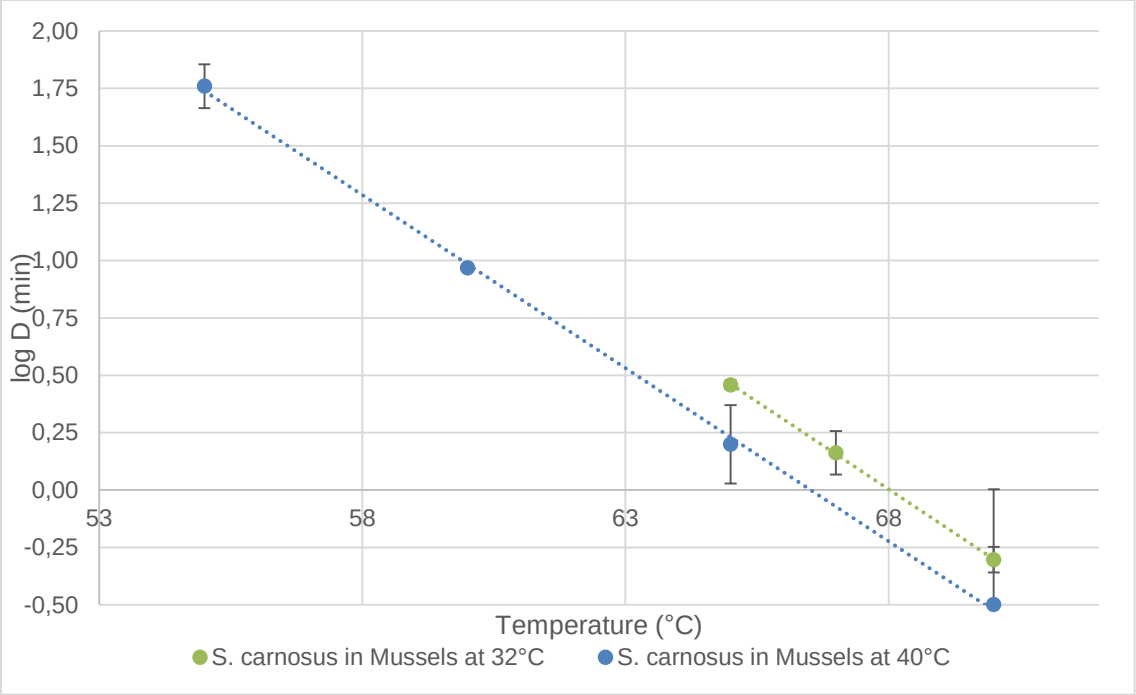


Figure 4.4 Thermal inactivation curves of *Staphylococcus carnosus* for the Weibull model [A] and first-order model [B] incubated at 32°C and 40°C in clams

A



B

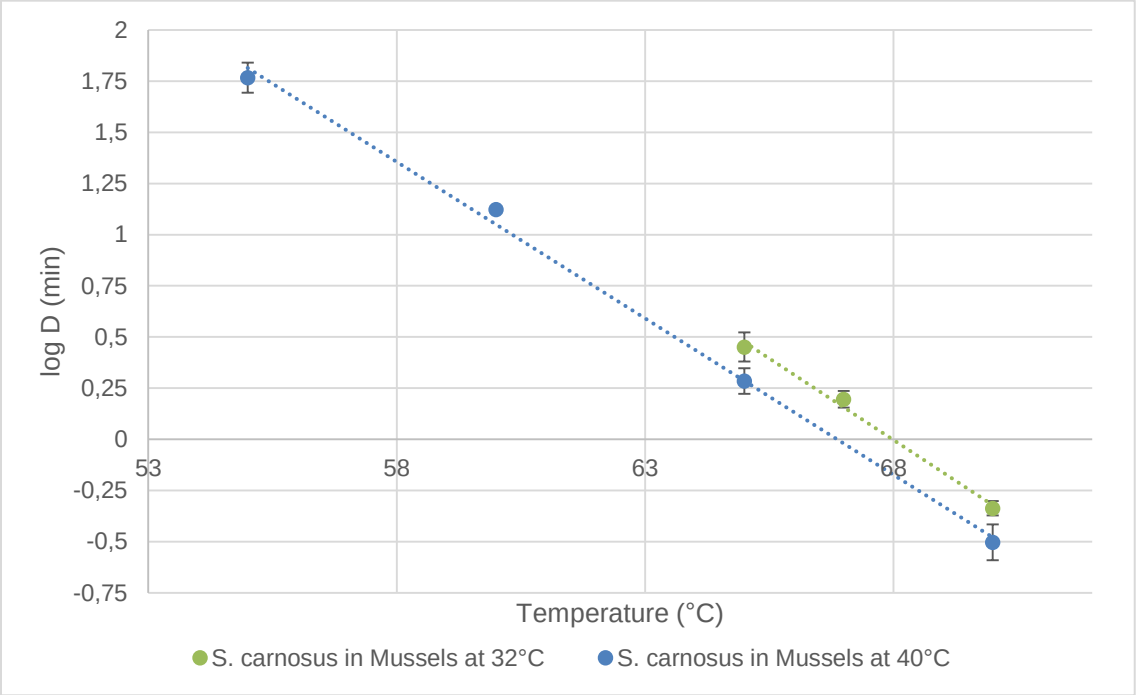


Figure 4.5 Thermal inactivation curves of *Staphylococcus carnosus* for the Weibull model [A] and first-order model [B] incubated at 32°C and 40°C in mussels

Vita

Marcel Schmidt was born in Bietigheim-Bissingen, Germany on July 31, 1990 to parents Dietmar and Petra Schmidt. He was raised as the oldest son of his family, who has two sons and one daughter. He attended elementary school in his home town, Sachsenheim, and high school in Bietigheim-Bissingen. He attended the University of Hohenheim, Stuttgart and received his B.S in Food Science and Biotechnology in 2014. During his undergraduate degree, Marcel conducted his Bachelor of Science thesis in collaboration with the University of Tennessee and studied abroad for 7 months. In 2015, Marcel Schmidt moved to the U.S. and started his Master of Science degree at the University of Tennessee, Knoxville. He will obtain his Master of Science degree in Food Science and Technology with a focus on Food Microbiology in August 2016.