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Research Paper

Growth of *Listeria monocytogenes* in Thawed Frozen Foods

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ABSTRACT

The growth characteristics of *Listeria monocytogenes* inoculated onto frozen foods (corn, green peas, crabmeat, and shrimp) and thawed by being stored at 4, 8, 12, and 20°C were investigated. The growth parameters, lag-phase duration (LPD) and exponential growth rate (EGR), were determined by using a two-phase linear growth model as a primary model and a square root model for EGR and a quadratic model for LPD as secondary models, based on the growth data. The EGR model predictions were compared with growth rates obtained from the USDA Pathogen Modeling Program, calculated with similar pH, salt percentage, and $NaNO_2$ parameters, at all storage temperatures. The results showed that *L. monocytogenes* grew well in all food types, with the growth rate increasing with storage temperature. Predicted EGRs for all food types demonstrated the significance of storage temperature and similar growth rates among four food types. The predicted EGRs showed slightly slower rate compared with the values from the U.S. Department of Agriculture Pathogen Modeling Program. LPD could not be accurately predicted, possibly because there were not enough sampling points. These data established by using real food samples demonstrated that *L. monocytogenes* can initiate growth without a prolonged lag phase even at refrigeration temperature (4°C), and the predictive models derived from this study can be useful for developing proper handling guidelines for thawed frozen foods during production and storage.

Key words: Frozen and thawed foods; Growth model; *Listeria monocytogenes*; Modeling; Ready-to-eat food

Listeria monocytogenes is a gram-positive, rod-shaped bacterium that causes the foodborne disease listeriosis in humans. Listeriosis can manifest as an invasive disease that can result in meningitis, pneumonia, septicemi, and death. Listerosis mainly affects the elderly, the immunocompromised *(20, 23, 27),* and pregnant women, who may develop flulike symptoms and experience miscarriage or stillbirth *(20, 23, 27)*. Although listeriosis is relatively rare, the mortality rate is high, and most patients are hospitalized. Scallan et al. *(33)* estimated that 1,600 cases of listeriosis occur annually in the United States, of which 250 cases are fatal. Although this pathogen is ubiquitous in the environment *(32),* it can be readily inactivated by pasteurization and cooking *(5)*. *L. monocytogenes* can grow at refrigeration temperatures, and refrigerated ready-to-eat foods that support the growth of *L. monocytogenes* have been associated with listeriosis outbreaks *(6, 14, 17, 20, 21, 40, 42)*. In the 1980s, the U.S. Food and Drug Administration and U.S. Department of Agriculture (USDA) Food Safety and Inspection Service established a "zero-tolerance" policy for *L. monocytogenes* in ready-to-eat foods *(35)*. Since then, several risk assessments have been conducted to better understand the risk of consuming food contaminated with *L. monocytogenes (14, 29, 39, 42)*.

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Freezing is an effective control to prevent the growth of pathogens, including *L. monocytogenes*. However, once a frozen food is thawed, it may be able to support the growth of *L. monocytogenes,* if present. Cooked and frozen shrimp and crabmeat, along with frozen green peas and corn, may be thawed and held refrigerated before consumption, and some consumers may eat them without cooking or reheating. Because *L. monocytogenes* can grow at refrigeration temperatures, holding these foods for extended periods may allow this pathogen to grow to levels that represent a public health concern. A survey of frozen vegetables conducted in Portugal showed that 14.8 to 22.6% of frozen vegetable samples were positive for *L. monocytogenes (24)*. Another investigation demonstrated that 26% of frozen seafood samples, including frozen cooked shrimp, cooked crabmeat, and raw seafood, were positive for *L. monocytogenes* overall *(43)*.

There is a gap in the knowledge concerning the growth kinetics of *L. monocytogenes* in frozen foods that are then thawed and held at refrigeration temperatures. The 2013 Food Code requires that foods that fall under the category of "time-temperature control for safety" be stored at <5°C for up to 7 days, based on limiting the growth of *L. monocytogenes* (to an increase of no more than 10-fold or 1 log) *(41)*. However, refrigerated temperature control can present a challenge in both retail and the consumer home setting. In a survey of product temperatures at retail locations, it was shown that 30.7% of products in retail

display and 9.4% of backroom refrigerators had temperatures higher than 5°C *(9)*. Cold salad bar settings may provide further opportunity for temperature abuse. For example, one study showed that the food surface of potato salad had temperatures of 13 to 16°C at salad bars, even though the units were set to the coldest setting *(37)*. The same study also showed food handling practices that might lead to prolonged display and storage of food items: for example, mixing fresh food into old batches of leftover food on the salad bar *(37)*. This study highlights the difficulties of controlling the food temperature in salad and food bar settings and in monitoring how long food products have been exposed to potential temperature abuse. Temperature abuse can also occur within the home; the abovementioned study showed that 16.8% of products tested within consumers' homes were stored at temperatures exceeding 5°C *(9)*.

The objective of this study was to investigate the growth kinetics of *L. monocytogenes* in thawed frozen foods (corn, green peas, crabmeat, and shrimp) stored at 4, 8, 12, or 20°C. The temperatures reflect recommended refrigeration temperature (4°C), elevated "abuse" refrigeration temperature (8 or 12°C), and room temperature (20°C). Furthermore, the growth curves derived were used to develop predictive models for the lag-phase duration (LPD) and the exponential growth rate (EGR) in those food types at 4 to 20°C. Knowledge of the length of the lag phase of this organism could provide more accurate handling guidance for frozen foods that are thawed and, subsequently, held at refrigeration temperatures.

MATERIALS AND METHODS

***L. monocytogenes* strains and culture conditions.** Twelve strains of *L. monocytogenes* from the Grocery Manufacturers Association culture collection (Washington, DC) were used in this study: N-7351 (1/2b, isolated from deli meat), N-7389 (1/2b, isolated from deli meat), N-7391 (1/2c, isolated from deli meat), N-7427 (4d, isolated from deli meat), N-7292 (4b, clinical isolate), N-7293 (4b, clinical isolate), N-7447 (1/2c, isolated from seafood salad), N-7497 (4b, isolated from seafood salad), N-7503 (1/2a, isolated from seafood salad), N-7601 (1/2b, isolated from seafood salad), N-7295 (4b, clinical isolate), and N-7296 (4b, clinical isolate). Working cultures were made from glycerol-frozen or lyophilized stocks stored in a −80°C freezer and maintained on tryptic soy agar (TSA; Difco, BD, Sparks, MD) with 0.6% yeast extract (YE; Difco, BD) slants at 4°C and transferred every 6 months. Before inoculation, a loopful of each strain was transferred in 10 mL of tryptic soy broth (TSB; Difco, BD) with 0.6% YE (TSB+0.6% YE) and grown aerobically at 35°C for 24 h (stationary-phase culture).

Preparation of inocula. One hundred microliters of each stationary-phase culture, approximately 10^9 CFU/mL, was transferred to an individual test tube containing 10 mL of sterile TSB+0.6% YE and incubated at 4°C for 7 days for cells to adapt to the cold *(32)*. After the 7-day incubation, each culture reached approximately 10^8 CFU/mL. All 12 strains of refrigeration temperature–adapted cultures were combined into a cocktail (2 mL of each culture) in a centrifuge tube. The cocktail, containing approximately 10^8 CFU/mL of *L. monocytogenes* cells, was serially diluted in 0.1% peptone water (pH 7.0; Fisher Scientific, Fair Lawn, NJ) to a desired inoculation level.

Source and inoculation of food. Four types of frozen food samples, blanched individually quick frozen corn, individually quick frozen green peas, cooked snow crabmeat, and cooked peeled shrimp, were obtained from a local grocery store and by mail order. Food samples were obtained frozen and held at −18°C prior to and during inoculation. Crabmeat from frozen cooked snow crab with shell was aseptically removed from shell as a part of sample preparation before the weighing process. Prior to each individual growth experiment, random samples from the four types of thawed frozen foods were tested for being *L. monocytogenes* negative by using VIDAS LMO2 (bioMérieux, Marcy l'Etoile, France). Aerobic plate counts were also performed with TSA plates incubated at 35°C for 48 h to obtain counts for background micoflora in each product, and the pH was determined by using a pH meter (Accumet Research AR 20, Fisher Scientific).

Test samples were weighed (25 g) into stomacher bags (Whirl-Pak, Nasco, Fort Atkinson, WI) while they were still frozen and inoculated with 100-μL aliquots of the culture cocktail that was distributed over the product surface by using a pipette. The initial inoculation level was approximately 10^3 CFU/g (confirmed immediately after the inoculation by plating, as described in the following). The inoculated product was stored frozen at −18°C for 7 days. Following frozen storage, the inoculated food samples were taken out of the freezer and transferred to air incubators set at 4, 8, 12, or 20°C. The initiation of the growth curves (time zero) was the time the food sample was transferred to 4, 8, 12, or 20°C (i.e., not the time the food sample reached temperature) as imitating consumer practices or practices potentially seen at food bars.

Enumeration of *L. monocytogenes*. At predetermined time intervals (established by preliminary experiments), samples were removed from incubation, and the samples were enumerated for *L. monocytogenes*. Samples were diluted 1:10 in buffered peptone water (3M, St. Paul, MN) and pulsified for 30 s (Pulsifier, Microgen Bioproducts, Ltd., Camberley, UK). If required, further decimal dilutions of samples were made with peptone water. The diluted samples were plated onto polymyxin–acriflavin–lithium chloride–ceftazidime–aesculin–mannitol agar (PALCAM; Difco, BD) by using a spiral plater (model AP 4000, Spiral Biotech, Norwood, MA). Preliminary experiments indicated that resuscitation steps for injured cells were not necessary. Plates were incubated for 48 h at 35°C. Cell counts were obtained by using a Q count system (model 510, Spiral Biotech). Three independent growth experiments, with one sample per replicate, were conducted for each food type at each storage temperature.

Curve-fitting and primary model. Data for each replicate were converted to log CFU per gram and iteratively fit to the two-phase linear growth equation *(4, 36)* to generate LPD and EGR by minimizing the residual sum of squares using the Solver function in Microsoft Excel, Version 1997 (Microsoft Corporation, Redmond, WA; worksheet provided by Dr. Richard Whiting [Exponent, Inc., Knoxville, TN]), in which an if–then statement defines the model:

$$N = N_0 + \mathrm{IF}[t < \mathrm{LPD}, N_0, \mathrm{EGR} \times (t - \mathrm{LPD})] \qquad (1)$$

where N is the log CFU/g at time t, N_0 is the initial log CFU/g, LPD is the lag-phase duration (h), EGR is the exponential growth rate ([log CFU/g]/h), and t is the elapsed time (h).

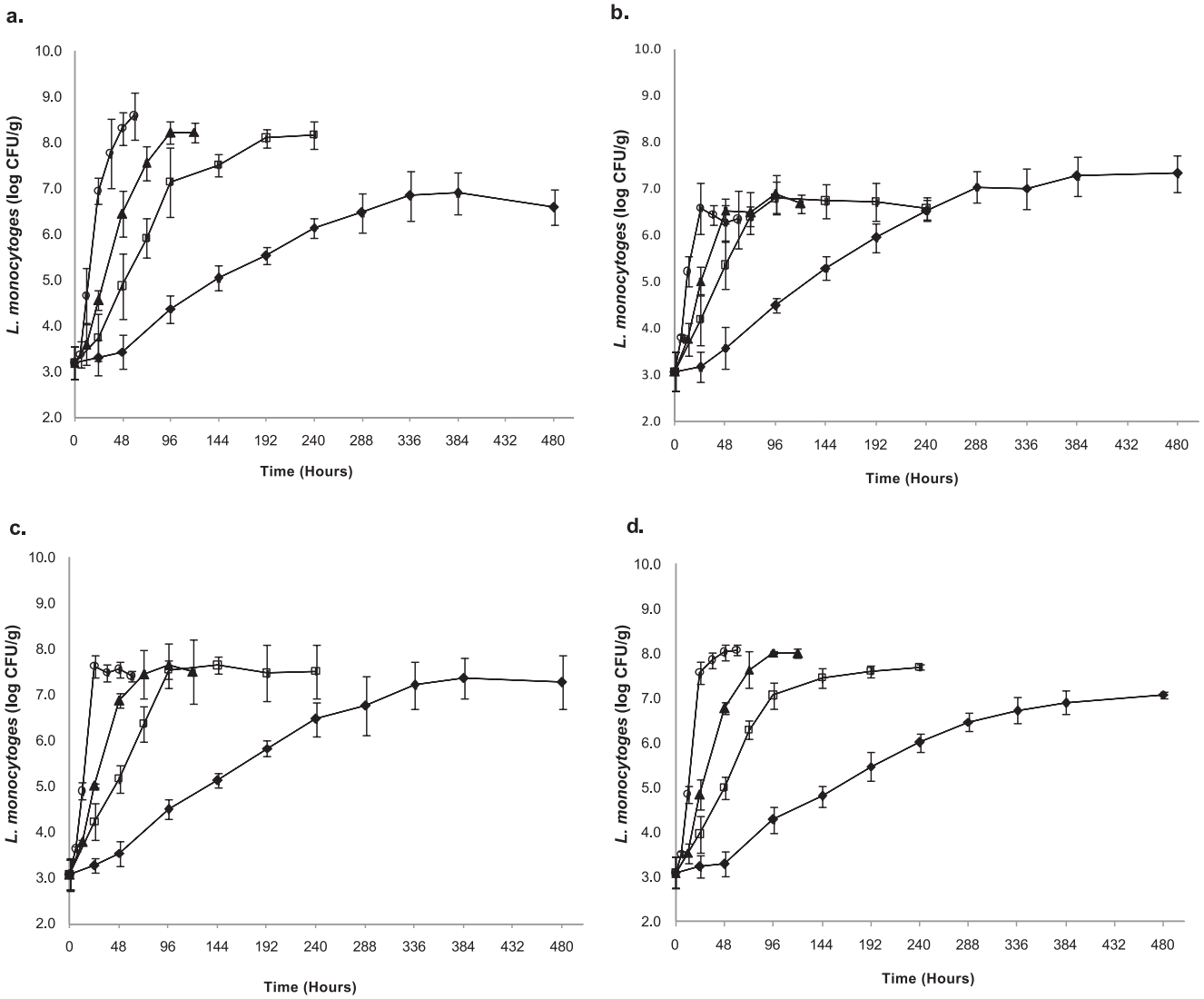

FIGURE 1. L. monocytogenes *growth curves of thawed frozen food samples, (a) crabmeat, (b) corn, (c) green peas, and (d) shrimp, performed in triplicate at four storage temperatures (♦, 4°C; □, 8°C; ▲, 12°C; ○, 20°C). The error bars indicate standard deviation.*

Secondary model for EGRs. To integrate the effect of storage temperature, EGRs were further calculated using data from the primary model (equation 1) with the square root model *(8, 30)*. The curve fitting was performed with the Excel Solver.

$$\sqrt{\mathrm{EGR}} = a(T - T_{\min}) \quad (2)$$

where a is the constant, T is the temperature, and $T_{\min}$ is the theoretical minimum temperature at growth that no growth is possible.

Secondary model for LPDs. To incorporate the effect of storage temperature on the LPD, the quadratic model was used to calculate LPD predictions *(31)*. LPDs were calculated by using data from the primary model (equation 1) with the quadratic model.

$$\mathrm{LPD} = p_1 + p_2T + p_3T^2 \quad (3)$$

$p_i = (i = 1, \ldots, 10)$ are coefficients to be estimated and T is the temperature.

Data analysis. The fit of models was evaluated by the residual mean squares (R^2) based on regression analysis *(15, 44)*. LPDs and EGRs derived from the secondary model were compared against a calculation on predictions made from the USDA Pathogen Modeling Program (PMP) *(38)* by using pH (7.0), NaCl (0.5%), and $NaNO_2$ (0%). These parameters were selected based on sample characteristics. For NaCl (percentage) and $NaNO_2$ (percentage), nutrient descriptions on the product label of each product were used.

RESULTS

Growth of *L. monocytogenes* in four types of thawed frozen foods. Frozen corn, green peas, crabmeat, and shrimp were obtained, and the pH values of the products were 7.2, 6.8, 7.2, and 7.5, respectively. Representative uninoculated samples were tested for *L. monocytogenes,* which was not detected. The products were inoculated with a cocktail of *L. monocytogenes* and held at −18°C for 7 days. Then, the inoculated samples were incubated at 4, 8, 12, or 20°C, and growth was monitored for up to 20 days.

L. monocytogenes grew to stationary phase in all products at all temperatures, as shown in Figure 1a through 1d. Growth of *L. monocytogenes* occurred much more

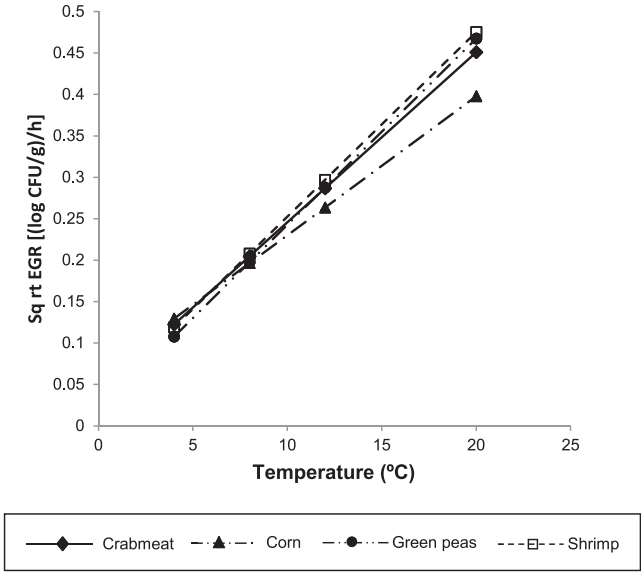

FIGURE 2. *Square root model predictions of the exponential growth rate (EGR; [log CFU per gram]/hour) calculated from the two-phase linear (primary) model for* L. monocytogenes *in four types of thawed frozen foods (crabmeat, corn, green peas, and shrimp) over the temperature range of 4 to 20°C.*

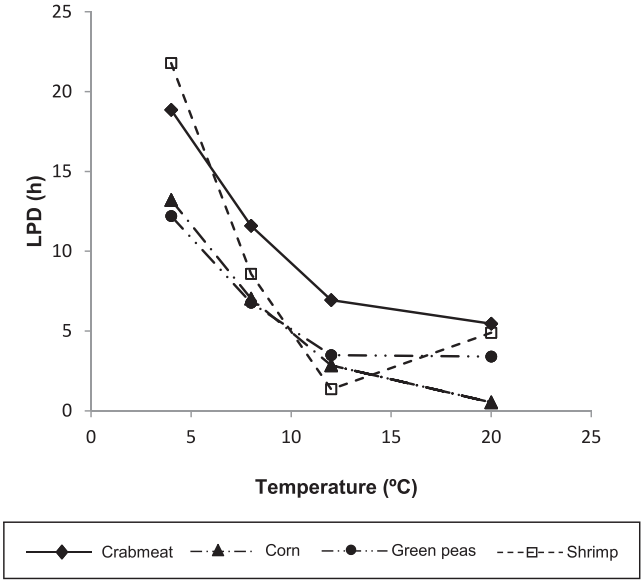

FIGURE 3. *Quadratic model predictions of the lag-phase duration (LPD; hours) calculated from the two-phase linear (primary) model for* L. monocytogenes *in four types of thawed frozen foods (crabmeat, corn, green peas, and shrimp) over the temperature range of 4 to 20°C.*

rapidly as the storage temperature increased for all foods. For example, *L. monocytogenes* reached stationary phase, when the growth curves appeared plateaued, after approximately 288 to 380 h in all four food types stored at 4°C, whereas the stationary phase was reached within 24 to 48 h, when samples were stored at 20°C. For all food types, the lag phase became shorter as the temperature increased. For instance, LPD was approximately 48 h for food samples stored at 4°C, whereas LPD was less than 12 h for food samples stored at 20°C. The final cell density was different in four types of foods. *L. monocytogenes* appeared to reach higher numbers in crabmeat and shrimp compared with corn and green peas overall, although statistical analysis was not conducted.

Calculation of LPD and EGR by using primary and secondary models. The LPD and EGR for *L. monocytogenes* in each food type at each storage temperature were generated by using two-phase linear models (equation 1), based on the log growth data of each replicate. Then, an EGR value was further calculated by using linear regression analysis of the square root model (equation 2), based on the outcome from equation 1, to incorporate the effect of storage temperature. The results revealed the relationship with storage temperature on EGRs of *L. monocytogenes,* with the square root of the EGRs becoming greater, indicating faster growth, as the growth temperature was increased for all food types (Fig. 2). The fit of the secondary model was good ($R^2 > 0.98$), as shown in Table 1.

For LPD, the quadratic model was used as the secondary model. Storage temperature had an impact on LPD, which generally decreased as the temperature increased in all food types (Fig. 3). However, the fit of the model was not ideal; R^2 values ranged from 0.23 to 0.71 (Table 1), due to high variability among replicates, indicating the model does not describe the lag phenomenon in these food samples precisely. Still, the model may be able to describe average LPD. The shape of the curve for shrimp was concave, with increasing the predicted LPD at 20°C, and high variability among replicates at 20°C.

Development of predictive models for *L. monocytogenes* in thawed frozen foods. A linear regression equation derived from the regression analysis of equation 2 or 3 for

TABLE 1. *Residual mean square (R^2) values and formulas for each thawed frozen food sample (crabmeat, corn, green peas, and shrimp), based on linear regression analysis for the predicted LPDs and square root of EGRs from square root models and actual* L. monocytogenes *growth data*

	LPD		Square root of EGR	
	R^2	Linear regression equation	R^2	Linear regression equation
Crabmeat	0.3748	$LPD = 28.73 - 2.792temp + 0.0814temp^2$	0.9839	$\sqrt{EGR} = 0.0205temp + 0.0403$
Corn	0.7117	$LPD = 21.41 - 2.300temp + 0.0628temp^2$	0.9816	$\sqrt{EGR} = 0.0168temp + 0.0623$
Green peas	0.3921	$LPD = 19.77 - 2.164temp + 0.0673temp^2$	0.9812	$\sqrt{EGR} = 0.0225temp + 0.0178$
Shrimp	0.2369	$LPD = 41.00 - 5.550temp + 0.1872temp^2$	0.9945	$\sqrt{EGR} = 0.0223temp + 0.0296$

TABLE 2. *LPD and EGR predictions for* L. monocytogenes *in each thawed frozen food calculated from the predictive models as compared with USDA PMP predictions calculated by using parameters similar to food samples*[a]

	Predicted LPD (h)				Predicted EGR ([log CFU/g]/h)			
	4°C	8°C	12°C	20°C	4°C	8°C	12°C	20°C
Crabmeat	18.86	11.60	6.94	5.45	0.015	0.042	0.082	0.203
Corn	13.21	7.03	2.85	0.53	0.017	0.039	0.070	0.159
Green peas	12.20	6.77	3.49	3.40	0.012	0.039	0.083	0.219
Shrimp	21.79	8.58	1.36	4.89	0.014	0.043	0.088	0.226
USDA PMP	62.03	32.00	17.56	6.35	0.027	0.056	0.107	0.303

[a] Aerobic, broth culture, pH (7.0), NaCl (0.5%), and $NaNO_2$ (0%).

each type of food is presented in Table 1. The aim of these equations is to predict EGRs or LPDs of *L. monocytogenes* in each type of the thawed frozen foods over the temperature range of 4 to 20°C. However, the LPD models were not reliable, which will be discussed subsequently.

Predicted EGRs and LPDs and comparison with PMP predictions. Predicted values of EGRs and LPDs for each food type from 4 to 20°C were calculated by using equations (established models: Table 1). A higher EGR value means a faster growth rate. Predicted EGR values demonstrated similar trends for all food types, with EGR increasing as storage temperature rose. The EGR values were slightly higher in crabmeat and shrimp than in green peas and corn. The predicted LPD values showed that a trend in which shorter LPDs were observed on the vegetables than the seafood, especially at lower temperatures (4 or 8°C). For example, thawed frozen vegetables had a predicted LPD of less than 13.2 h, and thawed frozen seafood had a predicted LPD of about 18 to 21 h at 4°C. These predicted LPDs and EGRs values were compared with the growth rates and lag phase derived from the USDA PMP (Table 2). At all four temperatures, the PMP predicted more rapid EGRs than were determined in the current study. However, the PMP predicted longer LPDs than those LPDs derived in this study.

Aerobic plate counts of uninoculated food samples. Corn and green pea samples had higher initial aerobic plate counts (time zero), with 4.05 and 2.87 log CFU/g, respectively, while the crabmeat and shrimp began with 1.65 and 2.18 log CFU/g, respectively.

DISCUSSION

Thawed frozen corn, green peas, crabmeat, and shrimp supported the growth of *L. monocytogenes* at each incubation temperature (4, 8, 12, or 20°C). The lag phase at 4°C was shorter than PMP predictions; however, the growth rates were consistent with PMP predictions and results in other publications. For example, Farber *(13)* showed 2- to 3-log growth of *L. monocytogenes* in 7 days in cooked shrimp and crabmeat incubated at 4°C; in the current study, a 2-log increase was seen in the same time frame. Hughey et al. *(18)* demonstrated 2 log of growth of *L. monocytogenes* in fresh corn and green beans in 9 days, which appears to be slightly less than the observation from the current study (i.e., approximately 3 log in 9 days in corn or green pea samples). These studies and our own results indicate that *L. monocytogenes* grows more rapidly in seafood products than in vegetables overall. There have been various studies on developing predictive models for growth of *L. monocytogenes* in laboratory media or foods *(7, 19, 22, 44),* and predictive models for growth of the organism in smoked salmon have been investigated extensively, as explored in Giménez and Dalgaard *(16)*. However, there is no study on a development of predictive models for the growth of this organism in thawed frozen foods. In the current study, growth parameters, LPDs and EGRs, of *L. monocytogenes* in thawed frozen foods were first calculated by using a two-phase linear model as a primary model based on experimental growth data. The two-phase model is a modified version of the three-phase linear model and allows the calculation of LPD and EGR without data from the stationary phase *(4)*. Several studies have been published using the two-phase linear model *(4, 10, 11, 25, 26)*. Secondary models were applied to incorporate the effect of storage temperature to EGRs and LPDs. In the current study, the square root model and quadratic model were used for prediction of EGR and LPD, respectively, based on the data obtained from a primary model. These models are simple and expandable to incorporate other factors and have been used in many published studies, as discussed in Ross and Dalgaard *(31)*.

The goodness of fit for EGR predictions was high based on R^2 values; hence, the equations derived from the regression analysis can be used to predict EGRs of *L. monocytogenes* between 4 to 20°C for those samples. On the contrary, the fit of model for LPDs was not ideal. Several models were used to fit data to predict LPDs, such as square root model and reciprocal model. None of the models provided an ideal fit. The quadratic model is one of empirical models, describing a set of data from experiments in a simple mathematical correlation *(31)*. However, the equations derived for predicting LPDs in this study are not adequate and are not reliable to predict precise LPD. Therefore, they should not be used to predict LPD.

A possible reason for the undesirable fit and outcome could be that not enough data points were collected during the growth experiment, particularly during the lag phase. This resulted in "no lag time (0 h)" calculation at the primary model step for some samples and caused high variability among replicates. In comparison to the development of growth rate models, creating lag time models

that estimate accurate lag phases are more difficult because the lag phenomenon is still not clearly understood *(1)*. There are many factors influencing lag behavior such as (i) adaptation mechanisms to a new environment, (ii) character and phenotype of the bacterium, (iii) physiological state of cells, (iv) physiological history of the cells, (v) inoculum size, or (vi) distribution condition in samples *(36)*. Therefore, whichever model is used, it is important to consider that models can only describe the simplified form of real phenomena and the imprecision of lag-time predictions *(1)*.

Despite being unable to adequately model the LPD, the growth curves showed that the lag phase was relatively short at each temperature, considering that the thawing process was included. Before conducting the experiment, it was hypothesized that freezing of the cultures may create an extended lag phase for this organism once the foods were thawed and held at refrigeration temperatures. However, this was not observed in the results. The short lag phases may indicate that there was no obvious effect of freezing and thawing to initiate growth of *L. monocytogenes* in thawed frozen foods incubated at 4 to 20°C. *L. monocytogenes* is known to be resistant to injury due to freezing in food and broth systems *(12, 28)*. Beauchamp et al. *(2)* also found that various methods of thawing frozen hot dogs had little effect on survival and growth of *L. monocytogenes* during refrigerated storage. Furthermore, the short lag phase observed here may have been due to the use of inocula that were acclimated to refrigeration temperatures by growing to stationary phase at 4°C prior to freezing in the food. Usage of environment-acclimated organisms when conducting laboratory challenge studies is recommended because those organisms may better replicate a real-world scenario *(34)*.

The predicted EGRs were compared with those from the USDA PMP. The values were in the same order of magnitude, but the PMP did produce slightly higher (i.e., rapid) EGR values. One reason why the PMP predictions were higher may be because the current study used actual food samples as growth media, which may be less supportive in nutrient composition for growth of this organism compared with laboratory broth media used to build the PMP predictions. Furthermore, the competing effect of background microflora is not incorporated in PMP predictions. Several researchers investigated inhibitory effect of spoilage organisms against *L. monocytogenes*. Buchanan and Bagi *(3)* demonstrated that growth of *L. monocytogenes* was inhibited due to coinoculation with *Pseudomonas fluorescens* in brain heart infusion broth with sodium chloride (5 and 25 g/L) at 4°C. In a study by Giménez and Dalgaard *(16)*, growth of *L. monocytogenes* was inhibited due to a cocktail of spoilage organisms (lactic acid bacteria, *Enterobacteriaceae,* and *Photobacterium phosphoreum*) in vacuum-packaged cold-smoked salmon slices at 5, 10, 17.5, or 25°C. General prediction models established, based on laboratory conditions (i.e., broth culture), may display predictions different from predictive models derived from data in real food having a complex matrix with competing microflora *(34)*.

The data generated in this study show that thawed frozen corn, green peas, crabmeat, and shrimp support the growth of *L. monocytogenes* in the temperature range of 4 to 20°C. Under the current experimental conditions, there was a relatively short lag phase, especially at the three higher temperatures (8, 12, and 20°C). Creating growth curves and subsequent predictive growth models of *L. monocytogenes* in these foods over a wide range of temperatures could aid in the development of specific handling and holding guidelines for the foods after thawing. Conducting additional research to obtain more data to develop predictive models for LPD would be highly desirable. Investigations of the prevalence and contamination level of *L. monocytogenes* in certain frozen foods could assist the industry to improve food safety and provide a better indication of the risk to public health.

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