DEVELOPMENT AND VALIDATION OF A PREDICTIVE MODEL FOR THE GROWTH OF *LISTERIA MONOCYTOGENES* IN EGG YOLK

by

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(Under the Direction of Manpreet Singh)

ABSTRACT

Listeria monocytogenes is a psychrotrophic pathogen that causes several foodborne outbreaks every year in the United States. In this study, a predictive model to study the growth of *L. monocytogenes* in egg yolk was developed and validated. The growth was studied and modeled at temperatures ranging from 0 to 45ºC. Baranyi model and Ratkowsky square root model were used to fit the growth data as primary and secondary models, respectively. Tertiary model was generated and validated using three dynamic temperature profiles of low (0-10ºC), ambient (10-25ºC) and high (25-40ºC).The primary and secondary models both provided coefficient of determination values of R^2=0.99. The validation results depict that at prior physiological state of bacteria (h_o) value of 0.01, more than 80% of the prediction errors lie within the Acceptable Prediction Zone (APZ) of 0.5>Prediction Error>-1.0. These predictions can help estimate the *L. monocytogenes* populations due to possible temperature abuse in egg yolk.

INDEX WORDS: *L. monocytogenes*, egg yolk, predictive modeling

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DEDICATION

To my mom and sister !

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CHAPTER 1

INTRODUCTION

Foodborne illness is a major public health challenge, where 31 known pathogens cause an estimated 9.4 million cases of foodborne illness annually in the United States (US), resulting in approximately 55,961 hospitalizations and 1,351 deaths. The pathogens reported amongst leading cause of death include *Listeria monocytogenes* and *Salmonella* spp., where *L. monocytogenes* was linked to 19% of the total deaths (CDC, 2011). It has a reported hospitalization rate of 94%. Incubation period for this pathogen typically range from 24 h to four weeks for different forms of listeriosis (USFDA, 2020).

Listeria monocytogenes is a ubiquitous organism found in soil, water, animals, and vegetation. It causes listeriosis and the symptoms include headache, vomiting and joint pain. It can cause deadly fetal-placental and central nervous system infections in pregnant women and immunocompromised populations (USFDA, 2020). A majority of the listeriosis cases are foodborne (CDC, 2021). *L. monocytogenes* implications in foodborne illnesses occurs worldwide but is most common in industrialized countries (Bille et al., 2003). The primary factors that lead to categorization of *L. monocytogenes* as major foodborne pathogen in industrialized countries include commercialization of food production resulting in large distribution of potentially contaminated foods, prevalence of refrigerated foods which allows *L. monocytogenes* growth and spike in patients undergoing immunosuppressive therapies along with extended life expectancy of people suffering from immunosuppressive disorders. All *L. monocytogenes* strains are serotyped

based on the variation in somatic (O) and flagellar antigens (H) (Seeliger, 1979). Serotype 1/2a is most isolated from food, but 4b is the one associated with a majority of human epidemics (Gilot et al., 1996).

Eggs are a highly nutritious food with high amount of protein content. It is widely consumed in the form of scrambled, fried, hard-boiled, pickled eggs and is used as an ingredient in many processed food products. Liquid eggs are out of shell contents of eggs that are sold separately as liquid whole egg, egg yolk and egg white, amongst other products. The United States Department of Agriculture (USDA) recommends pasteurization of eggs at minimum temperature of 61.1ºC for 3.5 min for plain egg yolk (USDA, 1980). However, post pasteurization contamination during storage or transportation can make the product microbiologically unsafe for consumption since *L. monocytogenes* can survive in refrigeration temperatures, where liquid eggs are stored. Thus, it is needed to systematically evaluate food safety hazards in the food supply chain and implement strategies to reduce and/ or eliminate the hazards. Predictive microbiology serves as a tool to help mitigate food safety risks as it can help risk assessors and managers to make informed decisions about handling of food products by understanding the potential behavior of a pathogen in food medium via mathematical models in various extrinsic and intrinsic factors. Pathogen Modeling Program (PMP) by USDA is one such tool that provides information on estimated growth and survival rates of various pathogens and spoilage organisms in a variety of environmental factors (USDA, 2016). Predictive models can be applied throughout the food chain, from raw material to final product. These can also be applied to risk analysis studies where dynamics of microbial population throughout the food chain are studied. A scientific validation for that particular food product and pathogen is required for safe application of predictive models (USDA, 2016).

Gumudavelli et al (2007) developed a dynamic predictive model to study the growth of *Salmonella* Enteritidis in egg yolk. Similarly, a predictive model to study the growth of *Salmonella* spp. in liquid whole egg has been developed (Singh et al., 2011). To develop a successful predictive growth model, the growth of a pathogen/ spoilage microorganism is studied in a controlled environment to generate data for mathematical modeling. Primary modeling is used to model the effect of an environmental factor on the growth of a microorganism of interest and secondary modeling to assess the relationship of parameters from primary model with the environmental factor. Model validation is performed as a follow-up, where predictions are compared with observations. The overall objective of this study is to develop a dynamic predictive model for the growth of *L. monocytogenes* in egg yolk with following sub-objectives:

1. To study the growth of *L. monocytogenes* at static temperature profiles of 0 to 45ºC and develop a dynamic predictive model for the growth of *L. monocytogenes* in egg yolk.
2. To validate the developed growth model in egg yolk and its comparison with salted and sugared egg yolk.

CHAPTER 2

LITERATURE REVIEW

General Overview of *Listeria monocytogenes*

Listeria monocytogenes is a rod-shaped, Gram-positive bacterium that causes listeriosis. It can grow at temperatures ranging from 1 to 45ºC with an optimum growth temperature ranging between 30 to 37ºC (Muriana & Kushwaha, 2010). It can survive in a pH range of 4-9.5 and can withstand water activity as low as 0.92 (Carpentier et al., 2011). *L. monocytogenes* has a poor ability to endure acidic conditions (Gray & Killinger, 1966), and no growth has been observed below pH 4 (Farber & Peterkin, 1991).

Listeria monocytogenes is a ubiquitous organism found in animals, birds, insects, soil, and vegetation (Dieterich et al., 2006). Every year, this bacterium infects around 1600 people and causes approximately 260 deaths, and most of these illnesses are foodborne (CDC, 2021). Foods that have been identified as a source of *L. monocytogenes* contamination include dairy products, soft cheeses, deli meat, fresh fruits, and vegetables, and ready to eat (RTE) products, amongst many others. Recent outbreaks of *L. monocytogenes,* as documented by the Centers for Disease Control and Prevention (CDC), include infections due to dairy products and fresh produce, including queso fresco in 2021 and enoki mushrooms in 2020 (CDC, 2021).

Listeria monocytogenes is a major foodborne pathogen that, although causing a lesser number of infections per year, has a mortality rate as high as 21% (Mead et al., 1999) and usually results in hospitalizations with a hospitalization rate as high as 94% ((USFDA, 2020).The symptoms of infections in healthy people are usually mild, including headache, vomiting, and joint pain, which

can occasionally get severe in immunocompromised people leading to encephalitis and meningitis. In pregnant women, symptoms are usually flu-like, including fever, fatigue, and muscle pain. However, apart from miscarriages and premature delivery, it can cause severe complications in the offspring, including stillbirths or life-threatening infections (US-FDA, 2020).

The potential of *L. monocytogenes* to survive in harsh conditions such as low pH, refrigeration temperatures, and high osmotic pressure, has made it a pathogen of concern in commercial food processing and preservation, as these are the commonly employed barriers to prevent the growth and transmission of foodborne pathogens (Melo et al., 2014).

All *L. monocytogenes* strains are serotyped based on the variation in somatic (O) and flagellar antigens (H) (Seeliger, 1979). Serotype 1/2a is most isolated from food, but 4b is the one associated with majority of human epidemics (Gilot et al., 1996). *Listeria innocua* is a microorganism that could be used as an indicator of *L. monocytogenes* (Greenwood et al., 1991) and as a surrogate to study the growth and transmission of the pathogen (Milly et al., 2008).

Listeria monocytogenes **in foods**

The majority of the listeriosis cases in humans are caused by RTE foods which are contaminated with high levels of *L. monocytogenes* at the time of consumption. According to a CDC report, the most recent outbreaks of *L. monocytogenes* in foods are in packaged salads, fully cooked chicken, queso fresco cheese and deli meats, all of which are RTE food products (CDC, 2022). The USDA and FDA has enforced a zero-tolerance policy for *L. monocytogenes* in RTE foods (Shank et al., 1996) as the organism, once contaminated can grow even at refrigeration temperatures in foods during storage.

The pathogen has been found to be able to survive on raw shell eggs with a potential to migrate to inner contents once broken. It can also survive shell egg washing and has been isolated from egg

wash water (Laird et al., 1990, Jones et al., 2006*). L. monocytogenes* can resist sunny side up frying and scrambling (Brackett and Beuchat, 1992). A prevalence rate of 15.5% of *L. monocytogenes* has been reported in laying hen flocks in France (Chemaly et al., 2008). *L. monocytogenes* can also proliferate during egg tempering steps when the temperatures are above 7.2^{o}C.

Listeria monocytogenes in liquid eggs

Also known as breaker eggs, these are the out-of-shell contents of eggs that are sold separately and are convenient for consumers. Liquid egg products available in the market include plain, salted, and sugared whole eggs and yolks, amongst many others. Plain egg albumen or egg whites are also commonly available commodities, that can be used for convenience in bakeries and homes. Egg yolk is a good emulsifier and is used in the production of mayonnaise, and salad dressings, amongst other food products. Other uses include making liqueurs like eggnog.

The pasteurization guidelines laid by the USDA include pasteurization at 60^{o}C for 3.5 min for plain whole egg and 61.1^{o}C for 3.5 min for plain egg yolk (USDA, 1980). *L. monocytogenes* has been found to be as high as eight times more heat resistant than *Salmonella* in eggs under identical conditions (Schuman et al., 1997). A study by Foegeding et al.,1990 indicated that pasteurization conditions suitable for eliminating *Salmonella* in liquid eggs might not be enough for similar reductions in *L. monocytogenes* populations, making it a pathogen of concern in further processing and handling stages. Furthermore, being a psychrotrophic organism, *L. monocytogenes* can survive and grow at refrigeration temperatures, at which liquid eggs are stored (Tasara and Stephan; 2006). *L. monocytogenes* has been isolated from raw liquid whole egg (Leasor et al., 1989) and has been

detected in 8.5% of the samples in five egg-breaking plants in France over a period of one year (Rivoal et al., 2013).

Albumen has several defenses against the microbial activity, with the high viscosity of the albumen obstructing bacterial movement from albumen to yolk (Board and Tranter, 1991). Also, lysozyme acts against gram-positive bacteria by lysing the cell wall. Lysozyme has been found to be effective in impeding *L. monocytogenes* growth (Wang and Shelef, 1991) and the antilisterial activity is supplemented by egg white proteins conalbumin and ovomucoid and alkaline pH. Other albumen proteins, including avidin, ovoinhibitor, and ovomacroglobulin, also have antimicrobial capabilities. All these defenses help egg albumen form barriers against bacterial activity. However, egg yolk is a good nutrient medium with high availability of iron for the growth of bacteria once it reaches egg yolk through the vitelline membrane during storage.

Economic Impact of Foodborne Illness

The estimated cost of foodborne illness has been found to be between $888 to $2591 per person in different states in the US. The national cost of foodborne illness is estimated between 55 and 93 billion US dollars (Scharff, 2015). A single foodborne outbreak cost a fast-food restaurant between 3968 to 1.9 million dollars and the cost goes higher for casual-dining and fine-dining restaurants. *L. monocytogenes* outbreaks are reported to be the costliest (Bartsch et al., 2018).

Predictive microbiology can provide estimates on the populations of a pathogen at different points in the food supply chain. This could help food processors determine the course of action in case of contamination about whether to destroy the batches contaminated or if it is still possible to reduce the pathogen levels. Predictive microbiology also aids in risk analysis of a given pathogen in a food to provide clear picture of foodborne risks associated with the food (Walls and Scott, 1997).

Predictive Microbiology and its Application in Food Safety

Predictive microbiology refers to the use of mathematical, statistical, and microbiological fundamentals to predict the behavior of microorganisms in various environmental conditions (Ross et al., 2000). For predictive microbiology, microbial behavior is generally assessed in controlled environmental parameters such as temperature, pH, water activity (a_w), sodium chloride concentration, antimicrobials, followed by results that are condensed in the form of mathematical equations. These equations help predict microbial behavior in unknown conditions by interpolation (Baranyi et al., 1996).

The basic structure of a mathematical model to study bacterial kinetics is

$$\underbrace{Y = \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3}_{\text{Deterministic}} + \underbrace{\varepsilon}_{\text{Stochastic}}$$

Where, Y is the response variable and X_1, X_2, X_3 are independent or explanatory variables. $\beta_1, \beta_2, \beta_3$ are regression coefficients obtained by the regression method. ε represents error factor which explains the observed variability in data. Therefore, deterministic part is that which describes the relationship between response and explanatory variables and the stochastic part represents observed data variability to which cannot be explained by deterministic part.

One classification of predictive models is based on information used to develop the model (McMeekin and Ross, 2002). Mechanistic models are built on a theoretical foundation, provide interpretation of the observed response in terms of the underlying mechanisms, and are more flexible to refinement as the system's knowledge changes. On the other hand, empirical models are simply developed to describe the observed phenomenon using mathematical equations.

Predictive microbiology can be used to study different microbial processes, referred to as, kinetic and probability modeling. Kinetic models pertain to predicting the degree and rate of microbial growth or decline, namely growth models, survival models and inactivation models (McMeekin et al., 1993), whereas probability modeling estimates the likelihood of occurrence of a microbial event such as toxin production by a bacteria or virus transfer (Pérez-Rodríguez et al., 2008). A vast majority of the models are kinetic models because of their intensive applications in the food industry. Kinetic models can be further classified as primary, secondary, and tertiary models. Primary modeling refers to estimating change in microbial concentration over a given time, secondary modeling pertains to estimating the relationship of parameters from primary models with environmental variables, while parameters from both primary and secondary models are integrated into computer-based tools to generate tertiary models.

Predictive microbiology has become a rapid answer to specific food safety questions in the food processing environment where microbial testing takes longer. Predictive microbiology coupled with user friendly software, help provide rapid estimations of effect of various factors like storage conditions and product formulations on the bacterial kinetics in food. It can be applied throughout the food chain from raw material accession to finished product release, has also been integrated into Hazard Analysis for Critical Control Point (HACCP) (McDonald and Sun, 1999) and is also a necessary element for Quantitative Microbial Risk Assessment (QMRA) (Lammerding and Fazil, 2000). It also serves as a useful tool in designing shelf-life studies on foods, making a sampling plan and development of new food products.

The United States Department of Agricultures' Agricultural Research Service (USDA-ARS) has developed a Pathogen Modeling program (PMP) which is a collection of models for both bacterial

growth and inactivation in various foods and broth media. Similarly, Combase Predictor consists of growth and survival curves for bacteria including *Listeria spp., Clostridium botulinum/perfringens, Salmonella spp., Escherichia coli, Staphylococcus aureus* (Baranyi & Tamplin, 2004).

Primary Modeling

Primary models aim to estimate kinetic parameters as a function of treatment time such as, maximal growth rate, lag phase, and inactivation rate, simulating a storage phase, processing, or heat treatment in model applications. Primary models first use a set of mathematical equations which are assumed to decipher the data, following which, these mathematical equations are fitted by regression into the observed microbial data. This provides values for the model parameters such as lag phase duration or rate of growth for growth models. The resulting predictive models are only applicable to the intrinsic and extrinsic factors in which the observed dataset was obtained.

Microbial growth is generally divided into four phases. The lag phase, also known as the adaptation period, is a period during which bacteria adapt to their surroundings and begin exponential growth (Buchanan and Klawitter, 1991). Microorganisms then grow exponentially (exponential phase) until they reach maximum population levels (stationary phase). The microbial population begins to decrease when the concentration of nutrients or the physiological state of cells drops (death phase). There are different growth models proposed by researchers that are differentiated by growth phases. Sigmoidal growth curve, with an immediate lag phase followed by an exponential and stationary phase, and finally death phase, is obtained when the logarithm of number of organisms is plotted against time. The three most common mathematical models for sigmoidal growth are the Gompertz model, Baranyi model, and logistic model.

Gompertz Model

Gibson et al., 1988 adapted the equations introduced by Benjamin Gompertz to fit microbial growth data since it consists of three phases, similar to microbial growth stages.

$$Y_t = Y_0 + C\{\exp[-\exp(-b(t-M))]\}$$

Equation A

Where Y_t =number of cells at time t (log CFU/g), Y_0 =lower asymptotic value as t approaches 0(log CFU/g), C=number of cells at stationary phase (log CFU/g) -Y_0 , in other words, the difference between upper and lower asymptote, M=time when growth is maximum and b=relative growth rate.

Zwietering et al., 1990 reparametrized the Gompertz equation, where the mathematical parameters were substituted with biological parameters. This reparameterization helps in determining start values for the parameters when they have a biological meaning, as well as finding 95% confidence intervals. The biological values used were specific growth rate= $\mu = \frac{bC}{e}$, lag phase duration= $\lambda = M - (\frac{1}{b})$, and generation time= $GT = Log(2).\frac{e}{bC}$

$$Y_t = Y_0 + A(\exp\left[-\exp\left[\left(\mu_m.\frac{e}{A}\right)(\lambda - t) + 1\right]\right])$$

Equation B

Where A = number of cells at stationary phase (log CFU/g), μ_m=specific growth rate (log CFU/h) and λ=lag phase duration.

Baranyi Model

Baranyi model is a mechanistic model introduced by Baranyi and co-workers in a series of reports (Baranyi et al. (1993, 1995); Baranyi and Roberts, 1994). Baranyi model is currently the most used primary model. This model assumes that bacteria need to produce an unknown substrate q that is required for growth during the lag phase. Once cells have adapted to their new environment, they will continue to grow exponentially until the growth medium imposes constraints. The model proposed has a set of differential equations:

$$\frac{dq}{dt} = \mu_{max} \cdot q(t)$$

Equation C

Where, q(t) = the concentration of limiting substrate, which changes with time; μ_{max}=maximum specific growth rate achieved.

The initial value of $q(q_0)$ describes the initial physiological state of the cells and therefore another form of the equation can be derived as below, where h_o represents interpretation of lag phase before it reaches the characteristic μ_{max} in that environment.

$$h_o = ln\left(1 + \frac{1}{q_0}\right) = \mu_{max}\lambda$$

Equation D

A more detailed version of Baranyi model has been derived as:

$$Y_t = Y_o + \mu_{max}F(t) - ln\left(1 + \frac{e^{\mu_{max}F(t)} - 1}{e^{y_{max} - y_o}}\right)$$

Equation E

Where,

$$F(t) = t + \frac{1}{v} log_e(e^{-vt} + e^{-h_0} - e^{(-vt-h_0)})$$

Equation F

Y_t= bacterial population at time t (ln CFU/g), Y_o = initial bacterial population at time $t = 0$, y_{max}=maximum bacterial population (achieved at stationary phase) (ln CFU/g), μ_{max}= maximum specific growth rate, v is the rate of increase of limiting substrate q, assumed to be equal to μ_{max}.

Logistic Model

Logistic model uses the following differential equation to model growth kinetics of microorganisms (Pearl,1927; Vadasz et al, 2001)

$$\frac{dN}{dt} = rN\left(1 - \frac{N}{N_{max}}\right)$$

Equation G

Where, N= microbial population at time t , r = rate constant which can also be referred to as maximum specific growth rate, N_{max} = maximum bacterial population (achieved at stationary phase).

New Logistic Model

Bacterial growth curves usually follow a sigmoid pattern. However, logistic model is unable to generate a sigmoid curve on a semi-logarithmic plot due to the absence of a lag phase. Therefore, it is not the best fit to model bacterial growth. Fujikawa et al., 2004 modified the traditional logistic model to a new logistic model which can be used to model bacterial growth

$$\frac{dN}{dt} = rN\left(1 - \frac{N}{N_{max}}\right)\left(1 - \frac{N_{min}}{N}\right)^c$$

Equation H

where a new factor N_{min}= minimum bacterial cell concentration (CFU/g) was introduced. N=bacterial concentration at time t and c = rate constant and is always ≥0.

Three-phase Linear Model

Proposed by Buchanan et al. (1997), three phase linear model employ three phases of bacterial growth curve viz. lag phase, exponential growth phase and stationary phase. Figure 2.1 depicts graphical representation of three-phase linear model.

In Lag phase, for $t \leq t_{lag}$,

$$N_t = N_0$$

Equation I

In Exponential growth phase, for $t_{lag} < t < t_{max}$

$$N_t = N_0 + \mu(t - t_{lag})$$

Equation J

In Stationary phase, for $t > t_{max}$

$$N_t = N_{max}$$

Equation K

Where, N_t = log population density at time t (log CFU/ml), N_0 =log of initial population density (log CFU/ml), N_{max} = maximum population density possible in that environment (log CFU/ml), t =time elapsed, t_{lag}= end of lag time (h), μ =maximum specific growth rate (log CFU/h).

The lag phase is divided into two time periods: t_a= time taken for the bacterial adaptation to the environment; t_m= the amount of time it takes for required energy to be generated to make biological components needed for cell reproduction. The growth rate is assumed to be highest between the end of the lag phase and the beginning of the stationary phase in this model whereas μ is set to zero during the stationary and lag phases.

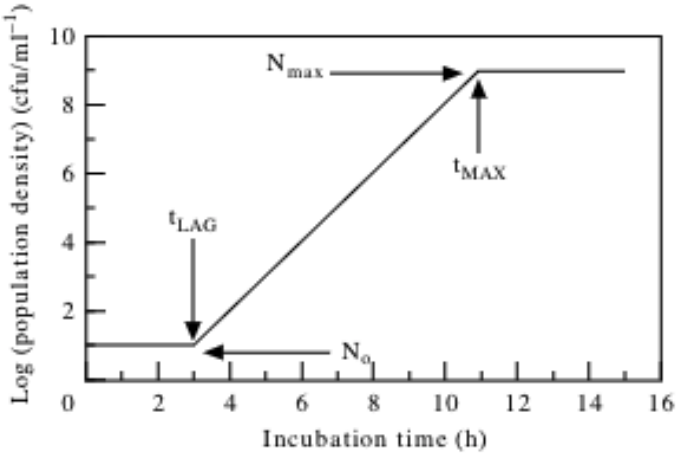

Figure 2.1 Representation of three-phase linear model graphically. Adapted from : Buchanan et al., 1997

Huang Model

A more recent model used for primary modeling is Huang model, proposed by Huang, 2013. A complete mathematical equation for the growth curve is written as:

$$Y = Y_0 + Y_{max} - ln\{e^{Y_0} + [e^{Y_{max}} + e^{Y_0}]e^{-\mu_{max} * B(t)}$$

Equation L

Where,

$$B(t) = t + \frac{1}{\alpha} ln\left[\frac{1 + e^{-\alpha(t-\lambda)}}{1 + e^{\alpha\lambda}}\right]$$

Equation M

Where, Y = bacterial population at time t (ln CFU/g), Y_0 = initial bacterial concentration(ln CFU/g), Y_{max}= maximum bacterial concentration (ln CFU/g), μ_{max} = specific growth rate , $B(t)$= transition function, α= lag phase transition coefficient (LPTC) which defines the shift from lag phase to exponential phase and λ= lag phase.

Secondary Modeling

Secondary models model the changes in the parameters of primary models such as bacterial growth rate and lag time with respect to various extrinsic and intrinsic factors.

There are two different outlooks for secondary modeling based the number of environmental factors:

In first approach, the effect of one environmental factor is modeled at a time and a general model describes the effects of all individual factors on the bacterial behavior as a combined whole. Common secondary models like square root model and cardinal parameter type models employ

this approach. The second method pertains to modeling the effect of multiple environmental factors simultaneously through a polynomial function.

Polynomial Models

Polynomial models predict the effects of multiple environmental factors on bacterial kinetics and are also known as response surface models. These can go from second order polynomial equations to quadratic and sometimes even higher than that. Polynomial models have been used to model the effect of pH, water activity, sodium nitrite concentration and temperature on growth kinetics of *L. monocytogenes* (Buchanan and Phillips, 1990) and others (Lebert et al. 2000).

A general equation for polynomial function is:

$$y = \beta_0 + \sum_{j=1}^{k} \beta_j X_j + \sum_{j=1}^{k} \beta_{jj} X_j^2 + \sum_{j \neq 1}^{k} \beta_{jl} X_j X_l + \varepsilon$$

Equation N

Where y= dependent variable and $\beta_0, \beta_j, \beta_{jj}, \beta_{jl}$ =regression coefficients and X_j, X_lbeing the independent variables (environmental factors), and ε = error term.

Polynomial models are easy to implement in the computer systems, however, unrestrained number of factors can result in incorrect predictions since errors can also be modeled. Another drawback is that the model lacks the terms that help in explaining biological behavior.

Square Root Type Models

As discussed before, square root type models employ first approach where the effects of a single extrinsic or intrinsic factor is modeled. It was initially proposed by Ratkowsky et al., (1982), after observing linear relationship between square root of the maximum specific growth rate and

temperature before it reaches optimum temperature for growth for that organism and environment. For Ratkowsky square root model:

$$\sqrt{\mu_{max}} = a(T - T_{min})$$

Equation O

Where, T_{min} =theoretical minimum temperature below which no growth is observed (it usually lies 2°C -3°C below the observed minimum temperature). T_{min} is generally calculated by a linear regression of the square root of maximum specific growth rate and temperature.

Ratkowsky et al., (1983) later modified the previous model to cover the whole temperature growth range.

$$\mu_{max} = a(T - T_{min})^2\{1 - exp[b(T - T_{max})]\}$$

Equation P

Where, T_{max} = temperature at which the growth is most rapid and b = fitting parameter for shape above T_{max}.

Ratkowsky square root model has also been adapted to model the effect of other environmental factors apart from temperature, such as pH and water activity (Ross et al. 2003).

Similarly, bacterial lag time can also be modeled using equation:

$$t_{lag} = b.(T - T_{min})$$

Equation Q

Tertiary Modeling

Modeling at the primary and secondary level make use of the static environmental conditions, say, isothermal temperature. Tertiary modeling refers to integration of primary and secondary models using computer programs for easy application. Examples of such software include Pathogen modeling program by USDA which use multivariant models based on Gompertz function and response surface analysis to predict bacterial behavior in food storage environment. Another such database is ComBase, which allows users to browse available data related to bacterial growth and inactivation along with developing new modeling techniques (Baranyi & Tamplin, 2004)

Validation Methods

Model validation is a crucial step in the field of predictive microbiology. Models cannot be used until they have gone through a validation procedure, which usually include testing the predictions using any quantitative approach (Dym 2004). Experimental analysis of the growth of the microorganism being studied in that food (as the model) is the basis of model validation where the observed (experimental growth) data is compared with the predicted (model predictions) (Gibson et al. 1988; Sutherland and Bayliss 1994).

There are usually two forms of model validation: internal and external validation. Internal validation is usually done when the actual challenge tests could not be performed due to economic or time constraints. Instead, data taken from other studies performed in similar environmental conditions as described in the model, is compared to the model predictions to accurately determine if the model is capable of adequately describing the experimental data.

Internal validation has been satisfactorily performed by (Garc´ıa- Gimeno et al. 2002).

On the other hand, external validation is performed when the actual independent challenge tests are employed to make comparisons between the model predictions and observed data (Ross et al., 2000). Additionally, data taken from scientific literature to validate the models also falls under external validation (Fernandez et al., 1997)

To determine goodness of fit of models to experimental data, Root mean square error (RMSE) and coefficient of determination (R^2) values are assessed where,

$$RMSE = \sqrt{\frac{\sum_{i=1}^{n}(\mu_{obs} - \mu_{pred})^2}{n}}$$

Equation R

With, μ_{pred}=maximum specific growth rate predicted; μ_{obs}= maximum specific growth rate observed and n=total number of data points. A low RMSE value depicts better fitting of the model (Sutherland et al., 1994).

Other indicators of better adequacy of models to explain observed data are bias factor which is the overall average of ratio of discrete model predictions to observations, and accuracy factors which is the absolute value of ratio of predictions to observations (Ross, 1996). Both of these values determine how close the model observations are to the predicted values.

$$B_f = 10^{[\Sigma\frac{log\ \mu_{max}pred/\ \mu_{max}obs}{n}]}$$

Equation S

$$A_f = 10^{[\Sigma\frac{|\log \mu_{max}pred/\mu_{max}obs|}{n}]}$$

Equation T

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CHAPTER 3

DYNAMIC PREDICTIVE MODEL FOR THE GROWTH OF *LISTERIA MONOCYTOGENES* IN EGG YOLK

Abstract

Listeria monocytogenes is a facultative anaerobic bacterium that can survive at refrigeration temperatures. It causes listeriosis and has been linked with several outbreaks in fresh produce, dairy products, eggs, and meat in the past decade. The current study was designed to develop a dynamic model to predict the growth of *L. monocytogenes* in egg yolk as a function of temperature. Liquid egg yolk was inoculated with approximately 2.5-3 log CFU/ml of a 6-strain *L. monocytogenes* cocktail and its growth at static temperatures of 0, 2, 5, 10, 15, 20, 25, 30, 35, 40, 42.5, 45ºC was studied to cover entire biokinetic range of *L. monocytogenes* growth. A primary model (Baranyi model) was used to fit the growth data from each static temperature and parameters from this model were fitted into a secondary model (Ratkowsky square root model) to estimate their relationship with temperature. R^2 values of higher than 0.9765 and RMSE values of lower than 0.5758 were obtained. The model developed for the growth of *L. monocytogenes* in egg yolk can deliver a rapid and cost-effective substitute for laboratory studies to determine the effects of storage temperature on *L. monocytogenes* growth characteristics in egg yolk and can also be implemented in microbial risk analysis to analyze egg safety.

Introduction

Listeria monocytogenes is a rod-shaped, gram-positive, and ubiquitous microorganism that can be found in soil, water, vegetation, humans, birds, and animals (Dieterich et al., 2006). *L. monocytogenes* causes listeriosis in humans and infects around 1600 people annually resulting in approximately 260 deaths (CDC, 2021). The pathogen has a mortality rate as high as 20-30% (Buchanan et al., 2004). Most listeriosis cases are foodborne and primarily effect children, pregnant women, and immunocompromised people. The symptoms of infections in healthy people are usually mild, including headache, vomiting, and joint pain, which can occasionally get severe in immunocompromised people leading to encephalitis and meningitis. In pregnant women, symptoms are usually flu-like, including fever, fatigue, and muscle pain. However, apart from miscarriages and premature delivery, it can cause severe complications in the offspring, including stillbirths or life-threatening infections (USFDA, 2020). These clinical infections reflect the ability of *L. monocytogenes* to cross tight barriers in human host including intestinal barrier, by crossing intestinal epithelium to gain access to internal organs; blood-brain barrier in case of severe infection, resulting in meningitis; and fetoplacental barrier resulting in fetal infection (Dworkin et al., 2006). *L. monocytogenes* strains are serotyped based on the variation in somatic (O) and flagellar antigens (H) (Seeliger, 1979). Serotype 1/2a is most isolated from food, but 4b is the one associated with majority of human epidemics (Gilot et al., 1996). *Listeria innocua* is a microorganism that could be used as an indicator of *L. monocytogenes* (Greenwood et al., 1991) and as a surrogate to study the growth and transmission of the pathogen (Milly et al., 2008).

Recently, multiple outbreaks involving *L. monocytogenes* were linked to ready-to-eat (RTE) packaged salads, cooked chicken, queso fresco cheese, deli meats and enoki mushrooms (CDC,

2021). It is a psychrotrophic pathogen which can grow at refrigeration temperatures and below. It can also grow at a wide pH range of 4 to 9.5 and a water activity as low as 0.92 (Buchanan et al., 2004). The ability of *L. monocytogenes* to survive in harsh conditions such as low pH, refrigeration temperatures, and high osmotic pressure, has made it a pathogen of concern in commercial food processing and preservation, as these are the commonly employed barriers to prevent the growth and transmission of foodborne pathogens (Melo et al., 2014).

A total of 96.9 billion eggs were produced in the United States (US) in 2020, with a per capita consumption of 286 eggs. Over 25% of all eggs are further processed by the industry (United Egg Producers, 2022). This includes production of liquid whole egg, salted, and sugared whole egg, liquid egg whites, liquid egg yolk, salted and sugared egg yolk, amongst many others. Egg products are used extensively as an ingredient in food products in bakeries, restaurants and processing facilities for their foaming and emulsification properties. According to the guidelines set by United States Department of Agriculture's Food Safety and Inspection Service (USDA-FSIS), shells eggs are required to be stored at 7.2ºC to minimize quality and microbial deterioration. Along with that, most eggs have an average sell by date of 30 days in the US (Bell et al., 2001). These long periods of storage can sometimes lead to temperature abuse or occasional cross contamination of the eggs. Since *L. monocytogenes* can move to inner contents of the eggshell once broken, it poses a food safety threat, especially with high water activity of the egg liquids, which facilitate bacterial growth. Furthermore, being a psychrotrophic organism, *L. monocytogenes* can survive and grow at refrigeration temperatures, at which liquid eggs are stored (Tasara and Stephan; 2006).

Listeria monocytogenes has been isolated from raw liquid whole egg (Leasor et al., 1989) and has been detected in 8.5% of the samples in five egg-breaking plants in France over a period of one year (Rivoal et al., 2013). Egg yolk is an iron rich nutrient media that lacks defenses against microbial activity like lysozyme and other antimicrobial proteins, thus providing a good environment for bacterial activity once it reaches egg yolk through the vitelline membrane during storage. Predictive modeling is a tool that uses mathematical and statistical concepts to quantitatively predict microbial behaviors in foods. Various intrinsic and extrinsic environmental factors can be modeled to study and predict microbial behavior including growth, inactivation, and survival. It is a useful reference tool for improving food safety, with applications in Hazard Analysis Critical Control Point (HACCP) development and risk analysis. It also provides operational support by providing insights on food safety hazards during processing steps, and incidental support in case of food safety concerns with products already released in the market. While there is several studies referencing growth characteristics of *Salmonella* in eggs, there is limited data on the behavior of *L. monocytogenes* in liquid eggs. Thus, this study was conducted to develop a dynamic predictive model for the growth of *L. monocytogenes* in egg yolk under varying temperature conditions.

Materials and Methods

Liquid egg yolk preparation

Shell eggs were obtained from a local grocery store (Grade A Large Eggs) on the day of each experimental setup and were sanitized by dipping in 70% ethanol for 15 min. The eggs were then broken using a sterile knife in a laminar flow biosafety cabinet (Labconco, Fort Scott, KS). Egg

yolk and albumen were separated aseptically using a sterile egg separator and egg yolk was collected in a sterile glass container.

Total solids, pH, and Water activity

Total solids content of the egg yolk was measured by following Association of Analytical Chemists methods of analysis (AOAC, 2019). The pH and water activity of the product were determined using a pH meter (Thermo Scientific, Waltham, MA) and water activity meter (Aqualab, Pullman, WA), respectively.

Bacterial Cultures

Six *L. monocytogenes* strains isolated from poultry environment (aviary forage drag swab, nest box shells, free range nest box shells, free range grass isolate, American Type Culture Collection (ATCC) 13932, stock pathogen *Listeria* control) were used. A day before use, each individual *Listeria* strain was transferred into 10 ml tryptic soy broth (TSB; Becton, Sparks, MD) and incubated for 20 h at 37ºC. Following incubation, 10 ml TSB suspension of each strain was transferred individually into a sterile centrifuge tube and centrifuged at 6000 × *g* for 10 min at 4ºC to concentrate bacterial cells. The supernatant was decanted, and the pellet was resuspended in 5 ml of 0.1% phosphate buffer saline (PBS; Fisher Scientific, Pittsburgh, PA) and recentrifuged. Finally, the pellet was suspended in 0. 5 ml 0.1% PBS. Each strain was centrifuged and suspended individually, following which the strains (0.5 ml) were mixed and vortexed to form a cocktail (3ml).

Sample Inoculation

The six-strain cocktail of *L. monocytogenes* was diluted, and appropriate volume was added to liquid egg yolk to obtain the final *L. monocytogenes* population of 2-3 log CFU/ml. The product was homogenized in a stomacher (Stomacher® 400 Circulator, Seward Laboratory Systems Inc., Islandia NY) at 100 rpm for 1 min. Vacuum pouches (6.35 cm x 12.7 cm; Prime Source, Kansas City, MO; 3 mil standard barrier nylon pouch, oxygen transmission rate of 3,000 cm^3/m^2/24 h at 23ºC and 1 atm) were filled with 5 ml portion of egg yolk and were heat sealed.

Isothermal and Dynamic Profiles

Listeria monocytogenes growth data was collected at isothermal profiles of 0, 2, 5, 10, 15, 20, 25, 30, 35, 40, 42.5 and 45ºC to account for the entire bio-kinetic zone of bacterial growth. Three biological replicates were performed for each isothermal profile. *L. monocytogenes* growth was followed until it reached stationary phase. For each isothermal profile, individual water baths with water circulation capabilities (Polyscience, Niles, IL) were set at the required temperature. Vacuum bags containing egg yolk were immersed in the water bath and after 10 min of immersion, a bag was removed to determine initial *L. monocytogenes* population and subsequent bags were removed at pre-determined time intervals until 3-4 data points in stationary phase of bacterial growth curve were obtained. Table 3.1 represents sampling intervals at each isothermal profile.

Microbial Enumeration

Following removal from the water bath, egg yolk bags were massaged for 2 min. for uniform mixing of the contents. The bag surface was sterilized by spraying 70% ethanol, wiped with paper towels, and opened using a flame sterilized scissor. Appropriate dilutions were performed on the

sample using 0.1% PBS and were spread plated on *Listeria* selective Modified oxford agar (MOX, Becton, Sparks, MD) for all temperature profiles. The plates were incubated at 37ºC for 48 h and bacterial colonies were counted and reported as $\log_{10}$ CFU/ml.

Mathematical Modeling

Primary Modeling

The Baranyi model (Baranyi & Roberts, 1994) was used to describe the growth kinetics of *L. monocytogenes* in egg yolk at various isothermal temperatures. The bacterial concentration was expressed as $\log_{10}$ CFU/ml for model fitting. The four-parameter Baranyi model includes the following equation:

$$y_t = y_o + \mu_{max} F(t) - ln\left(1 + \frac{e^{\mu_{max} F(t)} - 1}{e^{y_{max} - y_o}}\right)$$

Equation U

where $y(t)$ is concentration of bacterial cells at time t (log CFU/ml),

y_0 represents initial bacterial population (log CFU/ml),

u_{max} represents maximum specific growth rate (log CFU/h),

$y_{\max}$ is maximum concentration of bacterial cells (log CFU/ml).

The parameter $F(t)$ can be expanded as:

$$F(t) = t + \frac{1}{v} log_e(\, e^{-vt} + \, e^{h_0} - \, e^{(-vt - h_0)})$$

Equation V

where, h_0 represents previous physiological state of bacterial cells and λ portray lag phase duration of cells (h). The given equation can be used to quantify lag phase duration:

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

Equation W

For each individual static temperature, growth data of *L. monocytogenes* in egg yolk was plotted against time and the parameters were derived using MATLAB software (version R2021a, Mathworks, Natick, MA).

Secondary Modeling

Secondary modeling evaluates the relationship of parameters from primary models with various extrinsic and intrinsic factors. In this case, temperature was an environmental factor and thus Ratkowsky square root model was used which evaluates the relationship between temperature and parameters from primary model. Here, a maximum specific growth rate for each temperature estimated from Baranyi model was fitted into Ratkowsky square root model (Ratkowsky, Olley, McMeekin, & Ball, 1982).

$$\mu_{max} = a(T - T_{min})^2\{1 - exp[b(T - T_{max})]\}$$

Equation X

where, T is the ambient measured temperature (ºC), T_{min} is theoretical minimum temperature where growth is observed (ºC) and T_{max} is the maximum temperature of growth (ºC), a is a regression coefficient.

Zwietering et al. (1991) described a hyperbolic function to model lag phase duration with increment in temperatures of growth. The equation used was:

$$\lambda = e^{\frac{p}{T-q}}$$

Equation Y

where, T is the ambient measured temperature (ºC), q depicts the temperature where lag time is infinite (ºC) and p is the parameter to explain the decline in lag time with rise in temperature. MATLAB was used for the secondary model fitting.

Goodness of Fit of Primary and Secondary Models

The averages and standard errors of the *L. monocytogenes* populations in egg yolk at different time intervals were determined using Excel (version 2021, Microsoft, Redmond, WA). MATLAB was used for analyzing the goodness of fit of primary, secondary, and dynamic models, where the coefficient of determination (R^2) and Root Mean Square Error (RMSE) values were generated (Chai & Draxler, 2014). The equations used for calculating the same are:

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

Equation Z

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

Equation AA

where,

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

Equation BB

and

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

Equation CC

SSR is the regression sum of squares, SSTO is the total sum of squares, n represents sample size, y_i represents the observed value at i[th] item, $\bar{y}$ represents average response variable, $\hat{y}$ is the estimated value, and e_i stands for model prediction error at i^{th} term, which is represented by the difference between predicted and observed values.

Tertiary Modeling

Parameters from primary and secondary models are integrated to generate tertiary model in MATLAB software. Fourth order Runge-Kutta method was used to predict bacterial growth under non-isothermal conditions. Baranyi & Roberts (1994) put forward these two equations to model differential growth:

$$\frac{dy}{dt} = \frac{1}{1 + e^{-Q(t)}}\mu_{max}\,[T(t)]\left(1 - e^{y(t) - y_{max}}\right)$$

Equation DD

$$\frac{dQ}{dt} = \mu_{max}[T\ (t)]$$

Equation EE

where starting conditions were fixed at $y = y_0$, $t = 0$ and $Q(0) = log_e(Q_0)$, with Q_0 being the starting physiological state of the bacterial cells.

Results and Discussion

pH and Water Activity

Batches of egg yolk used in the study had an average pH of 6.24±0.20, water activity value of 0.99±0.01 and a total solids content of 65.00±0.69%. Each parameter was measured in triplicates and is represented as arithmetic mean±standard deviation. Table 3.2 depicts values of all measured parameters of egg yolk in the study.

Primary Growth Models in Isothermal Conditions

Table 3.3 shows the primary growth parameters of *L. monocytogenes* in egg yolk at the isothermal temperatures studied. While data were collected at temperatures ranging from 0 to 45ºC, data at 45ºC was excluded from the model fitting because the bacterial population decreased from an average 2.72 to 0.7 log CFU/ml over 84 h at this temperature. The lowest temperature at which growth was observed in the study was 0ºC, demonstrating the psychrotrophic nature of the bacteria, and highest was 42.5ºC. Figure 3.1 depicts the primary growth model at each temperature in the given range. The maximum specific growth rate (μ_{max}) of *L. monocytogenes* increased with increasing temperature up to 35ºC, which is within the reported optimum growth temperature range

of *L. monocytogenes* at 30-37ºC (Muriana & Kushwaha, 2010). The maximum specific growth rate expectedly declined at 40 and 42.5ºC temperatures, which are beyond the optimum growth temperature range. The μ_{max} was found to be 0.0052 log CFU/h at 0ºC, which was the lowest amongst all temperature profiles, gradually increasing to 0.4397 log CFU/h at 35ºC, the highest value of μ_{max} observed in the static temperature range. Similar relationship between μ_{max} and temperature was observed by Uhlich et al., (2006), who studied *L. monocytogenes* growth in queso blanco at temperatures ranging from 5-25ºC. Another study by Rodríguez et al., 2000 investigating *L. monocytogenes* growth in fresh green asparagus stored at temperatures 2-20ºC reported higher growth rates at higher temperatures.

The parameter h_0 remained consistent since the physiological state of the bacterial cells before inoculation was comparable, as recommended by Baranyi and Roberts (1994). As no relationship was observed between growth temperatures and h_0, this value was quantified by calculating mean of the individual h_0 values at each temperature. The data were again fitted using this new average h_0 (2.46) to estimate other parameters, (μ_{max}, y_{max} and λ). This value is in a similar range as observed by others including Juneja et al., (2022), who estimated an average h_0 value of 3.49 for *Clostridium botulinum* in cooked ground pork. Thomas, Tiwari, and Mishra (2019) used an average h_0 value of 2.47 to model growth of *L. monocytogenes* in queso fresco, while Singh et al. (2011) also set the value of h_0 at 3.51 to study *Salmonella* growth in liquid whole eggs, and Juneja et al. (2019) calculated a mean h_0 of 4.10 for *Bacillus cereus* during cooling of cooked rice. The minimal differences in h_0 could be due to different substrates and microbes studied.

The goodness of fit statistics for primary model fitting using the Baranyi equations were found to be between 0.9818 and 0.9959 for R^2 and from 0.1916 to 0.5758 for RMSE (Table 3.4). These values suggested acceptable goodness of fit with high R^2 and low RMSE values, also indicating

that the observed values were closer to the model predictions for *L. monocytogenes* growth under the given conditions.

Secondary Growth Models

Table 3.5 depicts the results from the Ratkowsky root square model fitting for the secondary growth model (Ratkowsky et al., 1982). Theoretical maximum temperature (T_{max}) of growth obtained was 45.35ºC, which was close to the experimental temperature at 42.5ºC. The theoretical minimum (T_{min}) temperature obtained while fitting the data was -0.3178ºC, that was near experimental minimum temperature of 0ºC. The difference in theoretical and experimental T_{max} and T_{min} is not unusual and generally lies between 5-10ºC (Ross & Dalgaard, 2004). When the values for T_{min} and T_{max} are placed in the Ratkowsky equation, the model is presented as:

$$\mu_{max} = 0.0006124\ (T - (-0.3178)^2\{1 - exp[0.08202(T - 45.35)]\}$$

Equation FF

The fitted model shown in Figure 3.2 displays positive linear relationship between μ_{max} and temperature up to optimum temperature of growth for *L. monocytogenes*. Similar observations were made by Xanthiakos et al., 2006, when using square root model to estimate relationship between temperature and μ_{max}. The fitted secondary model in this study has a R^2 value of 0.9949 and RMSE of 0.01399 as shown in Table 3.5. This is consistent with Juneja et al. (2019), where the model for *B. cereus* in cooked rice found the Ratkowsky model to provide the best fit for μ_{max} values generated from the Baranyi model.

Lag phase duration (λ) is the time it takes for the bacterial cells to adjust physiologically to the environment before the log phase (Baranyi and Roberts, 1994). Lag phase duration after substituting the values was calculated as:

$$\lambda = e^{\frac{50.45}{T+7.22}}$$

Equation M

The lag phase duration was observed to decrease with increase in temperature (Figure 3.3). This could be because *L. monocytogenes* cells were incubated in TSB at 37ºC prior to inoculation of the egg yolk, thus potentially aiding in quicker adjustment of the cells to the new environment at high temperatures (Métris et al., 2003).This explains the shortest lag phase of 5.59 observed at 35ºC.

Conclusion

The predictive model developed in this study can be used as a reference for the egg processing industry during long transport and storage periods and in case of any temperature abuse. *L. monocytogenes* populations in egg yolk grew to the maximum population density at all temperatures studied, from 0 to 42.5ºC. This observation highlights the importance of preventing contamination of eggshells with *L. monocytogenes*, since the bacteria can migrate into the inner contents of the egg. Liquid egg yolk has been found to be a good substrate for *L. monocytogenes* growth. These findings provide evidence to USDA-FSIS guidelines of maximum egg yolk storage period of 7 days for unopened packages at refrigeration temperatures. Apart from following the pasteurization guidelines set by USDA for egg yolks, prevention of post pasteurization contamination is necessary to make the product safe for use.

Table 3.1. Frequency of sampling to determine growth of *L. monocytogenes* at isothermal profiles

Temperature (ºC)	Time Intervals
0	Every 120 hours up to 1440 hours
2	Every 96 hours up to 1152 hours
5	Every 48 hours up to 624 hours
10	Every 24 hours up to 312 hours
15 and 42.5	Every 9 hours up to 117 hours
20	Every 7 hours up to 91 hours
25, 35 and 40	Every 6 hours up to 78 hours
30	Every 3 hours up to 36 hours

Table 3.2. pH, Water activity and Total solids (%) of various batches of egg yolk used for different isothermal profiles.

Batch number	pH*	Water activity *	Total solids (%) *
1	6.05±0.06	0.99±0.00	65.22±0.25
2	6.27±0.07	0.99±0.00	64.91±0.74
3	6.05±0.14	0.99±0.01	65.72±1.19
4	6.20±0.07	0.98±0.01	65.10±0.78
5	6.52±0.35	0.99±0.00	65.92±0.86
6	6.17±0.03	0.98±0.01	63.55±0.62
7	6.29±0.09	0.98±0.00	65.29±0.12
8	6.60±0.46	0.99±0.00	64.84±0.16
9	6.05±0.14	0.98±0.00	64.49±0.48

*Mean values± standard deviation

Table 3.3. Maximum specific growth rate (μ_{max}), lag phase duration (λ), and maximum population density (y_{max}) of *L. monocytogenes* in egg yolk at various isothermal temperatures.

Temp (ºC)	μ_{max}(log CFU/h)	λ (h)	y_{max} (log CFU/ml)
0	0.0052	473.323	9.29
2	0.0121	203.878	8.95
5	0.0231	106.667	9.37
10	0.0527	46.739	9.22
15	0.1275	19.300	9.07
20	0.2171	11.334	9.03
25	0.3438	7.1576	8.67
30	0.3822	6.438	8.14
35	0.4397	5.596	8.38
40	0.3585	6.864	8.32
42.5	0.2316	10.625	7.59

Table 3.4. Parameters from statistical analysis of fitted primary model indicating goodness of fit.

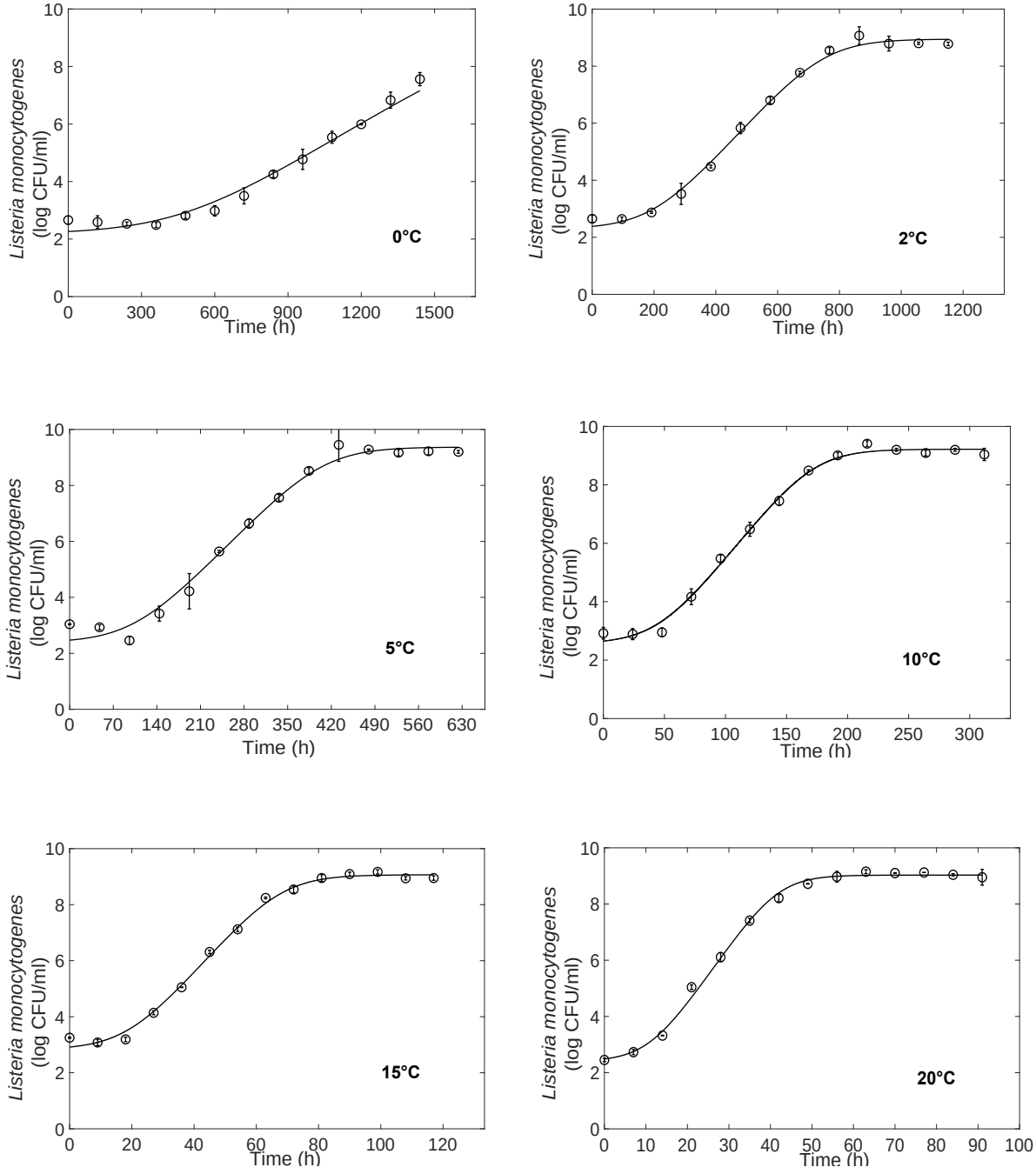
Temperature (ºC)	Coefficient of determination (R^2)	Root Mean Square Error (RMSE)
0	0.9848	0.2406
2	0.9956	0.1916
5	0.9876	0.3362
10	0.9955	0.1934
15	0.9959	0.1724
20	0.9972	0.1480
25	0.9938	0.1851
30	0.9818	0.3563
35	0.9765	0.3009
40	0.9912	0.1981
42.5	0.9002	0.5758

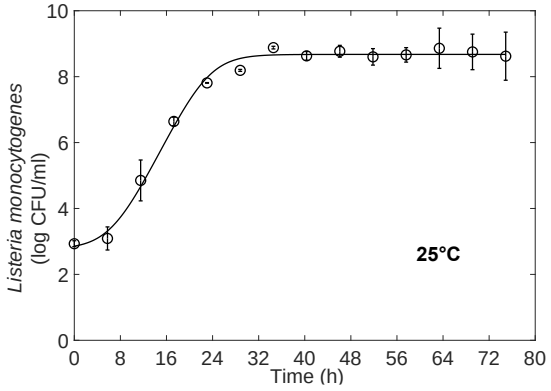
Table 3.5. Parameters from secondary model fitting for growth of *L. monocytogenes* in egg yolk.

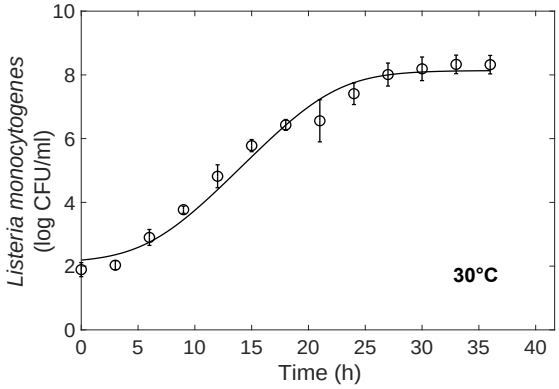
Secondary models	Model parameters	R^2	RMSE
Ratkowsky square root model	T_{min} =-0.3178ºC	0.9949	0.0139
	T_{max} = 45.35ºC		
	a = 0.0006124		
	b = 0.08202		
Hyperbolic function	p = 50.45	0.9794	21.7314
	q = -7.221		

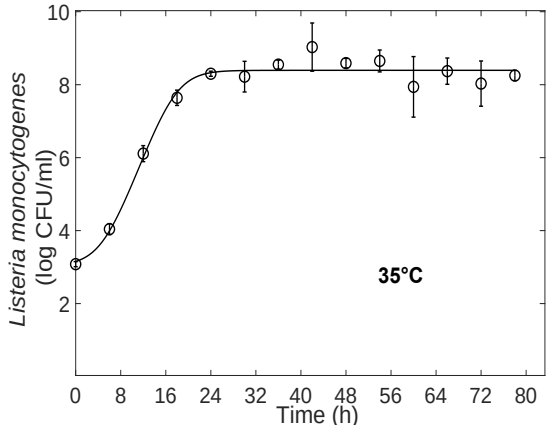
LIST OF FIGURES

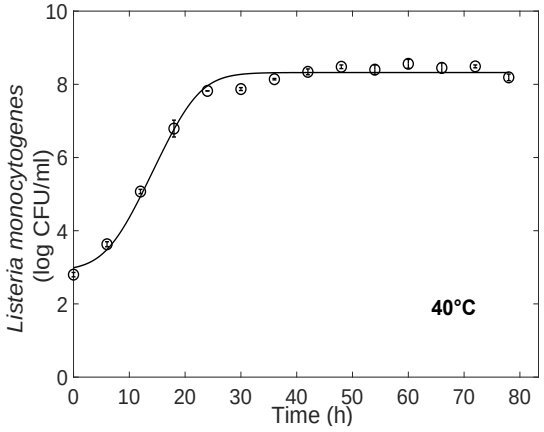
Figure 3.1




Listeria monocytogenes
(log CFU/ml)
Time (h)
25°C


Listeria monocytogenes
(log CFU/ml)
Time (h)
30°C


Listeria monocytogenes
(log CFU/ml)
Time (h)
35°C


Listeria monocytogenes
(log CFU/ml)
Time (h)
40°C

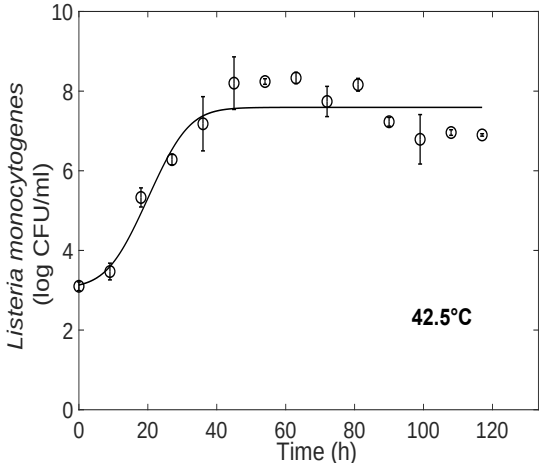

Listeria monocytogenes
(log CFU/ml)
Time (h)
42.5°C

Figure 3.2

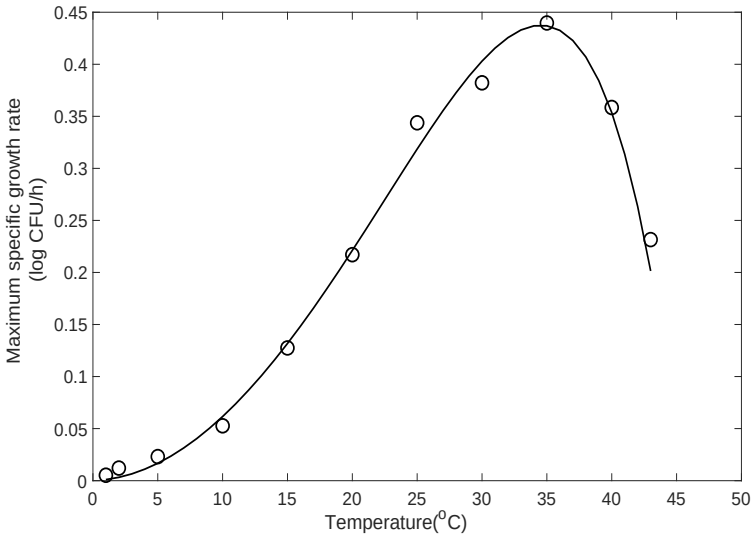
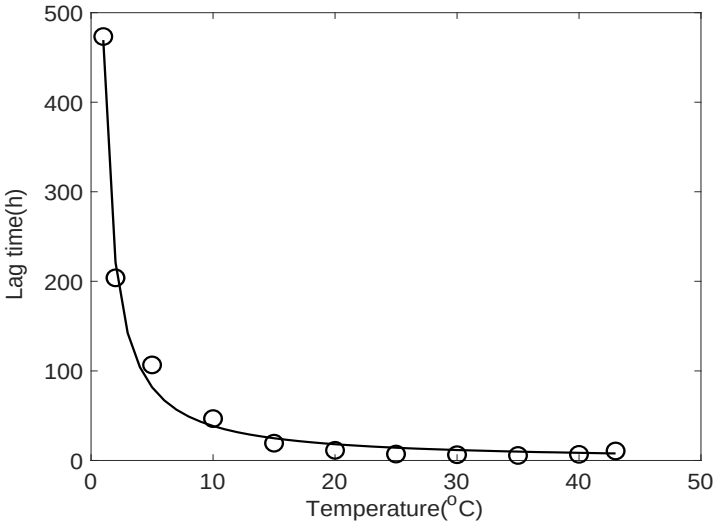

Maximum specific growth rate
(log CFU/h)
Temperature(°C)
0
0.05
0.1
0.15
0.2
0.25
0.3
0.35
0.4
0.45
0
5
10
15
20
25
30
35
40
45
50

Figure 3.3



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CHAPTER 4

VALIDATION OF PREDICTIVE MODEL FOR THE GROWTH OF *LISTERIA MONOCYTOGENES* IN EGG YOLK

Abstract

Listeria monocytogenes is a rod shaped, facultative anaerobic bacteria that causes listeriosis. Since most of the listeriosis cases are foodborne, a systematic evaluation of food safety hazards in food supply chain and implement strategies is needed to eliminate these hazards. A validated predictive model can be used to estimate the behavior of a pathogen at various points in a food supply chain. In this study, validation of the dynamic predictive model developed to study the growth of *L. monocytogenes* in egg yolk was performed. The growth characteristics of *L. monocytogenes* in egg yolk is studied at three different temperature profiles of low (0-10ºC), ambient (10-25ºC), and high (25-40ºC) temperatures and compared with the predicted growth from the dynamic predictive model. Furthermore, sugared, and salted egg yolk were also used to compare the growth of *L. monocytogenes* in these products at similar conditions as plain egg yolk. The indicators employed to validate the model were A_f, B_f and APZ analysis. The results indicated good fit for the model predictions in observed data with acceptable values of A_f, B_f and prediction errors.

Introduction

Validation of a predictive model is a crucial step in the field of predictive microbiology and provided help in estimating growth of pathogens in various environments and food matrices. Models cannot be used until validated, which usually include testing the predictions using a quantitative approach (Dym, 2004). Experimental analysis of the growth of a microorganism being

studied in that food (as the model) is the basis of model validation where the observed (experimental growth) data is compared with the prediction (model predictions) (Gibson et al. 1988; Sutherland and Bayliss 1994). Ross (1996) developed two statistical indicators to test the accuracy and applicability of the predictive models in real life conditions, namely accuracy factor (A_f) and bias factor (B_f). These accuracy factors were developed to compare the independent observations to the model predictions. The bias factor is an average of the ratio of discrete model predictions to observations, and a when the ratio of predicted and observed maximum specific growth rate is calculated, a B_f value of 1 indicates that the observations are equally distributed below and above predictions and thereby are in perfect agreement. A value >1 means fail-safe and a value <1 indicates fail-dangerous models. Models with excessively fail-safe predictions can lead to economic losses if applied in the food industry as it can lead to destruction of safe food which would otherwise be harmless for consumer consumption (Oscar , 2005). On the other hand, exceedingly fail-dangerous predictions are increasingly harmful and can lead to foodborne illness. Therefore, an acceptable prediction zone needs to be specified for each model assessed. The acceptable B_f range falls between 0.75-1.25. The A_f is an absolute value of ratio of model predictions to observations, determining closeness between the two. In this case, a value of 1 would mean perfect alignment between predictions and observations.

Despite their applications, A_f and B_f have some limitations which can lead to inaccurate evaluation of model performance. Some of these shortcomings include 1) A_f and B_f are mean values and therefore, can get affected by outliers in the data (Delignette-Mullerand others 1995), 2) In case of no growth, A_f and B_f are not calculated (Augustin and Carlier 2000), and 3) A_f and B_f being mean values can also get affected by systematic prediction bias, where overestimation in

one part of the model can get compensated by underestimation in other parts of the model (Ross 1996). To overcome these limitations, acceptable prediction zone (APZ) method was developed where predicted values from the model were subtracted from observed values to generate prediction errors. The acceptable prediction zone of -1 to 0.5 was determined, similar to B_f range of 0.7-1.15 (Oscar, 2005). The acceptable range leans more towards the fail-safe range (-1) than fail- dangerous (0.5) range as more errors can be tolerated in this case because, although it can lead to food wastage but ensures that the food is microbially safe. In case of underprediction (fail-dangerous), the actual bacterial population is more than expected and can give rise to food safety concerns (Ross et al., 2000). If 70% of the prediction errors fall within an APZ, the model was considered acceptable. Although a certain margin in predictions and observations is considered acceptable in A_f, B_f and APZ analysis methods, it is always recommended to have predictions as close as possible to observed data obtained.

In this study, validation of a dynamic model developed for the growth of *L. monocytogenes* in egg yolk was performed using three validation profiles where the temperature was constantly changing. These ranged from low temperature profile (0-10ºC), ambient temperature profile (10-25ºC) and high temperature profile (25-40ºC). For comparison, sugared and salted egg yolk were also tested along with the plain egg yolk. The salted and sugared product were not used for validation purposes and were only for comparison, as it is an extrapolation of the parameters originally used to develop the model (Buchanan et al., 1993).

Materials and Methods

Liquid egg yolk preparation

Shell eggs were obtained from a local grocery store (Grade A Large Eggs) on the day of each experimental setup and were sanitized by dipping in 70% ethanol for 15 min. The eggs were then broken using a sterile knife in the biosafety cabinet (Labconco, Fort Scott, KS). Egg yolk and albumen were separated aseptically using a sterile egg separator and plain egg yolk was collected in a sterile glass container. For salted egg yolk, salt on a 10% weight basis (10 g salt/ 90 g product) was added to the plain yolk. Similarly, sugar on a 10% weight basis was added to make sugared egg yolk.

pH, Water Activity and Total Solids

The pH and water activity of all three products were measured using a pH meter (Thermo Scientific, Waltham, MA) and water activity meter (Aqualab, Pullman, WA), respectively. Total solids content of was measured by following the American Association of Analytical Chemists methods of analysis (AOAC, 2019).

Bacterial Cultures

Six *L. monocytogenes* strains isolated from poultry environment (Aviary forage drag swab, nest box shells, free range nest box shells, free range grass isolate, American type culture collection (ATCC) 13932, Stock pathogen *Listeria* control) were used.A day before use, each *Listeria* strain was transferred into 10 ml tryptic soy broth (T.S.B.; Becton, Sparks, MD) and incubated for 20 h at 37ºC. After incubation, 10 ml TSB suspension of each strain was transferred individually to sterile centrifuge tube and centrifuged at 6,000 × *g* for 10 min at 4ºC to concentrate bacterial cells.

The supernatant was then decanted, and the pellet was resuspended in 5 ml of 0.1% phosphate buffer saline (PBS; Fisher Scientific, Pittsburgh, PA) and recentrifuged. Finally, the pellet was suspended in 0. 5 ml 0.1% PBS. Each strain was centrifuged and suspended individually, and the strains (0.5 ml) were mixed and vortexed to form a cocktail (3ml).

Sample Inoculation

The six-strain cocktail of *L. monocytogenes* was diluted, and appropriate volume was added to each egg yolk product to obtain the final *L. monocytogenes* population of 2-3 log CFU/ml. The products were homogenized in a stomacher (Stomacher® 400 Circulator, Seward Laboratory Systems Inc., Islandia NY) at 100 rpm for 1 min. Vacuum bags (6.35 cm x 12.7 cm; Prime Source, Kansas City, MO; 3 mil standard barrier nylon pouch, oxygen transmission rate of 3,000 cm^3/m^2/24 h at 23ºC and 1 atm) were filled with 5 ml portion of egg yolk products and heat sealed.

Validation Profiles

Listeria monocytogenes growth data was collected using three temperature profiles. These were low, ambient, and high temperature profile with temperature range of 0-10, 10-25 and 25-40ºC, respectively. Three biological replicates were performed for each product in each individual temperature profile. *L. monocytogenes* growth was followed till the stationary phase was attained.

For each validation profile, water baths with water circulation capabilities (Polyscience, Niles, IL) were set at the desired temperature profile. Required number of vacuum bags containing plain, sugared, and salted egg yolk were immersed in the water bath. After 10 min of immersion, a bag was removed to determine initial *L. monocytogenes* population and subsequent bags were removed

at pre-determined time intervals until 3-4 data points in the stationary phase of bacterial growth curve were obtained. Table 4.1 provides sampling intervals at each validation profile.

Microbial Enumeration

After removal from the water bath, egg yolk bags were massaged for 2 min for uniform mixing of the contents. The bag surface was sterilized by spraying 70% ethanol and opened using a flame sterilized scissor. Appropriate dilutions were performed using 0.1% PBS and 0.1 ml of the sample was spread plated on *Listeria* selective Modified oxford agar (MOX, Becton, Sparks, MD) for all profiles. The plates were incubated at 37ºC for 48 h and bacterial colonies were counted and reported as $\log_{10}$ CFU/ml.

Results and Discussion

Table 4.2 shows the parameters measured for plain, sugared, and salted egg yolk in this study. Figures 4.1, 4.2 and 4.3 show the results from the dynamic model validation for three profiles of low (0-10ºC), ambient (10-25ºC) and high (25-40ºC) temperatures, respectively. Figure 4.1 depicts underprediction in egg yolk in low temperature profile when an average prior physiological stage (h_o) value of 2.46 was used from primary modeling. However, when h_o= 0.01 was used, the prediction curve changed significantly, and lies close to the observed population density curve. Similar trends were observed in ambient and high temperature profiles. In figure 4.2, the prediction curve follows the observed population density closely when h_o was fixed at 0.01, similarly in figure 4.3, the prediction curve was observed in close proximity to the observed population density curve at $h_o = 0.01$. These findings indicate a possible difference between initial physiological conditions of the bacterial cells before inoculation. Similar results were obtained by Koseki and

Isobe, (2005), when modeling the growth of *Escherichia coli O157:H7, Salmonella* spp., *and Listeria monocytogenes* on iceberg lettuce in dynamic temperature conditions, where the model vastly overpredicted the growth of all three pathogens. The possible reason for this, as suggested by Bovill et al., (2000), is that the lag phase depends on prior physiological state of the bacteria which, in turn, is dependent on previous environment of the cells and cannot be estimated by data collection in other independent conditions in the lab such as at isothermal temperatures. Baranyi (2002) emphasized the need for a mathematical model to independently determine dynamics of bacterial cells.

In case of 10% sugared and salted egg yolk curves, which were plotted with the plain egg yolk curves for comparison purposes, sugared egg yolk shows better proximity than plain egg yolk between observed and predicted *L. monocytogenes* populations at $h_o = 2.46$ in ambient and high temperature profiles. However, at low temperature, observed *L. monocytogenes* population in sugared egg yolk again started deviating from predictions. Salted egg yolk showed no growth in low temperature conditions and a decline in *L. monocytogenes* concentration at ambient and high temperatures, which is not unexpected, given the average water activity of 0.83 in salted yolk which does not assist in bacterial growth (Farber et al.,1992).

The APZ method was employed to study the difference between predictions and observations in the validation study, as shown in figures 4.4 and 4.5. It was observed that 14 out of 17 from the low temperature, 13 out of 16 from ambient, and 15 out of 16 prediction errors (PE) from high temperature profile were within the acceptable range of 0.5>Prediction Error>-1.0, at $h_o = 0.01$. For a model to be considered acceptable, 70% of the PE need to be within the APZ (Oscar, 2005). Similar results have been observed by Juneja et al., (2022) when using APZ analysis. At $h_o =$

2.46, majority of the values from the low temperature and approximately one third from the ambient and high temperature profile lie outside the APZ. Table 4.3 represents A_f and B_f values from the validation profiles. Oscar, (2005) and Ross, (1996) suggested that B_f values between 0.7 and 1.15 can be interpreted as acceptable when describing a pathogen growth. The A_f values ranged from 1.05 to 1.08 and B_f from 0.99 to 1.02 for $h_o = 0.01$ in all three profiles, indicating concordance between of the model predictions and observations.

CONCLUSION

The models developed in this study can predict the growth of *L. monocytogenes* in varying temperature conditions that can possibly be encountered by egg yolk during transport and storage. The pathogen is found to be growing fast in egg yolk, reaching maximum population densities in each temperature profile. Along with that, the comparison studies done with sugared and salted egg yolk provides some insight into the shelf life of these products at dynamic temperatures where sugared egg yolk is found to be highly susceptible to bacterial growth once contaminated. On the other hand, salted egg yolk is established as a highly sheltered food from the bacterial activity. The disagreement observed between predicted and observed *L. monocytogenes* populations in egg yolk warrant further research to analyze the possible causes. The products used in this study were fresh with no preservatives added to extend shelf life, however the results could vary in case of any addition of preservatives like potassium sorbate which necessitate future studies.The general comparison between *L. monocytogenes* activity in salted, sugared, and plain egg yolk in validation profiles developed for plain egg yolk suggest further exploring separate dynamic modeling for these two products. This could aid in the successful implementation of those predictive models for egg processors.

Table 4.1. Sampling frequency at validation profiles

Product	Validation profile (ºC)	Time Intervals
Plain egg yolk	Low temperature	Every 24 hours up to 504 hours
	Ambient temperature	Every 12 hours to 144 hours
	High temperature	Every 4 hours up to 60 hours
Salted egg yolk	Low temperature	Every 72 hours up to 936 hours
	Ambient temperature	Every 24 hours up to 336 hours
	High temperature	Every 4 hours up to 68 hours
Sugared egg yolk	Low temperature	Every 48 hours up to 600 hours
	Ambient temperature	Every 12 hours up to 144 hours
	High temperature	Every 4 hours up to 68 hours

Table 4.2. pH, Water activity and Total solids (%) of various batches of egg yolk used for different dynamic profiles.

Batch	Product	pH*	Water activity *	Total solids (%) *
1	Plain egg yolk	6.38±0.04	0.97±0.00	64.91±0.16
2	Plain egg yolk	6.74±0.13	0.96±0.00	64.43±0.60
3	Plain egg yolk	6.27±0.00	0.97±0.00	64.61±0.28
1	Sugared egg yolk	6.39±0.02	0.94±0.01	62.48±0.32
2	Sugared egg yolk	6.62±0.18	0.95±0.00	62.89±0.42
3	Sugared egg yolk	6.20±0.00	0.94±0.00	62.79±0.50
1	Salted egg yolk	6.24±0.02	0.83±0.01	62.31±0.30
2	Salted egg yolk	6.19±0.03	0.83±0.01	62.70±0.19
3	Salted egg yolk	6.02±0.02	0.82±0.01	62.90±0.32

*Mean values± standard deviation

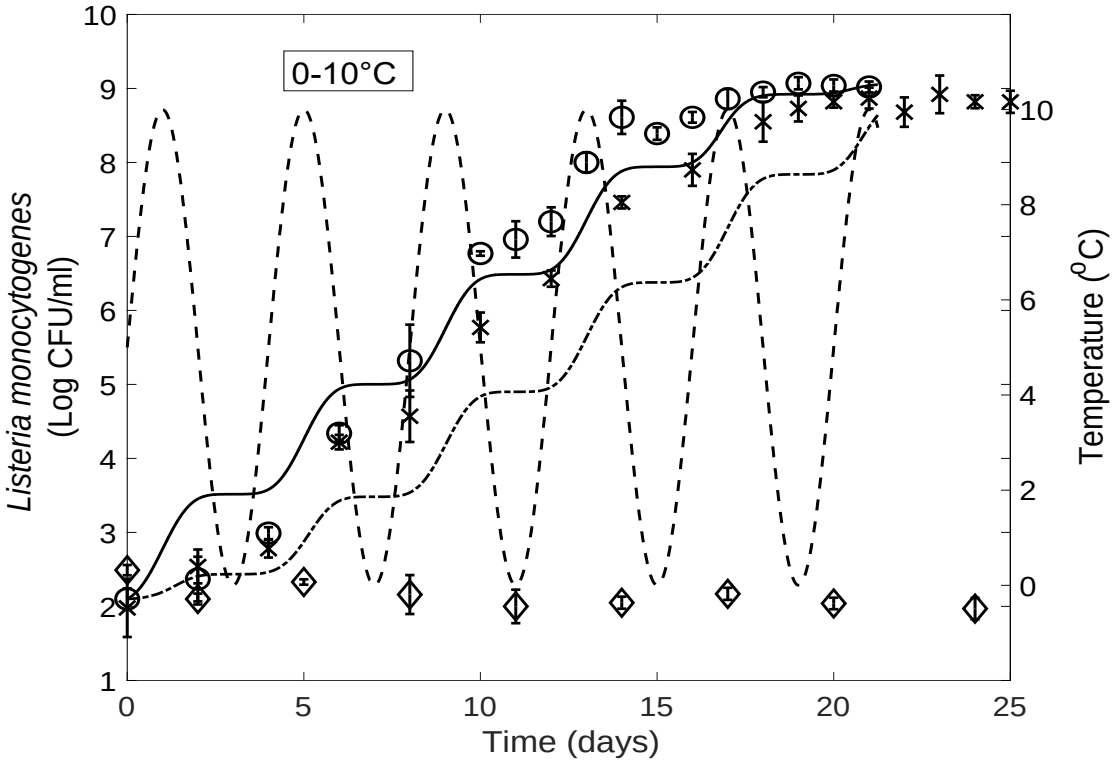

Figure 4.1. Validation of dynamic growth model of *L. monocytogenes* in egg yolk at low temperature profile.

——— : Prediction line at $h_o = 0.01$, ○: observed growth in plain egg yolk

– · – · · : Prediction line at h_o=2.46, ✕ : observed growth in sugared egg yolk

◊ : observed growth in salted egg yolk, ------: temperature curve

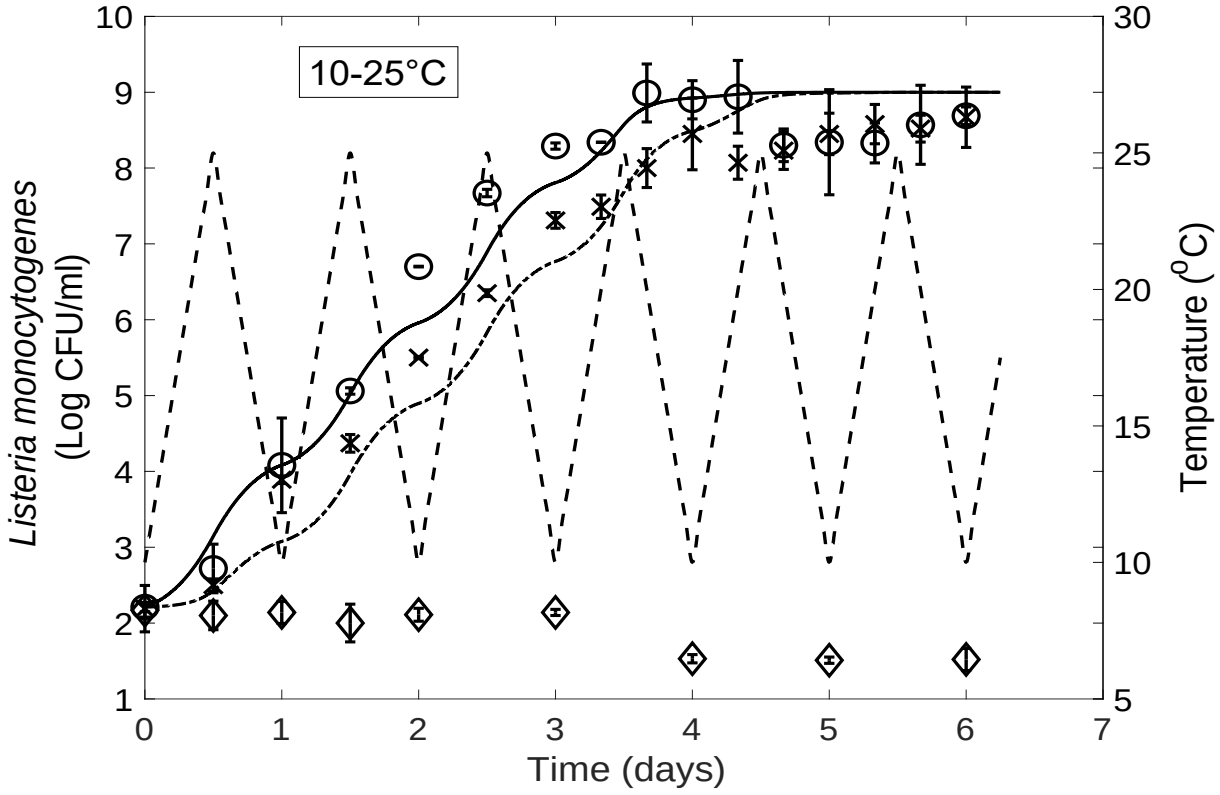

Figure 4.2. Validation of dynamic growth model of *L. monocytogenes* in egg yolk at ambient temperature profile.

—— : Prediction line at $h_o = 0.01$, ○: observed growth in plain egg yolk

- · - - · · : Prediction line at h_o=2.46, ✕ : observed growth in sugared egg yolk

◊ : observed growth in salted egg yolk, ------: temperature curve

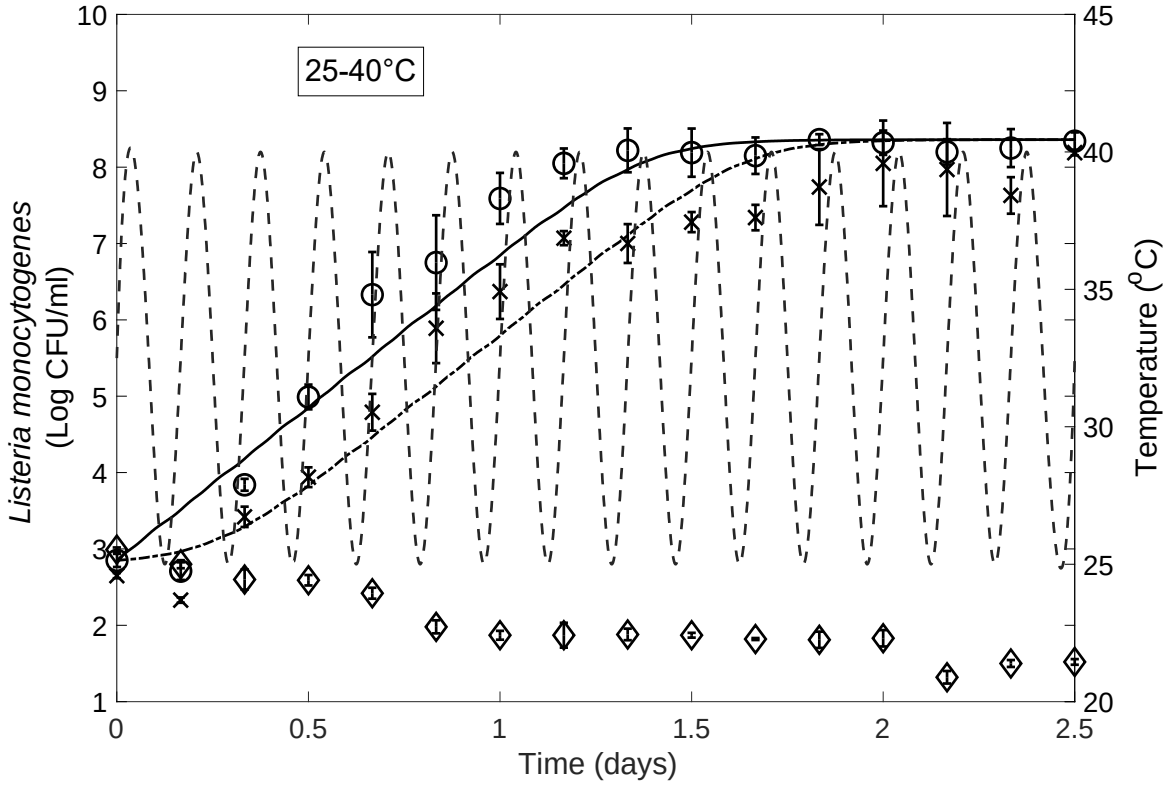

Figure 4.3. Validation of dynamic growth model of *L. monocytogenes* in egg yolk at high temperature profile.

——— : Prediction line at $h_o = 0.01$, ○: observed growth in plain egg yolk

– · – · · : Prediction line at h_o=2.46, ✕ : observed growth in sugared egg yolk

◊ : observed growth in salted egg yolk, ------: temperature curve

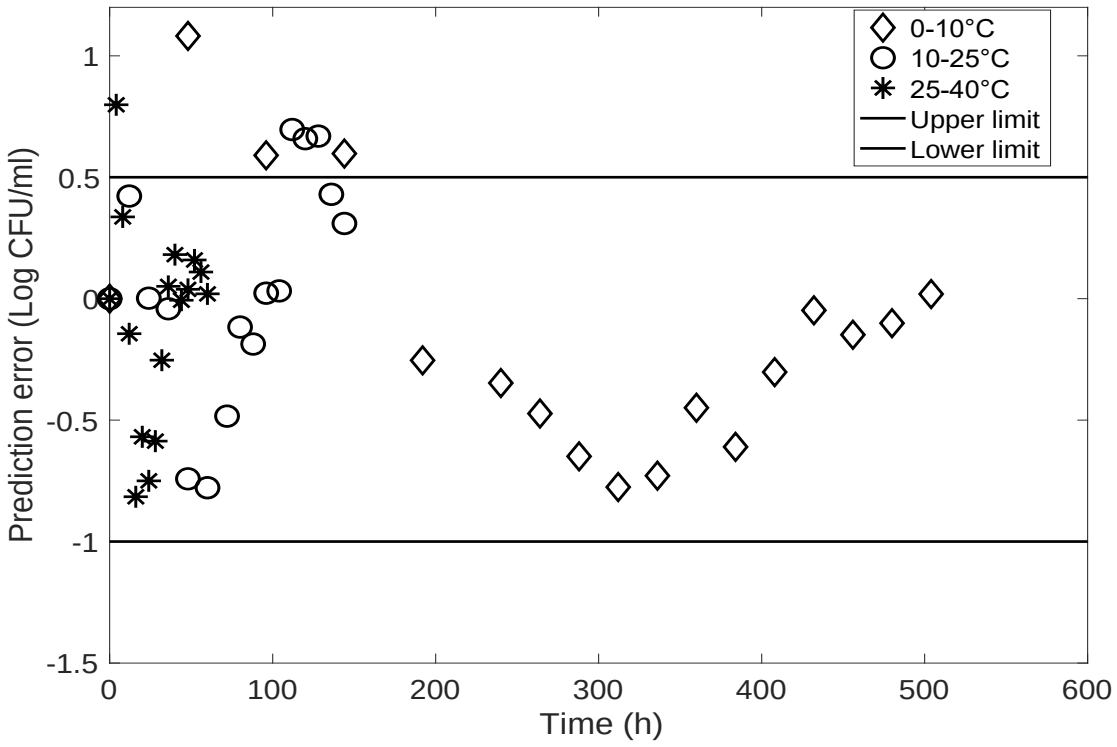

Figure 4.4. Acceptable prediction zone (APZ) analysis of prediction error for growth of *L. monocytogenes* in egg yolk for three different temperature profiles at $h_o = 0.01$.

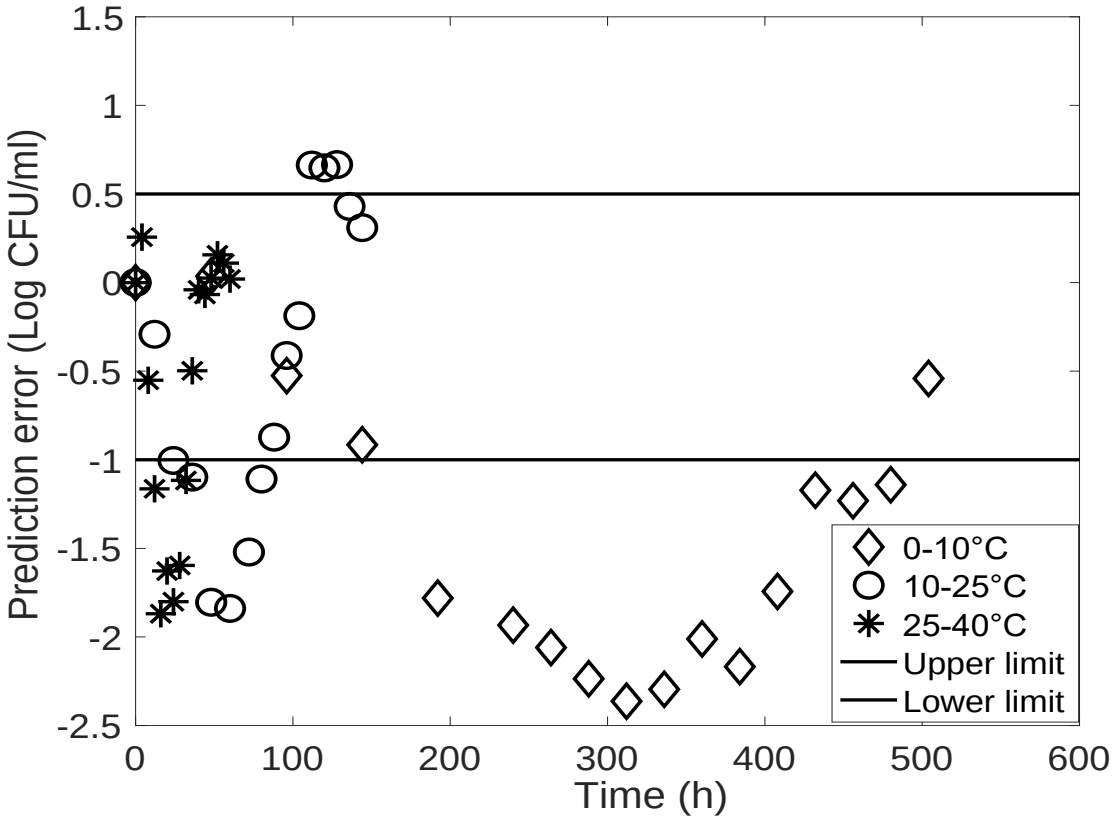

Figure 4.5. Acceptable prediction zone (APZ) analysis of prediction error for growth of *L. monocytogenes* in egg yolk for three different temperature profiles at $h_o = 2.46$.

Table 4.3. Accuracy factor (A_f) and Bias factor (B_f) values at three different temperature profiles.

Profile	Title	h_o=0.01	h_o=2.46
0-10ºC	A_f	1.08	1.25
	Bf	1.00	0.79
10-25ºC	A_f	1.05	1.13
	Bf	1.02	0.92
25-40ºC	A_f	1.05	1.12
	Bf	0.99	0.90

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CHAPTER 5

OVERALL CONCLUSIONS

In this study, the growth of *L. monocytogenes* in dynamic temperature conditions in egg yolk was modeled and validated. Based on the parameters set in this study the following was concluded:

1. An increase in maximum specific growth rate and a decrease in lag phase duration of *L. monocytogenes* in egg yolk was observed with an increase in temperature till the optimum temperature of growth was achieved.
2. The predictive models developed were acceptable with all goodness of fit parameters falling within acceptable ranges. These models can be used in predicting *L. monocytogenes* growth in egg yolk in case of temperature deviations in the supply chain.
3. The growth rates observed in this study highlight the importance of preventing any cross-contamination with *L. monocytogenes* as even at low initial populations and low temperatures, *L. monocytogenes* populations grew to the maximum population density.

CHAPTER 6

FUTURE RESEARCH

Further research can be conducted to observe the growth of *L. monocytogenes* in egg yolk supplemented with various shelf-life extending preservatives like potassium sorbates to further evaluate the safety of egg yolk against pathogen activity. Additionally, the effects of other non-pathogenic foodborne bacteria on the growth of *L. monocytogenes* could also be studied to determine if non-pathogenic foodborne bacteria compete with *L. monocytogenes* for nutrients and thus help diminish its growth. The general comparison between *L. monocytogenes* activity in salted, sugared, and plain egg yolk in validation profiles developed for plain egg yolk suggests further exploring separate dynamic modeling for these two products. This could aid in the successful implementation of those predictive models for egg processors.