PREDICTIVE MODEL OF GROWTH OF *LISTERIA MONOCYTOGENES* IN QUESO FRESCO CHEESE

by

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(Under the Direction of Abhinav Mishra)

ABSTRACT

Listeria monocytogenes is a hardy psychotropic pathogen that has been historically linked to soft and fresh cheese related outbreaks in the U.S. This study developed mathematical models that can predict the growth of *L. monocytogenes* in queso fresco. Samples of freshly prepared queso fresco were vacuum-packaged and stored at 4, 10, 15, 20, 25 and 30°C. The Baranyi and Ratkowsky square root models were fitted to the growth data at each temperature as the primary and secondary model, respectively. These primary and secondary models provided coefficient of determination values of R^2=0.93 and 0.97, respectively. Tertiary models for non-isothermal profiles were developed and validated with the Acceptable Prediction Zone (APZ) method. Greater than 70% of the observations were within the APZ (0.5>Prediction Error>-1.0). These predictions will help in estimating the risk of listeriosis due to possible extended storage with temperature abuse scenarios for queso fresco.

INDEX WORDS: *Listeria monocytogenes*, microbial growth, queso fresco, predictive microbiology

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DEDICATION

I would like to dedicate my thesis to God, my family and those who have helped shape me into the person that I am today. I am very thankful for the endless support and dedication that my parents and sisters provided throughout my graduate studies. They helped me overcome all the battles I faced while finishing my studies with their words of encouragement, humor, emotional support and prayers.

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CHAPTER 1

INTRODUCTION

Purpose of the Study

The aim of this study was to develop mathematical models that can predict the growth of *Listeria monocytogenes* in a Mexican style cheese known as queso fresco and to validate these models using non-isothermal time-temperature profiles. Foodborne illness is a known concern, but due to better detection and information gathering methods, awareness and concern has grown in developed countries like the United States *(76)*. However, obstacles like scarcity of epidemiological data in developing countries have made it difficult to implement food safety control and intervention *(76)*. *L. monocytogenes* can cause severe complications and even be fatal for certain populations *(19, 30)*. *L. monocytogenes* is a threat to the dairy industry with several outbreaks of *L. monocytogenes* in soft and fresh cheeses *(13)*. According to the Centers for Disease Control and Prevention (CDC), there have been confirmed cases with 70 outbreaks, 865 illnesses, 620 hospitalizations and 132 deaths caused by *Listeria monocytogenes* during 1998-2016. The CDC estimates that about 1,600 people become ill of listeriosis each year and about 260 people die of listeriosis. Most of these cases were foodborne and many of those occurred due to the consumption of fresh cheeses including Mexican style cheese or queso fresco *(13)*.

Predictive microbiology can be implemented as a tool for food safety. Modeling the growth of bacteria like *L. monocytogenes* can be used to predict parameters like growth rate, lag phase duration and the maximum population density of bacteria under certain environmental conditions *(57)*. This study used the Baranyi model *(5)* as a primary model and the Ratkowsky model *(53)* as the secondary model to predict the growth of *L. monocytogenes* in queso fresco. These models were combined to develop a tertiary model that ultimately predicts and validates the growth of *L. monocytogenes* in non-isothermal conditions.

How this study is original

While there have been studies on modeling the growth of *L. monocytogenes* on fresh cheeses, these studies are limited to just a few temperature points *(31, 70)*. For instance, a recent study by Leggett et al. *(31)* modeled the growth of *L. monocytogenes* in queso fresco at 4 and 10°C. The present study focuses on modeling the pathogen growth at six different temperature points which provides a wider range when modeling the pathogen growth in non-isothermal conditions. Many past studies also focused on different types of fresh cheese, like acid-set queso blanco, while this study focuses primarily on rennet-set and vacuum packaged queso fresco *(17, 31, 70)*.

The inoculation method in this study is different from previous studies due to the fact that pasteurized milk was inoculated with the five-strain cocktail before the cheese was made. This may represent the real-life scenarios of post-processing contamination of milk and the survivability of *L. monocytogenes* throughout processing and storage *(17, 31, 77)*.

CHAPTER 2

LITURATURE REVIEW

L. monocytogenes and Dairy

L. monocytogenes is an environmental and foodborne pathogen that is known to cause listeriosis in humans and animals *(46, 61)*. This microorganism is a gram-positive, psychotropic rod that is found throughout the environment *(19)*. *L. monocytogenes* can be isolated from vegetation, soil, sewage, stream water, slaughter house waste, milk and the feces of humans *(15)*. Listeriosis can affect the general population, but it can cause severe complications and be fatal for a specific group of people: the elderly, pregnant women, young children and the immunocompromised *(60)*. Complications from this invasive bacterium include septic abortion, newborn and adult septicemia, and meningitis or meningoencephalitis *(19, 75)*.

Due to the hardy nature of *L. monocytogenes*, this pathogen can be persistent in the environment and ready-to-eat foods like deli meats, melons, and soft cheeses *(28, 29)*. The Centers for Disease Control and Prevention (CDC) started electronic documentation of the foodborne outbreaks from 1998 to 2017 *(13)*. Their data suggests that *L. monocytogenes* has been implicated with outbreaks associated with cantaloupes, bean sprouts, ready-to-eat meat products and various dairy products including soft and fresh cheeses. Raw milk from cows may contain isolates of *L. monocytogenes (6)*. Contamination of raw milk is mainly due to fecal excretions from dairy cows but this microorganism may also occur intracellularly in the udder of the dairy cow which may be difficult to detect *(15)*. Unpasteurized milk has been and is still being used for making cheese, especially in small farms that manufacture artisan cheeses. This practice is being done not only in many parts of the world but also in the United States. The U.S. Food and Drug Administration (FDA) enacted the “60 days rule” in 1949 which allows cheese manufactures to produce raw milk cheeses as long as the cheeses are aged for at least 60 days to allow the aging process to kill pathogens that may be present in the cheese *(69)*. Recent sampling of raw milk cheeses by the FDA detected 10 samples out of 6,606 tested to be *L. monocytogenes* positive even after 60 days of ageing *(69)*. The use of raw, unpasteurized milk for dairy products greatly increases the risk of pathogenic bacteria contaminating the final product *(38)*. The possibility of raw milk containing *L. monocytogenes* makes pasteurization an important step in milk processing for consumption. Processed dairy products such as cheeses are also susceptible to *L. monocytogenes* contamination because this pathogen can grow even at refrigerated temperature *(61)*.

Outbreaks of *L. monocytogenes* in Cheese

The first reported case of human listeriosis in the U.S. was due to the consumption of soft cheese in 1985. Table 1 provides a list of *L. monocytogenes* outbreaks that occurred in the U.S. from 2003 to 2017 that were reported to the CDC *(13)*. Table 1 illustrates the frequency of cheese related outbreaks in the United States *(13)*. It is important to reduce future outbreaks by evaluating dairy and cheese production and determining the growth niches and harborage sites of *L. monocytogenes*. Among all the *L. monocytogenes* related outbreaks reported to the CDC, soft and fresh cheeses are found to be most frequently contaminated with *L. monocytogenes*.

L. monocytogenes and Queso Fresco

Queso fresco is a popular Latin American fresh cheese that has a mild, salty flavor and a crumbly texture. Queso fresco is traditionally made using raw milk from cows or goats, but due to food safety concerns in the United States, milk should be pasteurized before making commercially available queso fresco *(67)*. *L. monocytogenes* has been associated with queso fresco along with other Mexican-style cheeses *(61)*. Because *L. monocytogenes* has been implicated in many foodborne illness outbreaks related to queso fresco, this fresh cheese is among foods with the highest risk of *L. monocytogenes* contamination *(65)*. Queso fresco is also processed at a temperature range of 32 to 37°C which provides suitable growth temperatures for *L. monocytogenes (61)*. Combined with the survivability of *L. monocytogenes* throughout processing and storage, queso fresco is a major food safety concern.

Queso fresco is susceptible to *L. monocytogenes* growth, not only due to the temperature during processing and storage, but also due to the makeup of the cheese. Since queso fresco usually has a high water activity and near neutral pH, this fresh cheese has conditions favorable for the growth of *L. monocytogenes (32, 62)*. A study by Schvartzman et al. *(64)* observed the growth of *L. monocytogenes* in relation to pH and water activity. This study reported that *L. monocytogenes* grew at a water activity of 0.975 and a pH of 5.6. Although queso fresco is susceptible to *L. monocytogenes* growth, there are ways to alleviate the risk by assessing the sources of contamination and examining processing steps that are critical to food safety.

Sources of Contamination

There are some sources of contamination when it comes to *L. monocytogenes* in dairy products like queso fresco and many of these sources are found in the farm environment *(12)*. Microbiological contamination of milk from *L. monocytogenes* can start from the inside of the udder to the utensils, equipment and surfaces in the milking shed *(11)*. *L. monocytogenes* is usually found in bulk tanks of milk and feces of healthy cows *(12)*. *L. monocytogenes* can easily colonize in the mammary gland and especially in cows with a bacterial infection known as mastitis, which increases the chance of spreading the bacteria to the milk *(63)*.

Although contaminated raw milk may be a source of *L. monocytogenes*, processed milk and dairy products can also carry *L. monocytogenes* through environmental contamination and post-processing contamination. Pasteurization of dairy milk is an essential kill step and is necessary to kill a number of vegetative cells *(73)*. Issues arise when pasteurized milk is contaminated post processing. This may cause pathogens like *L. monocytogenes* to grow at refrigeration temperatures at which milk is stored *(39)*. According to the CDC, there have been outbreaks from the consumption of pasteurized fluid milk. An outbreak occurred in 2008 due to post-processing contamination when pasteurized milk from a small dairy plant caused 5 cases due to *L. monocytogenes (49).*

Predictive Microbiology

While steps can be taken to reduce the risk of bacterial contamination and growth, it is helpful to have a tool that can predict the growth of bacteria in varying conditions. This tool can provide useful information in either times of distress when electrical power fails or when attempting to maintain the cold chain. Throughout the history of food microbiology, qualitative approaches were used to determine microbial population before the “modern” quantitative method of predictive microbiology came about *(42)*. Early mathematical modeling began with the use of empirical models with unknown parameters and eventually progressed into developing models that show biological interpretations of the parameters *(16)*. Predictive microbiology is beneficial to food safety since modeling the growth of bacteria and spoilage microorganisms can be used as a tool in the food industry to predict microbial degradation of products *(26)*.

It is essential to model the growth of *L. monocytogenes* in queso fresco because processers can use the information to predict the growth and behavior of this bacterium under certain conditions that may be encountered during processing, distribution and storage *(57)*. *L. monocytogenes* can grow in conditions of low pH, high water activity and low temperature and queso fresco is more susceptible to environmental contamination due to frequent worker handling during processing *(31, 70)*. Since temperature is an important factor that can alter microbial growth dynamics, this parameter is taken into account when creating predictive models *(35)*. It is also necessary to be able to predict the growth of *L. monocytogenes* at different growth temperatures. *L. monocytogenes* can potentially grow in the temperatures range of 4 to 37°C which includes storage and processing temperatures for queso fresco *(61)*. In a study conducted by Uhlich *(70)*, there was a positive correlation between the temperature and the growth rate of *L. monocytogenes* in fresh cheese. As the temperature increased, the specific growth rate increased as well. There are three different levels of predictive modeling: primary, secondary and tertiary.

Primary Models

Primary models portray the change in the bacterial population, whether it be survival, growth or inactivation, under certain environmental conditions over a period of time *(10)*. The objective of the primary model is to fit experimental growth curves and estimate parameters like growth rate, maximum population density and lag phase duration *(78)*.

There are several models that are used to predict the growth of microorganisms in isothermal and non-isothermal conditions *(51)*. Some of these models include the Baranyi and Roberts model *(5)*, the Modified Gompertz model *(79)*, and the Logistic model *(79)*. In a study conducted by Longhi et al. *(35)*, these models were compared. These authors reported that, although there was not much of a difference between the results of these models, the Baranyi growth model displayed consistency, a narrow range of bias factors and the best accuracy factor. Other studies have shown consistent results of the Baranyi model displaying the best behavior for growth curves under multiple criteria *(26, 36)*.

Secondary Models

Secondary models describe the relationship between one or more of the primary model parameters, such as growth rate, with respect to a conditions like temperature, pH, or water activity (a_w) *(35)*. Parameters from the primary model are used to develop secondary models *(21)*. A widely used secondary model is the Ratkowsky square root model which displays a linear relationship of growth rates and temperature *(53, 79)*. Other popular secondary models include the Arrhenius and response surface (polynomial) models *(16, 40)*. Mechanistic models, such as Ratkowsky square root model are known to provide high quality of fit due to the ability to interpret the parameters biologically

(43). Conversely, equations such as polynomial models are developed using an empirical approach *(18)*.

Tertiary Models

Tertiary models are developed by integrating primary and secondary models using computational methods for predicting the growth of microorganisms under certain environmental conditions *(10)*. Computer programs are presently being used to compute and develop these tertiary models instead of computation by hand. The United States Department of Agriculture (USDA) Food Safety Research Unit has developed the Pathogen Modeling Program (PMP) using experimental data collected from observing microbial activity in liquid media *(9, 21, 40)*. The PMP software uses multivariant models based on the use of a combination of Gompertz function and response surface analysis *(9, 40)*. Although PMP provides models well used within food processing, this software is not as customizable due to limited access to raw data. Another software package known as ComBase provides a database with over 50,000 records which describe growth and inactivation of microbes in culture media or in food *(4, 66)*. Due to the extensive database, Combase allows users to develop new modeling techniques, keep permanent records of experimental design and data and increase efficiency for data analysis *(4)*.

Validation Methods

There may be differences between observed and predicted growth due to growth history of inoculum, naturally-occurring food microflora, intrinsic and extrinsic factors that may influence microbial activities *(21)*. Validation of predictive models can be conducted with experimental data. Some methods of analysis include bias factor, accuracy factor and acceptable prediction zone (APZ) method.

Bias factor and accuracy factory are complementary measures that are simple indicators of the performance of models. The indices assess the level of confidence one can have in the predictions of the model and whether the model displays any bias which could lead to ‘fail-dangerous’ predictions *(55)*. Bias factor illustrates the difference between over predicted and under predicted:

$$B = 10^{(\Sigma(\mu_{max}\, predicted/\mu_{max}\, observed)/n)} \tag{1}$$

A perfect agreement between predicted and observed will give a bias factor of 1, but this can be misleading since under- and over-prediction will tend to cancel each other out due to the ratios having opposite signs. Accuracy factor addresses this issue by looking at the absolute value of predicted over observed *(55)*:

$$B = 10^{(\Sigma\ /\mu_{max}\, predicted/\mu_{max}\ observed)\ //n)} \tag{2}$$

A perfectly accurate model will have an accuracy factor of 1.0.

APZ method calculates prediction error or relative error (RE) of individual prediction cases *(50)*:

$$RE = \frac{Observed - Predicted}{Predicted} \tag{3}$$

An RE less than zero indicates fail-safe while an RE greater than zero indicates fail-dangerous *(50)*. Boundaries for APZ were set by Ross *(56)* with fail-safe being 0.70 and fail-dangerous being 1.15. Other studies have set the boundaries for APZ from -1.0 to 0.5 with the wider margin for the fail-safe *(41, 47)*. A highly fail-safe model is considered better than a fail-dangerous because the over-prediction of growth will ensure that the food is safe for consumption *(50)*. An acceptable model has at least 70% of the prediction errors falling within the prediction zone *(50)*.

Table 1. Outbreaks in cheese due to *L. monocytogenes* in the United States

State	**Month Year**	**Type of Cheese**	**Cases/ Deaths**
Texas	February 2003	Queso fresco, unpasteurized	12/1
Oregon	August 2006	Other cheese pasteurized	3/1
Multistate	October 2008	Mexican style cheese pasteurized	8/0
Multistate	June 2009	Mexican style cheese	18/0
Michigan	August 2011	Ackawi cheese, pasteurized; chives cheese, pasteurized	2/1
New Jersey	July 2011	Mexican style cheese pasteurized	2/0
Multistate	October 2011	blue-veined cheese, unpasteurized	15/1
Multistate	March 2012	Ricotta Salata cheese	23/5
Multistate	May 2013	Cheese-le frère	6/1
Multistate	August 2013	Latin style soft cheese	8/1
Multistate	September 2013	Mexican style cheese (pasteurized)	9/1
Multistate	March 2014	Queso fresco	8/1
Washington	November 2014	Mexican style cheese (pasteurized)	3/1
Multistate	September 2015	Soft cheeses distributed by Karoun Dairies, Inc.	30/3
New York	October 2015	American cheese (pasteurized)	2/1
Multistate	September 2016	Artisanal soft cheese (unpasteurized)	10/2
New York	March 2017	Soft raw milk Cheese from Vulto Creamery	8/2
Multistate	May 2017	Pasteurized cheese	6/0
Rhode Island	August 2017	Queso fresco (unspecified)	2/0

CHAPTER 3

MATERIALS AND METHODS

Bacterial Strains and Inoculation

Inoculant was prepared by mixing five strains of *L. monocytogenes.* The five strains were previously isolated from Brie cheese, milk, a patient during outbreak in Massachusetts due to improper pasteurization of milk (*L. monocytogenes* Scott A), Mexican-style cheese from California, and a patient during a coleslaw outbreak (*L. monocytogenes* G1091). All strains were stored in Microbank™ beads for long-term storage at -80°C. When ready to be used, each bead was suspended in 9 ml tubes of tryptic soy broth (TSB) and incubated at 37°C for 24 h. After growth, the five tubes were sub-cultured to new TSB tubes using sterile loops. Fresh cultures were grown for approximately 18-24 h before inoculating the whole pasteurized milk.

Whole pasteurized milk, procured from a local grocery store in Athens, GA was inoculated before making cheese. The inoculant cocktail was prepared by mixing 2 ml of each strain into one sterile tube and vortexed to mix. To obtain about 1-2 log CFU/g of *L. monocytogenes* in the final queso fresco product, the cocktail was diluted 1:100 times in 0.1% peptone water and 0.75 ml of inoculant was added to ten liters of pasteurized whole milk before processing.

Queso Fresco Preparation

Ten liters of inoculated whole pasteurized milk was poured into a 12 L stainless steel container and placed in warm water bath at 32°C. A temperature probe was placed in the middle of the container to measure the temperature throughout the cheese-making process. When the temperature of the milk reached 32°C, the following ingredients were added into the milk in amounts: 269 mg of mesophilic starter culture (*Lactococcus lactis* subsp. lactis, *Lactococcus lactis* subsp. cremoris, *Lactococcus lactis* subsp. lactis biovar. diacetylactis, *Streptococcus thermophilus*) (Danisco, France), 3.26 ml of liquid animal rennet (Cultures for Health, Morrisville, NC), and 3.26 ml of calcium chloride mixed with 156 ml of sterile cold deionized water. Each ingredient was mixed into the milk separately, and the milk was covered with a stainless-steel lid and allowed to set for 30-45 min *(27)*. Once the milk was set and had coagulated, a sterilized cheese knife was used to cut the curd into approximately 1mm x 1mm squares. The cut curd was left to cook at 32°C for another 50 min before straining. Curds were poured out into sterile colanders lined with sterile 50 grade cheesecloths to separate the curds and the whey. The curds were drained and mixed often to facilitate straining. Strained curds were weighed out and salted evenly at 3 g of salt per 100 g of curd *(27)*. The queso fresco was pressed using a stainless-steel Dutch style cheese press (The Sausage Maker, Buffalo, NY) and weighed down with 4.5 kg weight.

Packaging and Storage of Queso Fresco

After pressing the queso fresco, approximately 10 g samples were stored in sterile filtered polyethylene stomacher bags (WVR®, Radnor, PA) and vacuum-packaged using the LEM® MaxVac machine (LEM®, West Chester, OH). Sample bags were separated according to the specific storage temperatures: 4, 10, 15, 20, 25 and 30°C. Bags were immediately stored in incubators at specific storage temperatures until sampled. For each temperature, one bag was assigned for each of the sampling points.

pH and Water Activity

The pH and water activity (a_w) of the milk and the final queso fresco product were measured using a pH meter (Oakton pH Spear Waterproof Pocket pH Tester™ 35634-40) and water activity meter (AQUALAB 4TE S40002162), respectively. After initial readings were taken, the pH of the vacuum packaged cheese was taken on sampling days throughout the storage period.

Enumeration of Bacterial Populations

Sampling times were determined by previous studies and expected growth at storage temperatures *(31, 70)*. During sampling, one bag was removed and the queso fresco sample was diluted 1:10 times with 0.1% peptone water. Samples were stomached using the Seward Stomacher® 400, 27105 (Seward, England) for 60 sec to make sure the samples were thoroughly and uniformly blended. Ten-fold dilutions of samples were plated on to *Listeria* selective Modified Oxford (MOX) Agar (Oxoid Limited, Hampshire, United Kingdom). Samples were plated three times per dilution to reduce statistical variability and sampling errors. These plates were incubated at 37°C for 24-48 hours before colonies were counted *(68)*.

Primary Models

Baranyi model is a common primary model used to observe how bacteria grow in different conditions *(5, 25)*. This model describes the growth of *L. monocytogenes* as a function of time at a constant temperature *(5)*:

$$y(t) = y_0 + \mu_{max}F(t) - \ln\left(1 + \frac{e^{\mu_{max}F(t)} - 1}{e^{(y_{max} - y_0)}}\right), \tag{4}$$

Where

$$F(t) = t + \frac{1}{v}\ln\left(e^{-vt} + e^{-h_0} + e^{(-vt-h_0)}\right). \tag{5}$$

The parameter $y(t)$is the $\log_{10}$ CFU/g of the *L. monocytogenes* cell population at time t; y_0 is the initial cell population in $\log_{10}$CFU/g; y_{max} is the maximum population density of the cells in $\log_{10}$CFU/g; μ_{max} is the maximum specific growth rate in $\log_{10}$CFU/h; h_0 describes the prior physiological state of the cells; λ is the lag phase duration (h). Lag phase duration was calculated from the following relationship:

$$\lambda = \frac{h_0}{\mu_{max}} \tag{6}$$

A regression curve that fits the sigmoidal growth of *L. monocytogenes* was fitted using MATLAB software (version R2018a, MathWorks, Natick, MA) for each storage temperature.

Secondary Models

The parameters estimated from the primary Baranyi model were used to fit secondary models which represent the relationships between these parameters and temperature. The maximum specific growth rates (μ_{max}) corresponding to each temperature were fitted into the Ratkowsky square root model *(53, 79)*:

$$\mu_{max} = a(T - T_{min})^2 \tag{7}$$

where T is the temperature (°C), T_{min} is the theoretical minimum temperature (°C) and a is the regression coefficient. The lag phase duration for each temperature was modeled by fitting the lag phase for each temperature with a hyperbolic function *(79)*:

$$\lambda = e^{\frac{r}{T-s}} \tag{8}$$

Where T is the temperature (°C); r is the parameter for the decrease in lag phase duration as the temperature increases; s represents the temperature (°C) when lag time is infinite. Secondary models were fitted using MATLAB (Version R2018a, MathWorks, Natick, MA).

Statistical Analysis

The averages and standard errors of the pH and the population counts of *L. monocytogenes* in queso fresco during storage were calculated using Microsoft Excel (Version 2016, Microsoft, Redmond, VA). Goodness-of-fit statistics were used for both primary and secondary model performances. Performances were compared using the coefficient of determination (R^2) and root mean square error (RMSE) values *(14)*. The RMSE value can be calculated using the following equation:

$$RMSE = \sqrt{\frac{1}{n} \sum_{i=1}^{n} e_i^2} \tag{9}$$

The R^2 is represented by the following equation *(52)*:

$$R^2 = \frac{SSR}{SSTO} \tag{10}$$

Where

$$SSR = \sum_{i=1}^{n} (\hat{y}_i - \bar{y})^2 \tag{11}$$

and

$$SSTO = \sum_{i=1}^{n} (y_i - \bar{y})^2 \tag{12}$$

Where, n is the sample size, e_i represents the model prediction error at the i^{th} term y is the response variable (cell population of *L. monocytogenes*), $\hat{y}$ is the estimated regression and $\bar{y}$ is the average response variable. The model prediction error is given by the predicted minus observed value. SSR represents the regression sum of squares and SSTO represents total sum of squares. The values for goodness-of-fit were generated from MATLAB.

Tertiary Models

The tertiary model was developed by integrating the primary and secondary models using the MATLAB® software. The following two first-order differential equations were proposed by Baranyi and Roberts *(5)* to model differential growth:

$$\frac{dy}{dt} = \frac{1}{1+e^{-Q(t)}}\mu_{max}\big(T(t)\big)\big(1 - e^{y(t)-y_{max}}\big) \tag{9}$$

$$\frac{dQ}{dt} = \mu_{max}\big(T(t)\big) \tag{10}$$

Where

$$Q(t) = \ln q(t) \tag{11}$$

where $y(0)= y_0$ and $Q(0)= \ln (q_0)$ are the initial conditions. The parameter q_0 represents the physiological state of the cells *(5)*. These equations were solved by applying the fourth-order Runge–Kutta method in MATLAB.

Validation of Models

Separate experiments were conducted to validate the tertiary model. These experiments were conducted using non-isothermal sinusoidal time-temperature profiles (frequency of 24 h) over a period of time: 4 to 20°C for 19 days and 15 to 30°C for 11 days. The queso fresco was prepared using the same recipe as mentioned in the section "Queso Fresco Preparation". Pasteurized whole milk was inoculated using the same five strains of *L. monocytogenes* that were mentioned in the section "Bacterial Strains and Inoculation" to attain 2-3 log CFU/g of *L. monocytogenes* in the final product. Samples were stored in Percival Scientific Intellus incubator (Percival Scientific, Perry, IA) which was programmed for the sinusoidal time-temperature profiles. Samples were enumerated on *Listeria* selective Modified Oxford (MOX) Agar (Oxoid Limited, Hampshire, United Kingdom) plates using the Eddy Jet 2 spiral plater 611 (Neu-tec group, Farmingdale,NY).

Predicted populations of *L. monocytogenes* were compared to the observed data from the validation study using Acceptable Prediction Zone (APZ) method where the observed values were subtracted from the predicted values to generate the prediction error (PE) *(50)*. The Acceptable Prediction limits for the APZ were set between -1.0 and 0.5 $\log_{10}$CFU/g *(41, 47)*.

CHAPTER 4

RESULTS AND DISCUSSION

pH and Water Activity

The pH and water activity (a_w) of the milk before making the queso fresco was 6.74 and 0.9963 respectively. The initial pH and a_w values of queso fresco after pressing were 6.40 and 0.967, respectively. These results are in close agreement with other studies which reported that cheeses like queso fresco have pH ≥ 6.10 and a_w ≥0.962 *(31, 71)*. Due to the near-neutral pH and high a_w, pathogens like *L. monocytogenes* can potentially grow on this cheese *(70)*.

For all the constant temperatures studied (4-30°C), the pH values decreased as time progressed (Table 2). This decrease in pH may be due to the lactic acid buildup in the packages when residual lactose in the cheese is converted to lactic acid by lactic acid bacteria (LAB) *(44)*. Queso fresco that was stored at relatively higher temperatures (15-30°C) had a faster pH decrease than the cheese stored at lower temperatures (4 and 10°C). This greater decrease in pH at higher temperatures may be due to the elevated temperatures facilitating the fermentation process because the enzymes of LAB are optimum at around 35°C *(44)*. Another reason for this pH decrease may be due to the presence of *L. monocytogenes* since the bacterium can use lactose as a sugar for fermentation to produce lactic acid under anaerobic conditions *(20, 60)*.

Primary Growth Models in Isothermal Conditions

L. monocytogenes survived through queso fresco processing and continued to grow during storage. Past studies have reported the survival of *L. monocytogenes* during cheese processing and storage *(33, 34)*. Survival and growth of *L. monocytogenes* throughout processing and storage may be due to the hardy nature of this bacterium and its ability to survive and grow under different unfavorable conditions, including low temperature.

The maximum specific growth rate (μ_{max}) of *L. monocytogenes* increased with the increase in storage temperature. Queso fresco that was stored at 4°C had a μ_{max} of 0.0131 $\log_{10}$CFU/h which is lower than 0.0898 $\log_{10}$CFU/h which is the μ_{max} of queso fresco that was stored at 30°C (Table 3). This relationship between temperature and maximum specific growth rate was also observed in a previous study conducted by Uhlich *(70)* where slices of queso blanco were inoculated with *L. monocytogenes* and stored at 5, 10, 15, 20 and 25°C. Another study by Xanthiakos et al. *(77)* observed similar pattern when modeling the growth of *L. monocytogenes* in pasteurized milk at different temperatures from 1.5 to 16°C. A study conducted by Lobacz et al. *(34)* reported a similar trend as well when modeling the growth of *L. monocytogenes* in mold ripened cheeses like Camembert and blue cheese at temperatures from 3 to 15°C. This relationship between temperature and maximum specific growth rate is not only seen in dairy products but also in other food systems. In a study by Rodríguez et al. *(54)* the growth of *L. monocytogenes* on packaged fresh green asparagus was modeled and the growth rate was higher at higher temperatures. This positive relationship between the maximum specific growth rate and the temperature observed with different types of foods is due to *L. monocytogenes* having optimal growth temperatures from 32 to 37°C *(8)*.

The primary models in the present study assumed that the h_0 was constant because the physiological state of the bacteria was the same at the time of inoculation. The values of h_0 before taking the average are listed in Appendix A. The individual h_0

ranged between 0.56 and 4.60, and there was no clear pattern between h_0 and storage temperature. Therefore, the arithmetic mean of h_0 was used for further model development. The arithmetic mean value of h_0 was calculated as 2.47 and the other parameters were re-estimated according to the new h_0 value *(25)*. Juneja et al. *(22)* modeled the growth of *Bacillus cereus* during cooling of cooked rice, and h_0 was set at an average constant at 4.10. Another study by Jenuja et al. *(24)* reported an average h_0 of 1.70 for *Salmonella* in chicken. A study done by Vulgoti et al.*(72)* set the average h_0 as 2.14 for *Salmonella* in ground sterile pork. These h_0 values are all different due to differences in microorganisms and the food matrices.

Contrary to the behavior of *L. monocytogenes* in previous studies, the maximum population density (y_{max}) did not exceed 6 $\log_{10}$ CFU/g at any of the temperatures *(1, 31, 70)*. The lack of oxygen along with the presence of the mesophilic starter culture may have facilitated the buildup of lactic acid *(17, 59)*. This buildup of acid can lower the pH of the product, limiting the growth of *L. monocytogenes*. As mentioned in the previous section (pH and Water Activity), the pH values decreased at all temperatures especially at the higher temperatures (15, 20, 25 and 30 °C). Another reason for this low maximum population density may be due to the processing techniques and varying amounts of ingredients used in each recipe; ingredients like salt vary and may be a stressor for *L. monocytogenes (27, 31)*.

The Baranyi model that was fitted to the isothermal growth data showed goodness-of-fit statistics with a coefficient of determination (R^2) of 0.9282 and a root-mean-square error (RMSE) of 0.3173 (Table 4). Higher R^2 value and lower RMSE values indicate a strong goodness-of-fit which means that the observed values were close to the model predictions *(14, 31)*. Similar to the present study, previous studies have also reported that the Baranyi model has proven to have an acceptable goodness-of-fit when compared to other models *(22, 26, 34, 37)*.

The present study modeled the growth of *L. monocytogenes* using the Baranyi model because this model is mechanistic and strives to account for the biological factors that bacteria experience during adaptation and growth. By accounting for biological factors, the Baranyi model can characterize the transition between lag phase and exponential phase *(5)*. A study conducted by Juneja et al. *(23)* used Gompertz, Huang and Baranyi models to model the growth of *Bacillus cereus* on cooled cooked pasta. When comparing the models, these authors found that Baranyi model preformed the best for primary modeling and proved to be well equipped for predicting bacterial growth in dynamic conditions.

Secondary Growth Models and Lag Phase Duration

Figure 2 illustrates the secondary growth rate model (Ratkowsky square root model) of for all the temperatures included in this study. The Ratkowsky square root model was chosen for this study because it provides a high quality of fit due to the model's ability to interpret the parameters biologically *(55)*. The square root transformation was performed to stabilize variance in maximum specific growth rate *(79)*. The T_{min} that was used to fit the data was -18.6 which is much lower than the minimum growth temperature of *L. monocytogenes* because this parameter is a theoretical or notational minimum growth temperature *(19, 58)*. The use of a very low minimum temperature is seen in other studies when modeling the growth of *L. monocytogenes (1, 56, 74)*. Due to the inherent nature of the Ratkowsky model, the T_{min} is the y intercept of the linear model which happens to be -18.6.

$$\mu_{max} = 0.0051(T + 18.6)^2 \tag{12}$$

There was a positive linear relationship between $\sqrt{\mu_{max}}$ and temperature *(25, 77)* with a coefficient of determination of R^2=0.9745. Xanthiakos et al. *(77)* observed a similar relationship between $\sqrt{\mu_{max}}$ and temperature when modeling with the Ratkowsky square root model. The Ratkowsky square root model was an acceptable fit for the growth rate data which was indicated by the high R^2 value (Figure 2).

Lag phase duration (λ) is the time it takes for the bacterial cells to adjust physiologically to the environment before the log or exponential growth phase *(5)*. Lag phase duration was calculated with the following equation:

$$\lambda = e^{\frac{297.5}{T+52.54}} \tag{13}$$

Lag phase duration decreased with increasing temperature with a high R^2 =0.9866 *(70, 77)* (Figure 3). This relationship between lag phase duration and temperature could be due to the fact that the *L. monocytogenes* cells were grown in nutrient broth at 37°C before inoculation. The bacterial cells adjusted quicker to the higher temperatures of storage due to the environment the inoculant was kept in prior to inoculation *(3, 45)*. The 30°C samples had the shortest lag phase duration which was 27.6 hours while those of 4°C had the longest lag phase duration which was 189.1 hours (Figure 3). There was no clear trend between maximum population density and temperature which has also been reported in previous studies of *L. monocytogenes (17, 70, 77)*.

Growth Models in Non-isothermal Conditions and Validation

Figure 4 displays the tertiary models for two temperature profiles (4 to 20°C and 15 to 30°C) along with the observed growth from the validation experiment. Figure 4 shows that there is over-prediction toward the end of the validation experiment. For both temperature profiles in Figure 4, the experimental maximum population density that was observed during the validation experiment did not reach the predicted maximum population density. This may be due to the fact that the average of the maximum population densities during isothermal conditions was used to develop the tertiary models before validation. This lower maximum population density may also be due to a decline in pH caused by lactic acid buildup due to the temperature and storage of the vacuumed packed queso fresco *(44)*. In another study by Bovill et al. *(7)*, the growth of *L. monocytogenes* was compared in both milk and broth medium. It was reported by these authors that growth was much slower in the milk due to the drop in pH from the production of LAB during incubation at 4 and 30°C. The natural microflora within a food can affect the growth of pathogens due to competition. This phenomenon is known as the Jameson effect or the competition between groups of microorganisms within a system to obtain nutrients necessary for growth *(2, 34, 48)*.

The Acceptable Prediction Zone (APZ) method has been widely used for validation of growth models of bacteria in several foods *(22, 25, 47)*. The prediction errors from the APZ analysis (Figure 5) suggest that the tertiary models are acceptable. This is due to all 11 observed points from the low temperature profile and 9 out of 12 of the observed points in the high temperature profile being within the APZ (0.5>Prediction Error>-1.0). If 70% of the prediction errors are within the acceptable prediction zone, then the model is considered acceptable *(50)*. Similar studies using APZ have reported similar results *(22, 25, 47)*. Because the APZ analysis was based on observed minus predicted populations of *L. monocytogenes*, we set the APZ bounds to be from -1 (fail-safe) to 0.5 (fail-dangerous). Fail-safe cases indicate that there was over-prediction and fail-dangerous means that there is under-prediction of the bacterial populations *(25, 37, 47, 50)* The uneven margins for the APZ analysis (a wider range for fail-safe side than fail-dangerous side) is because prediction error can be tolerated in the fail-safe direction when using models to predict the bacterial growth for food safety *(56)*. Having an overly fail-safe model means that there might be destruction of food that might have been perfectly safe for consumption. On the other hand, an overly fail-dangerous model can result in the consumption of unsafe foods which can increase chances of foodborne illness *(50)*.

Figure 5 shows that, although the data points stay within the APZ, most of the prediction errors are in the fail-safe zone towards the latter portion of the experimental study. This is especially true for the temperature profile of 15 to 30°C which indicates an overall over-prediction. This was expected for the APZ analysis since Figure 5 shows that the growth of *L. monocytogenes* did not reach the expected maximum population density for both temperature profiles.

Table 2. pH values of contaminated queso fresco samples during storage at different temperatures

Time (hours)	4°C	10°C	15°C	20°C	25°C	30°C
0	6.40	6.40	6.40	6.40	6.40	6.40
24	6.45	6.23	6.15	5.89	5.55	5.19
72	6.39	6.10	5.75	5.47	5.37	5.17
120	6.43	6.05	5.39	5.19	5.11	4.90
264	6.10	5.71	5.15	4.90	5.20	5.12
288	-	-	5.11	5.11	5.21	N/A
336	-	-	5.24	5.08	5.25	5.15
384	6.18	5.62	-	-	-	-
408	6.16	5.83	-	-	-	-
456	6.13	5.65	-	-	-	-
480	6.31	5.76	-	-	-	-

-: pH was not recorded

Table 3. Maximum specific growth rate (μ_{max}), lag phase duration (λ) and maximum population density (y_{max}) of *L. monocytogenes* in queso fresco stored at different temperatures

Temperature (°C)	Maximum Specific Growth Rate ($\log_{10}$ CFU/h)	Lag phase duration (hours)	Maximum Population Density ($\log_{10}$ CFU/g)
4	0.0131	189.07	5.48
10	0.0194	127.47	5.67
15	0.0318	77.76	5.10
20	0.0400	61.78	5.26
25	0.0545	45.34	5.68
30	0.0898	27.55	4.41

Table 4. Goodness-of-fit parameters for the fitted Baranyi models for different storage temperatures

Temperature (°C)	Coefficient of determination (R^2)	Root mean square error (RMSE)
4	0.9578	0.2665
10	0.9433	0.3318
15	0.9339	0.2523
20	0.8804	0.4100
25	0.9631	0.2573
30	0.8909	0.3862
Average	**0.9282**	**0.3173**

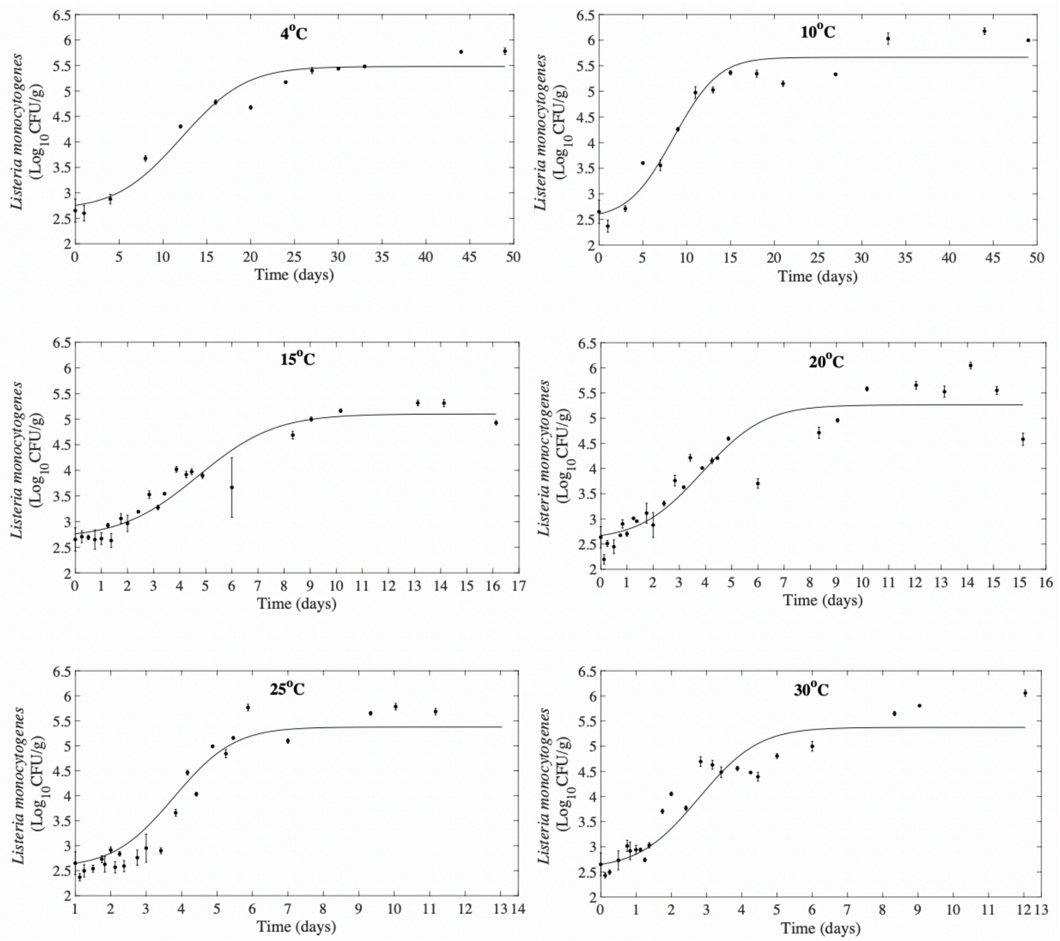

Figure. 1. Growth of *L. monocytogenes* in queso fresco cheeses at different storage temperatures. Points are *L. monocytogenes* populations at different time intervals, and the curves are the fitted Baranyi models to the *L. monocytogenes* populations at different temperatures.

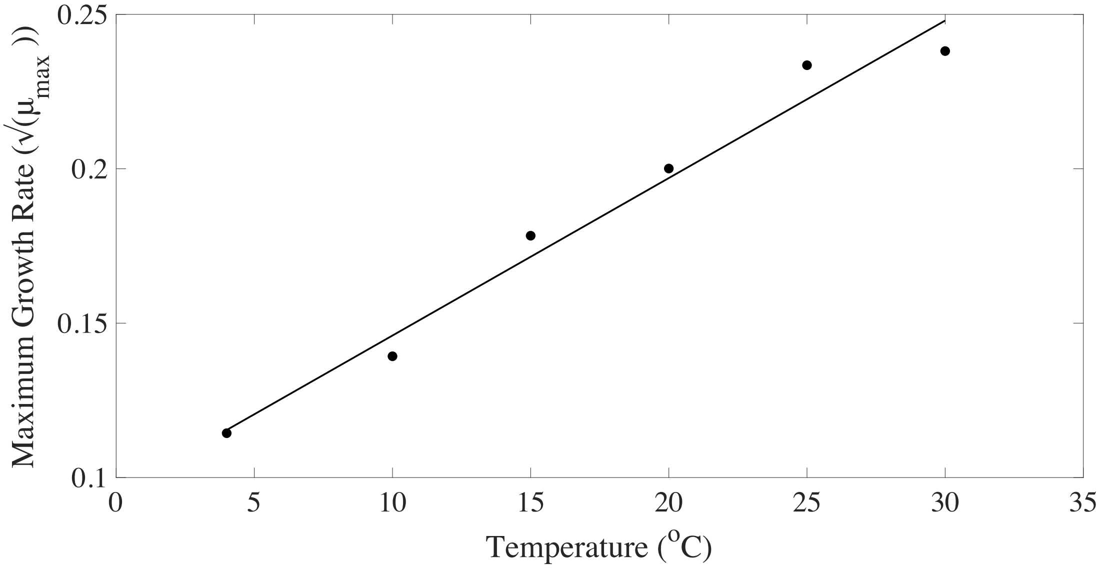

Figure. 2. Ratkowsky square root model of maximum specific growth rate ($\sqrt{\mu_{max}}$) of *L. monocytogenes* in queso fresco as a function of storage temperature

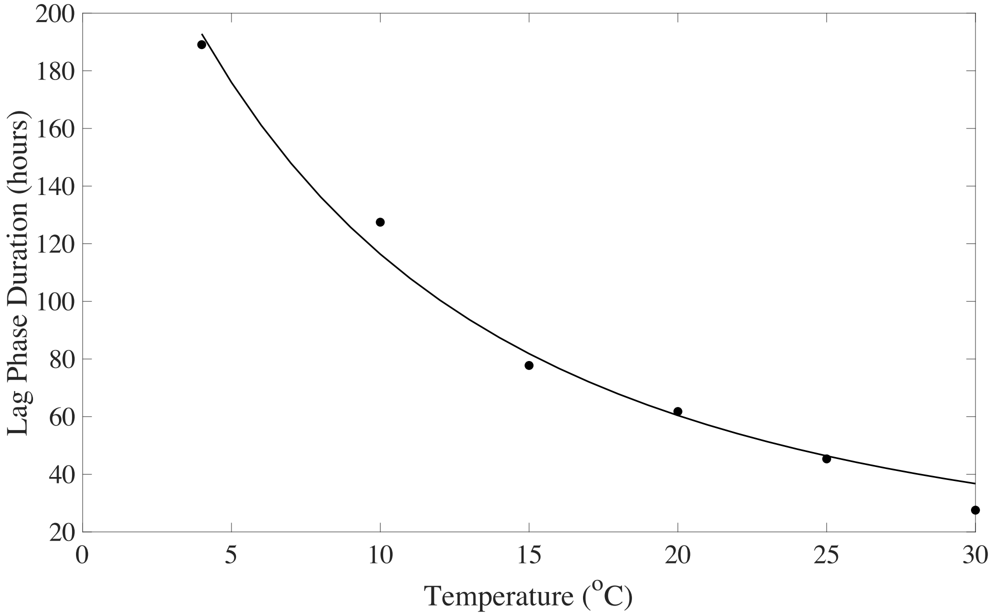

Figure. 3. Lag phase duration (λ) of *L. monocytogenes* in queso fresco as a function of storage temperature

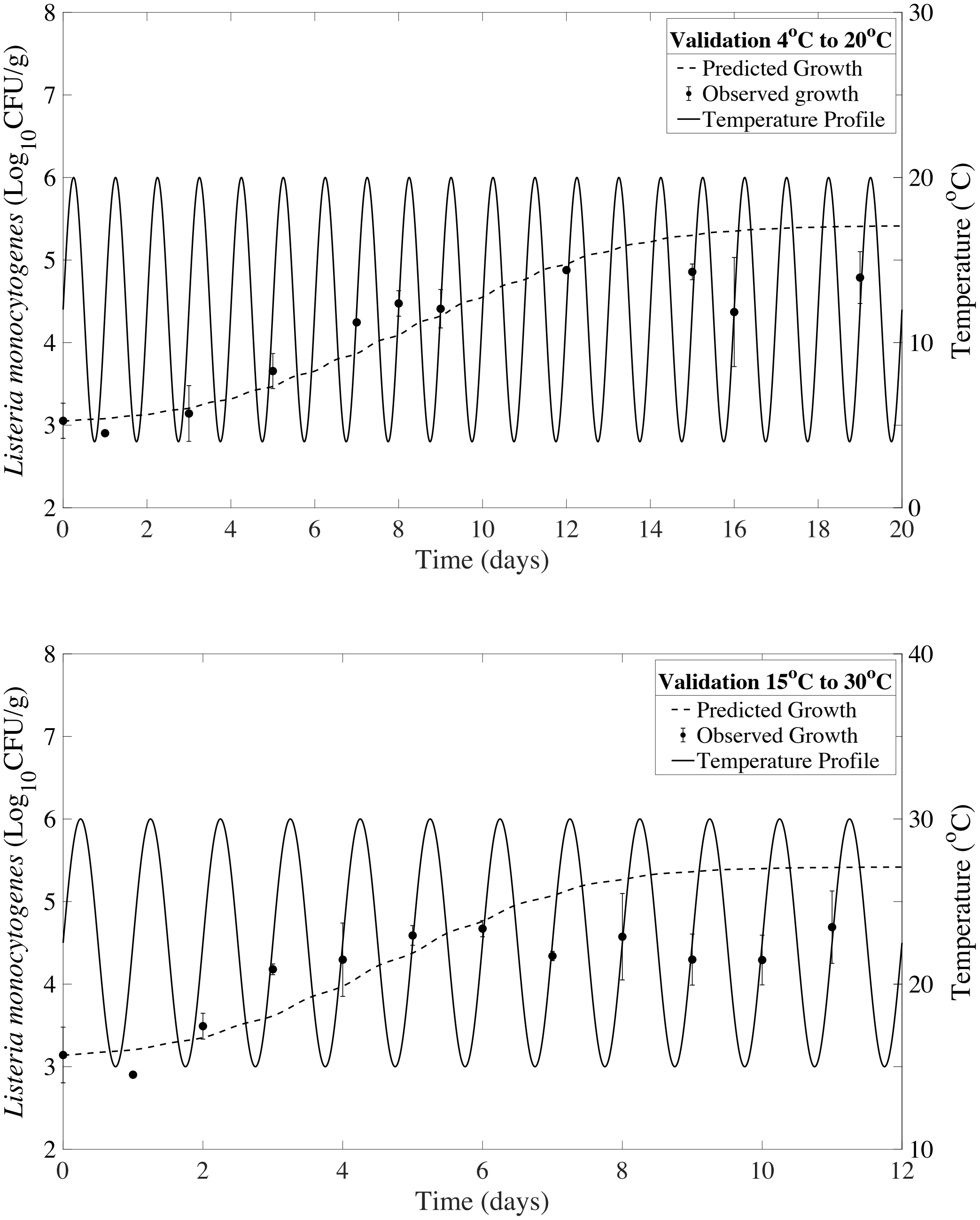

Figure. 4. Validation for predicting growth of *L. monocytogenes* in queso fresco cheeses stored in non-isothermal conditions

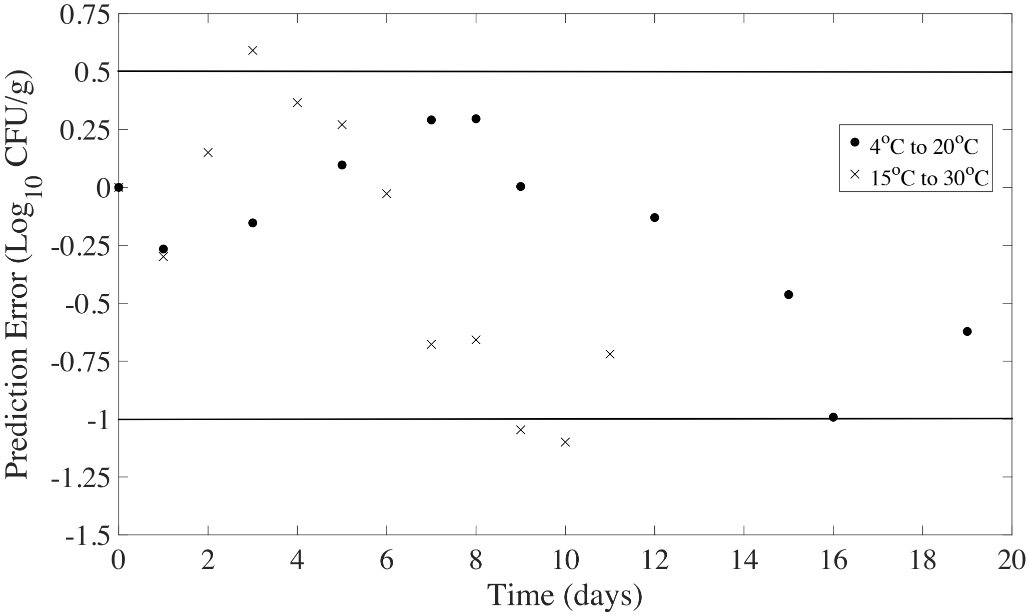

Figure. 5. Acceptable Prediction Zone (APZ) analysis of the prediction error (PE) for *L. monocytogenes* for non-isothermal temperature profiles of 4 to 20°C and 15 to 30°C. APZ within 0.5>PE>-1.0.

CHAPTER 5

CONCLUSIONS

In conclusion, an increase in temperature caused an increase in maximum specific growth rate and a decrease in lag phase duration of *L. monocytogenes* in queso fresco. The predictive models developed in this study were acceptable and can be used by the food industry to predict the growth of *L. monocytogenes* in queso fresco during long storage periods and cases of temperature abuse. These models can be used to understand the importance of maintaining the cold temperature during transport and storage of cheeses. The growth rates found in this study reiterate the importance of eliminating cross-contamination of foods with *L. monocytogenes*. Even at low temperatures and low inoculation levels, the bacteria grew to the maximum population density.

Further research can be conducted to observe the growth of *L. monocytogenes* on different formulations of fresh cheeses or cheeses that are not pasteurized. Because of the effect of temperature on pH observed in this study, further studies can be conducted to develop models that can be fitted to both temperature and pH data at the same time.

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APPENDIX A

PARAMETERS FOR BARANYI MODEL BEFORE h_0 WAS SET CONSTANT

Temperature (°C)	h_0	μ_{max}	y_0	y_{max}
4	1.00	0.0086	2.65	5.61
10	0.57	0.0119	2.40	5.78
15	0.56	0.0181	2.55	5.25
20	1.00	0.0255	2.55	5.38
25	4.59	0.0789	2.58	5.59
30	4.16	0.1250	2.63	4.39

APPENDIX B

MATLAB PROGRAM FOR VALIDATING GROWTH OF *L. MONOCYTOGENES* IN QUESO FRESCO STORED IN NON-ISOTHERMAL TIME-TEMPERATURE PROFILES

For 4°C-20°C

```
clc;
clear;
t=[0:1:480]; %in hours
t=t*60;
T=12 + 8*sin(2*pi*t/(60*24));


Tempvec = [t' T'];
primod = [3.05, 5.42, 2.473];
secmod = [-18.6, 0.0051^2];
TIME_min = Tempvec(:, 1);
Temp_C = Tempvec (:, 2);
Tmin_C = secmod(1);

a = secmod(2);

y0 = primod(1)*log(10);
ymax = primod(2)*log(10);
h0 = primod(3);

% Define Time Step
dt_min = 1;
dth = dt_min/60;
% Create time vector - user input - it is assumed in uniform time steps
t = TIME_min/60;
nt = length(t);
tt = (t(1):dth:t(nt));
csT = spline(t,Temp_C);
TT = ppval(csT,tt);

n = length(TT);
tfinal = tt(n);
```

```
% SOLVE DYNAMIC BARANYI'S MODEL USING RUNGE-KUTTA 4th ORDER
% Spline interpolation of temperature to obtain midpoints of each time interval
% Oversampling the time and temperature vectors by a factor of two for
% calculation purposes
csT = spline(tt,TT);
t1 = [t(1):dth/2:tfinal]; % new time vector containing midpoint values
T1 = ppval(csT,t1); % new temperature vector containing midpoint values
nn = length(t1);
% Discretized Secondary Model
% secondary model predicts growth rate as a function of temperature
% determine the growth rate for the oversampled temperature vector.
% initialize growth rate vector
mumax = [];
for i = 1:length(T1)
 if T1(i) >= 4
 mumax(i) = a*(T1(i) - Tmin_C)^2;
 elseif T1(i) < 4
 mumax(i) = 0;
 end
 end
% Numerical Solution of dQ/dt using RK4
% Define Initial Condition
% "work to be done", constant for given microorganism & given substrate
% h0 = 1.7788;
alpha = exp(-h0); % initial physiological state of cells
q0 = 1/(exp(h0)-1); % initial bottleneck substance
Q = [];
Q(1) = log(q0); % initial condition for dQ/dt
% Runge-Kutta algorithm
i = 1;
for j = 2:n
% PREDICTION
k1 = dth*(mumax(i));
k2 = dth*(mumax(i+1));
k3 = dth*(mumax(i+1));
k4 = dth*(mumax(i+2));
i = i+2;
% CORRECTION
Q(j) = Q(j-1) + ((1/6)*(k1 + (2*k2) + (2*k3) + k4));
end
% Numerical Solution of dy/dt using RK4
% Define Initial Condition
y = [];
y(1) = y0; % initial condition for dy/dt - user input
% Define Maximum Cell Population - constant for given microorganism
% & given substrate.
ymax = ymax;
```

```
% Spline interpolation of Q to obtain midpoints of each time interval
% Oversample or increase the length of Q vector by a factor of two
csQ = spline(tt,Q);
t1 = [t(1):dth/2:tfinal]; % new time vector containing midpoint values
QQ = ppval(csQ,t1); % new Q vector containing midpoint values


% Runge-Kutta algorithm
i = 1;
for j = 2:n
% PREDICTION
k1 = dth*(mumax(i)*(1/(1+exp(-QQ(i))))*(1-exp(y(j-1)-ymax)));
k2 = dth*(mumax(i+1)*(1/(1+exp(-QQ(i+1))))*(1-exp((y(j-1)+(k1/2))-ymax)));
k3 = dth*(mumax(i+1)*(1/(1+exp(-QQ(i+1))))*(1-exp((y(j-1)+(k2/2))- ymax)));
k4 = dth*(mumax(i+2)*(1/(1+exp(-QQ(i+2))))*(1-exp((y(j-1)+(k3))- ymax)));
i = i+2;
y(j) = y(j-1) + ((1/6)*(k1 + (2*k2) + (2*k3) + k4));
end
ypred = y./log(10);
out = [tt' ypred'];
Tv=[0
1
3
5
7
8
9
12
15
16
19];
Yv=[3.053604985
2.903089987
3.141650614
3.655664976
4.245512668
4.474909605
4.410350256
4.878369768
4.856929999
4.370339213
4.788073888];
Err=[0.21286035
0
0.33737567
0.21286035
0
0.15474554
```

```
0.23311555
0.01625208
0.09524628
0.66079019
0.31427512];
% FIGURE
[AX,H1,H2]=plotyy(tt/24,ypred,tt/24,TT,'plot');
ylim(AX(1), [2 8]);
ylim(AX(2),[0 30]);
hold all;
errorbar(Tv,Yv,Err);
set(get(AX(1),'Ylabel'),'String','\itListeria monocytogenes \rm(Log CFU/g)','FontSize',20,
'Color', 'k');
set(AX(1),'FontSize', 20,'YColor','black');
set(AX(1),'YTick',[2, 3, 4, 5, 6, 7,8,9])
set(get(AX(2),'Ylabel'),'String','Temperature (^oC)') ;
set(AX(2),'FontSize', 20,'YColor','black');
datetick(AX(2),'keeplimits')
set(AX(2),'YTick',[0,10,20,30,40,50,60])
xlabel('Time (h)')
```

For 15°C-30°C

```
clc;
clear;
t=[0:1:288]; %in hours
t=t*60;
T=22.5 + 7.5*sin(2*pi*t/(60*24));


Tempvec = [t' T'];
primod = [3.14, 5.42, 2.473];
secmod = [-18.6, 0.0051^2];
TIME_min = Tempvec(:, 1);
Temp_C = Tempvec (:, 2);
Tmin_C = secmod(1);

a = secmod(2);

y0 = primod(1)*log(10);
ymax = primod(2)*log(10);
h0 = primod(3);

% Define Time Step
dt_min = 1;
dth = dt_min/60;
% Create time vector - user input - it is assumed in uniform time steps
t = TIME_min/60;
```

```
nt = length(t);
tt = (t(1):dth:t(nt));
csT = spline(t,Temp_C);
TT = ppval(csT,tt);

n = length(TT);
tfinal = tt(n);

% SOLVE DYNAMIC BARANYI'S MODEL USING RUNGE-KUTTA 4th ORDER
% Spline interpolation of temperature to obtain midpoints of each time interval
% Oversampling the time and temperature vectors by a factor of two for
% calculation purposes
csT = spline(tt,TT);
t1 = [t(1):dth/2:tfinal]; % new time vector containing midpoint values
T1 = ppval(csT,t1); % new temperature vector containing midpoint values
nn = length(t1);
% Discretized Secondary Model
% secondary model predicts growth rate as a function of temperature
% determine the growth rate for the oversampled temperature vector.
% initialize growth rate vector
mumax = [];
for i = 1:length(T1)
 if T1(i) >= 4
 mumax(i) = a*(T1(i) - Tmin_C)^2;
 elseif T1(i) < 4
 mumax(i) = 0;
 end
 end
% Numerical Solution of dQ/dt using RK4
% Define Initial Condition
% "work to be done", constant for given microorganism & given substrate
% h0 = 1.7788;
alpha = exp(-h0); % initial physiological state of cells
q0 = 1/(exp(h0)-1); % initial bottleneck substance
Q = [];
Q(1) = log(q0); % initial condition for dQ/dt
% Runge-Kutta algorithm
i = 1;
for j = 2:n
% PREDICTION
k1 = dth*(mumax(i));
k2 = dth*(mumax(i+1));
k3 = dth*(mumax(i+1));
k4 = dth*(mumax(i+2));
i = i+2;
% CORRECTION
Q(j) = Q(j-1) + ((1/6)*(k1 + (2*k2) + (2*k3) + k4));
end
```

```
% Numerical Solution of dy/dt using RK4
% Define Initial Condition
y = [];
y(1) = y0; % initial condition for dy/dt - user input
% Define Maximum Cell Population - constant for given microorganism
% & given substrate.
ymax = ymax;
% Spline interpolation of Q to obtain midpoints of each time interval
% Oversample or increase the length of Q vector by a factor of two
csQ = spline(tt,Q);
t1 = [t(1):dth/2:tfinal]; % new time vector containing midpoint values
QQ = ppval(csQ,t1); % new Q vector containing midpoint values


% Runge-Kutta algorithm
i = 1;
for j = 2:n
% PREDICTION
k1 = dth*(mumax(i)*(1/(1+exp(-QQ(i))))*(1-exp(y(j-1)-ymax)));
k2 = dth*(mumax(i+1)*(1/(1+exp(-QQ(i+1))))*(1-exp((y(j-1)+(k1/2))-ymax)));
k3 = dth*(mumax(i+1)*(1/(1+exp(-QQ(i+1))))*(1-exp((y(j-1)+(k2/2))- ymax)));
k4 = dth*(mumax(i+2)*(1/(1+exp(-QQ(i+2))))*(1-exp((y(j-1)+(k3))- ymax)));
i = i+2;
y(j) = y(j-1) + ((1/6)*(k1 + (2*k2) + (2*k3) + k4));
end
ypred = y./log(10);
out = [tt' ypred'];
Tv=[0
1
2
3
4
5
6
7
8
9
10
11];
Yv=[3.141650614
2.903089987
3.491135617
4.179424095
4.296465698
4.589545988
4.671999535
4.340620619
4.574301327
```

```
4.297527545
4.292165612
4.690105621];
Err=[0.337375675
0
0.156870755
0.064891453
0.444338074
0.119353982
0.098909451
0.055989596
0.523515478
0.308788297
0.301205379
0.438256834];
% FIGURE
[AX,H1,H2]=plotyy(tt/24,ypred,tt/24,TT,'plot');
ylim(AX(1), [2 8]);
ylim(AX(2),[10 40]);
hold all;
errorbar(Tv,Yv,Err);
set(get(AX(1),'Ylabel'),'String','\itListeria monocytogenes \rm(Log CFU/g)','FontSize',20,
'Color', 'k');
set(AX(1),'FontSize', 20,'YColor','black');
set(AX(1),'YTick',[2, 3, 4, 5, 6, 7,8,9])
set(get(AX(2),'Ylabel'),'String','Temperature (^oC)') ;
set(AX(2),'FontSize', 20,'YColor','black');
datetick(AX(2),'keeplimits')
set(AX(2),'YTick',[0,10,20,30,40,50,60])
xlabel('Time (h)')
```