CPG Sec. 555.320 Listeria monocytogenes

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Compliance Policy Guide
Guidance for FDA Staff
Sec. 555.320
Listeria monocytogenes

DRAFT GUIDANCE

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For questions regarding this draft document, contact the Center for Food Safety and Applied Nutrition (CFSAN) at 301-436-1400.

U.S. Department of Health and Human Services
Food and Drug Administration
Center for Food Safety and Applied Nutrition
Office of Regulatory Affairs
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I. INTRODUCTION:

The purpose of this Compliance Policy Guide is to provide guidance to FDA Staff on FDA's enforcement policy for *Listeria monocytogenes* (*L. monocytogenes*) in foods.

FDA's guidance documents, including this guidance, do not establish legally enforceable responsibilities. Instead, guidances describe the Agency's current thinking on a topic and should be viewed only as recommendations, unless specific regulatory or statutory requirements are cited. The use of the word *should* in Agency guidances means that something is suggested or recommended, but not required.

II. BACKGROUND:

L. monocytogenes is a pathogenic bacterium that is widespread in the environment and may be introduced into a food processing facility. *L. monocytogenes* can contaminate foods and cause a mild illness (called listerial gastroenteritis) or a severe, sometimes life-threatening, illness (called invasive listeriosis). Foods that have been implicated in outbreaks of invasive listeriosis have been foods that are ready-to-eat (RTE).

RTE foods can be contaminated if ingredients in the foods are contaminated with *L. monocytogenes* and are not treated to destroy viable cells of this pathogen, or if *L. monocytogenes* is allowed to contaminate the RTE food because of improper sanitary conditions or practices. Most RTE foods do not contain detectable numbers of *L. monocytogenes*. For many RTE foods, contamination with *L. monocytogenes* can be avoided – *e.g.*, through the application of current good manufacturing practice requirements that establish controls on ingredients, listericidal processes, segregation of foods that have been cooked from those that have not, and sanitation. Sanitation controls include effective environmental monitoring programs designed to identify and eliminate *L. monocytogenes* in and on surfaces and areas in the plant.

In 2003, FDA and the Food Safety and Inspection Service of the United States Department of Agriculture, in consultation with the Centers for Disease Control and Prevention of the United States Department of Health and Human Services, released a quantitative assessment (the Risk Assessment) of relative risk associated with consumption of certain categories of RTE foods that had a history of contamination with *L. monocytogenes*, or that were implicated epidemiologically with an outbreak or a sporadic case of listeriosis. The Risk Assessment estimated that the risk of listeriosis would vary widely among these food categories.

According to the Risk Assessment, foods estimated to pose the highest risk of being associated with listeriosis are RTE foods that support the growth of *L. monocytogenes*. Examples of RTE foods that support the growth of *L. monocytogenes* include:

- Milk;
- High fat and other dairy products (*e.g.*, butter and cream);
- Soft unripened cheeses (greater than 50 percent moisture) (*e.g.*, cottage cheese and ricotta cheese);
- Cooked crustaceans (*e.g.*, shrimp and crab);
- Smoked seafood (*e.g.*, smoked finfish and mollusks);
- Raw seafood that will be consumed as sushi or sashimi;
- Many vegetables (such as broccoli, cabbage, and salad greens);
- Non-acidic fruit (such as melon, watermelon, and papaya); and
- Some deli-type salads and sandwiches (particularly those containing seafood and those prepared at retail establishments without acidification and/or the addition of antimicrobial substances).

In contrast, the foods estimated to pose the lowest risk of being associated with listeriosis are foods that, because of intrinsic factors, extrinsic factors, and/or processing factors do not support the growth of *L. monocytogenes*. Intrinsic factors include chemical and physical factors that are normally within the structure of the food, *e.g.*, pH and water activity. Extrinsic factors are those that refer to the environment surrounding the food, e.g., storage temperature. Processing factors include substances added to adjust the pH of food (e.g., acids) and substances that, alone or in combination with other substances, have antimicrobial properties (e.g., sorbates and benzoates). It is well established that *L. monocytogenes* does not grow when:

- The pH of the food is less than or equal to 4.4;
- The water activity of the food is less than or equal to 0.92; or
- The food is frozen.

Foods may naturally have a pH or water activity that prevents growth of *L. monocytogenes* or processing factors may be deliberately used to achieve those characteristics (*e.g.*, by adding acid to deli-type salads to bring the pH to less than or equal to 4.4). At pH values above 4.4, processing factors generally are used in combination to prevent the growth of *L. monocytogenes* (e.g., sorbates or benzoates may be used in combination with organic acids such as acetic acid, lactic acid, and citric acid in foods such as deli-type salads). The effectiveness of a particular listeristatic control measure in preventing growth in a particular RTE food generally is determined case-by-case, for example, using the results of growth studies specific to the food matrix.

Examples of RTE foods that generally are considered to not support the growth of *L. monocytogenes* include:

- Fish that are preserved by techniques such as drying, pickling, and marinating;
- Ice cream and other frozen dairy products;
- Processed cheese (*e.g.*, cheese foods, spreads, slices);
- Cultured milk products (*e.g.*, yogurt, sour cream, buttermilk);
- Hard cheeses (less than 39 percent moisture) (*e.g.*, cheddar, colby, and parmesan);
- Some deli-type salads, particularly those processed to a pH less than 4.4 and those containing antimicrobial substances such as sorbic acid/sorbates or benzoic acid/benzoates under conditions of use documented to be effective in preventing the growth of *L. monocytogenes*;
- Some vegetables (such as carrots); and

- Crackers, dry breakfast cereals, and other dry foods.

Fruits, vegetables, and cheeses (e.g., soft and semi-soft cheeses) not listed in this CPG may include some products that support growth as well as other products that do not support growth.

III. **POLICY:**

FDA will review the available evidence on a case-by-case basis to determine if a food is a RTE food that supports growth or a RTE food that does not support growth.

A. **Ready-to-Eat Food**

"Ready-to-eat food" (RTE food) means a food that is customarily consumed without cooking by the consumer, or that reasonably appears to be suitable for consumption without cooking by the consumer.

A food may be considered to be suitable for consumption without cooking by the consumer, and thus a RTE food, even though cooking instructions are provided on the label. For examples, fresh and frozen crabmeat and individually quick frozen (IQF) peas and corn may be RTE foods. Some consumers eat such products without cooking, because they appear to be ready-to-eat.

B. **Ready-to-Eat Foods that Support Growth of *L. monocytogenes***

Generally, we intend to consider that a RTE food will support the growth of *L. monocytogenes* if it does not meet the characteristics of a RTE food that does not support growth, as indicated in section III.C.

FDA may regard a RTE food that supports growth of *L. monocytogenes* to be adulterated within the meaning of section 402(a)(1) of the Federal Food, Drug, and Cosmetic Act (the Act; the FD&C Act) (21 U.S.C. 342(a)(1)) when *L. monocytogenes* is present in the food based on the detection method indicated in section IV.A.

C. **Ready-to-Eat Foods that Do Not Support Growth of *L. monocytogenes***

A RTE food does not support the growth of *L. monocytogenes* if the food:

- Has a pH that is less than or equal to 4.4; or
- Is customarily held and consumed in a frozen state; or
- Has a water activity that is less than 0.92; or
- Is processed using an effective listeristatic control measure (e.g., an antimicrobial substance or a combination of factors such as pH, water activity, and antimicrobial substances).

FDA may regard a RTE food that does not support the growth of *L. monocytogenes* to be adulterated within the meaning of section 402(a)(1) of the Act (21 U.S.C. 342(a)(1)) when *L. monocytogenes* is present at or above 100 colony forming units per gram of food (cfu/g)

IV. **REGULATORY ACTION GUIDANCE:**

A. **Ready-to-Eat Foods that Support Growth of *L. monocytogenes***

The following represents criteria for recommending legal action to CFSAN/Office of Compliance/Division of Enforcement (HFS-605):

- *L. monocytogenes* is detected in one or more subsamples of a RTE food that supports the growth of *L. monocytogenes*.

Use Bacteriological Analytical Manual Online, Chapter 10 - "*Listeria monocytogenes*," "Detection and Enumeration of *Listeria monocytogenes* in Foods" as the method for detecting and confirming presence of *L. monocytogenes* (available at **http://www.cfsan.fda.gov/~ebam/bam-10.html (http://www.cfsan.fda.gov/~ebam/bam-10.html)**).

B. **Ready-to-Eat Foods that Do Not Support Growth of *L. monocytogenes***

Consult with CFSAN/Office of Compliance/Division of Enforcement (HFS-605) before recommending legal action for RTE foods that do not support the growth of *L. monocytogenes*. Use ISO 11290-2:1998(E) "Microbiology of food and animal feeding stuffs - Horizontal method for the detection and enumeration of *Listeria monocytogenes* - Part 2: Enumeration method" as the method for enumerating *L. monocytogenes*. (ISO 11290-2:1998/Amd. 1:2004(E) "Microbiology of food and animal feeding stuffs - Horizontal method for the detection and enumeration of *Listeria monocytogenes* - Part 2: Enumeration method AMENDMENT 1: Modification of the enumeration medium" amends ISO 11290-2:1998(E). The amendment uses ALOA agar instead of PALCAM agar. If ALOA agar is not commercially available in the United States, use PALCAM according to ISO 11290-2:1998(E)). ISO methods are available from the International Organization for Standardization at **http://www.iso.org/iso/en/ISOOnline.frontpage (http://www.iso.org/iso/en/ISOOnline.frontpage)**.

Use rapid biochemical test kits according to the Bacteriological Analytical Manual Online, Chapter 10 – "Detection and Enumeration of *Listeria monocytogenes* in Foods" Section E-11 (available at **http://www.cfsan.fda.gov/~ebam/bam-10.html (http://www.cfsan.fda.gov/~ebam/bam-10.html)**), instead of ISO 11290-2:1998(E) Section 9.5, for confirmation of *L. monocytogenes* isolates.

C. **Foods that are Not RTE Foods**

Consult with CFSAN/Office of Compliance/Division of Enforcement (HFS-605) when *L. monocytogenes* is present in a food that is not a RTE food.

D. **Other Considerations**

The criteria in this guidance do not establish an acceptable level of *L. monocytogenes* in food. FDA may choose to take legal action against adulterated food that does not meet the criteria for recommending legal action to CFSAN.

Further, the criteria in this guidance do not excuse violations of the requirement in section 402(a)(4) of the Act (21 U.S.C. 342(a)(4)) that food may not be prepared, packed, or held under insanitary conditions or the requirements in FDA's good manufacturing practices regulation (21 CFR part 110). As set out in 21 CFR 110.80, food manufacturers must take "[a]ll reasonable precautions … to ensure that production procedures do not contribute contamination from any source."

V. **SPECIMEN CHARGES:**

A. **Domestic Seizure**

The article of food was adulterated when introduced into and while in interstate commerce and is adulterated while held for sale after shipment in interstate commerce within the meaning of the Act, 21 U.S.C. 342(a)(1), in that it bears and contains a poisonous or deleterious substance, namely *Listeria monocytogenes*, which may render it injurious to health.

B. **Import Detention**

The article of food is subject to refusal of admission pursuant to section 801(a)(3) of the FD&C Act in that it appears to be adulterated within the meaning of section 402(a)(1) of the FD&C Act in that it bears and contains a poisonous or deleterious substance, *Listeria monocytogenes*, which may render it injurious to health.

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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).

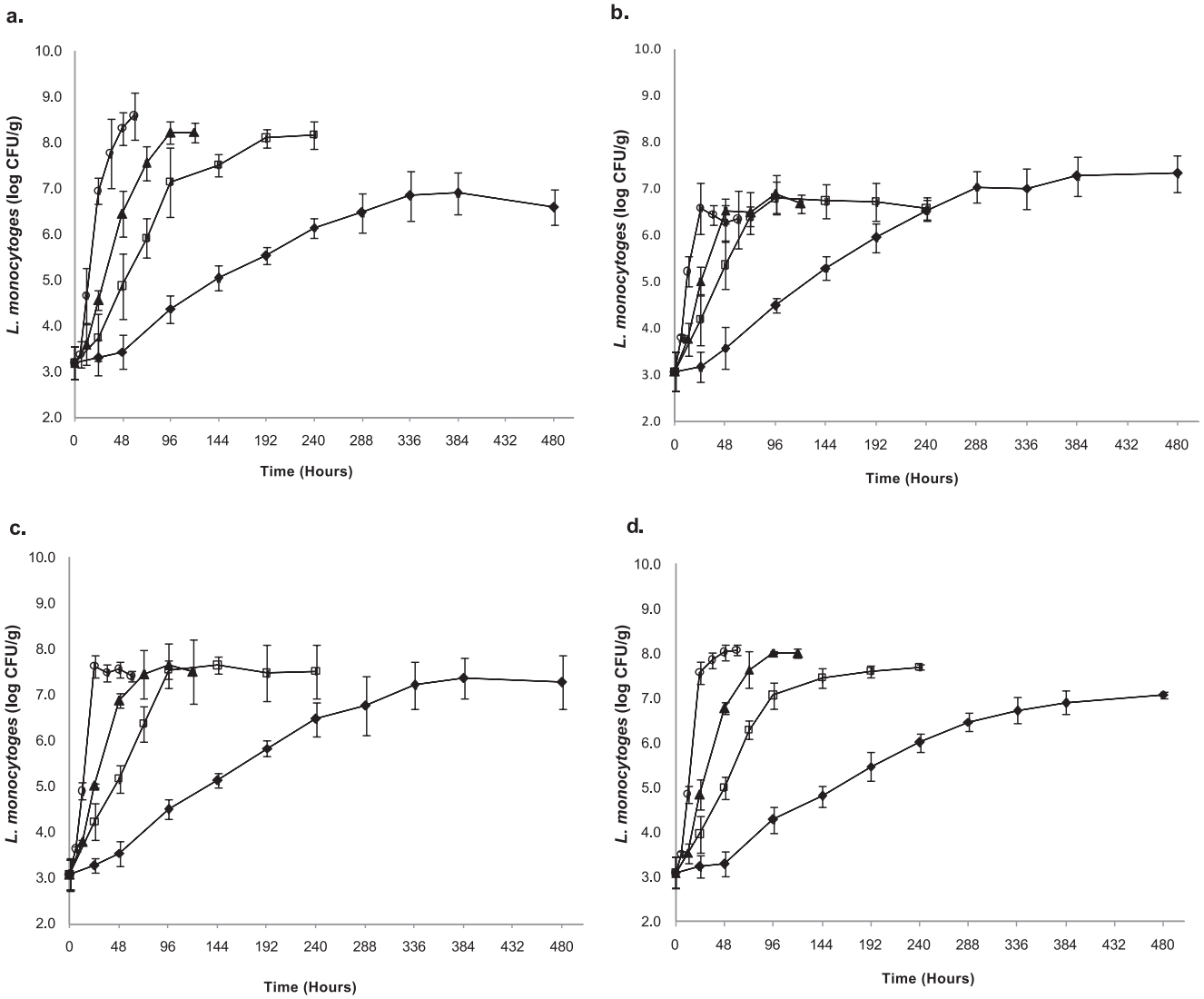

FIGURE 1. L. monocytogenes *growth curves of thawed frozen food samples, (a) crabmeat, (b) corn, (c) green peas, and (d) shrimp, performed in triplicate at four storage temperatures (♦, 4°C; □, 8°C; ▲, 12°C; ○, 20°C). The error bars indicate standard deviation.*

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

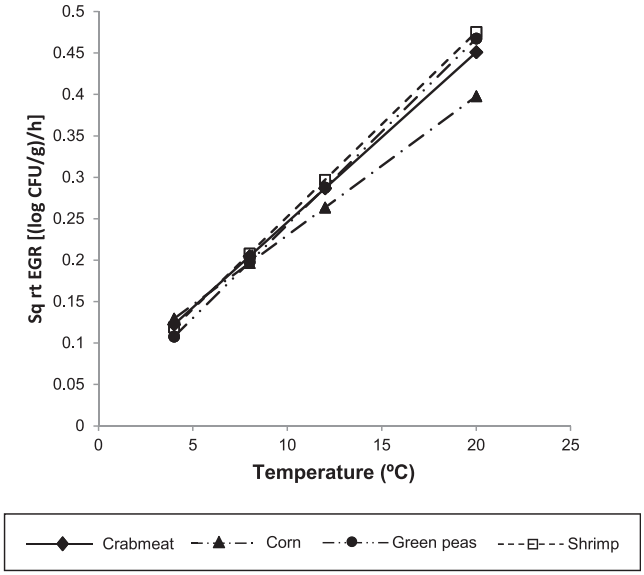

FIGURE 2. *Square root model predictions of the exponential growth rate (EGR; [log CFU per gram]/hour) calculated from the two-phase linear (primary) model for* L. monocytogenes *in four types of thawed frozen foods (crabmeat, corn, green peas, and shrimp) over the temperature range of 4 to 20°C.*

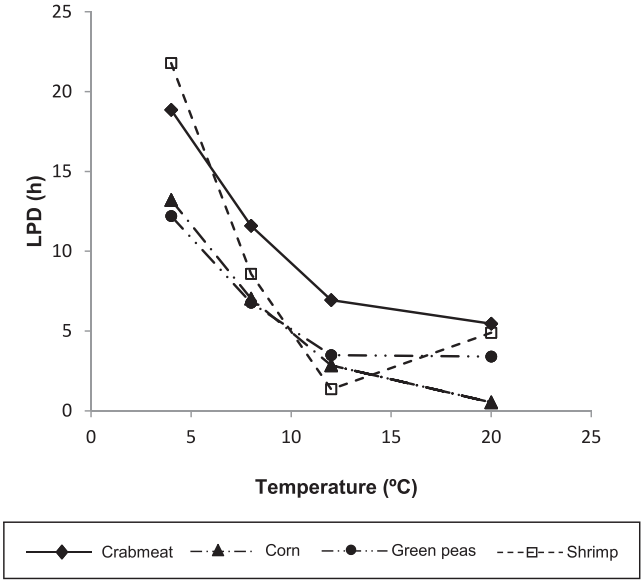

FIGURE 3. *Quadratic model predictions of the lag-phase duration (LPD; hours) calculated from the two-phase linear (primary) model for* L. monocytogenes *in four types of thawed frozen foods (crabmeat, corn, green peas, and shrimp) over the temperature range of 4 to 20°C.*

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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A predictive microbiology approach for thermal inactivation of Hepatitis A virus in acidified berries

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ABSTRACT

Hepatitis A virus (HAV) is a food-borne enteric virus responsible for outbreaks of hepatitis associated with consumption of raw vegetables. Soft fruits, such as red berries, exposed to faecal contamination are increasingly responsible for collective food-borne illnesses associated with HAV, when eaten raw or used in unprocessed foods. Heat is the most effective measure for the inactivation of HAV. Thermal treatments are used on fruits as a decontamination method, but they have to be adapted to product characteristics; indeed, factors such as sugar or pH may have an impact on the viral sensitivity to thermal treatments. A model was developed for the inactivation of HAV in red berries without supplemented sugar and with different pH values. Nonlinear inactivation curves in acidified raspberries were modelled using an integrated model, with a single equation nesting secondary models of temperature and pH in the primary model. Model predictions were then confronted to experimental results obtained in another laboratory on other berries with different pH values. Excellent predictions were obtained in most cases, while failed predictions provided safe results, with the model predicting higher residual virus titres than what was observed.

1. Introduction

Hepatitis A virus (HAV) is an enteric virus, mainly transmitted via faecal–oral route, either through person-to-person contact or by contaminated water and food. A large percentage of food-borne outbreaks are caused by HAV and numerous epidemiological studies have particularly associated viral hepatitis with the consumption of raw vegetables (Kuritsky et al., 1985; Rosenblum et al., 1990; Warner et al., 1991; Niu et al., 1992; Hernandez et al., 1997; Gaulin et al., 1999; Nygard et al., 2001; Long et al., 2002; Le Guyader et al., 2004). Indeed, soft fruits and vegetables may be exposed to faecal contamination during irrigation with contaminated water, fertilization with inadequately composed manure, or handling by infected persons with poor hygiene (Ward et al., 1982; Niu et al., 1992; Deng and Cliver, 1995; Bidawid et al., 2000a; Calder et al., 2003; Koopmans et al., 2003; Todd et al., 2007). Although raw vegetables are usually consumed after a washing step, pathogenic bacteria, parasites and enteric viruses can survive this minimal treatment (Beuchat et al., 1998; Gulati et al., 2001; Butot et al., 2008). Frozen raspberries, strawberries and blueberries are being recognized as vehicles for hepatitis A virus and responsible for collective food-borne toxi-infections, when used in unprocessed foods (Noah, 1981; Reid and Robinson, 1987; Ramsay and Upton, 1989; Niu et al., 1992; Hutin et al., 1999; Calder et al., 2003). The potential of gamma irradiation and sanitation with low chlorine level to inactivate HAV has been investigated on experimentally contaminated samples of berries (Sattar et al., 2000; Butot et al., 2008). Heating appears as the most effective measure for the inactivation of HAV (Siegl et al., 1984; Murphy et al., 1993). There are many reports that describe the heat tolerance and survival of HAV in various food matrices, including shellfish (Millard et al., 1987; Croci et al., 1999, 2005; Hewitt and Greening, 2006), dairy products (Parry and Mortimer, 1984; Bidawid et al., 2000b) and fruit-based products (Deboosere et al., 2004; Butot et al., 2009). Food constituents and factors can have an impact on the viral sensitivity to thermal treatments: high sugar concentrations can induce a protective effect for enteric viruses (Deboosere et al., 2004), and low pH increased thermal inactivation (Salo and Cliver, 1976). Therefore industrial heat treatment can be used on raw fruits to secure the finished products for consumption in terms of viral risk, but they have to be adapted to product characteristics.

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Likewise, efficiency of treatments depends on the target virus. HAV is an RNA non-enveloped virus, structurally similar to noroviruses, enteroviruses and astroviruses. However numerous studies have addressed the higher stability of HAV under denaturising environmental conditions (McCaustland, 1982; Siegl et al., 1984; Scholz et al., 1989; Hollinger, 1996). A cell-culture-adapted HAV strain (HM175/18f) seemed to be a "relevant" virus in studies aiming at validating the virus inactivation procedures used in agro-food industry (Bidawid et al., 2000b; Deboosere et al., 2004; Butot et al., 2008, 2009), when efforts to cultivate human norovirus have been unsuccessful (Duizer et al., 2004). Indeed, in the absence of a model virus or model system, food safety guidelines need to be based on studies that have been performed with the most resistant enteric RNA viruses. HAV may be thus considered as a good indicator virus or as a surrogate for enteric viruses.

Mathematical models to predict the thermal inactivation of food pathogens during industrial process assist in assessing the risk of contamination for the consumer. A model for the effect of sugar concentration and pH on heat inactivation of HAV in strawberry preparations was published by Deboosere et al. (2004). The results have shown a significant protective effect of sugar contents on the heat resistance of the virus between 80 and 90 °C and a significant effect of pH (range 3.3–4.3) on the D-value at 85 °C. However, high temperatures can not be applied on some fruit matrices (fruit breaks, coulis) for which no sugar is added. In the present paper, we describe a new predictive microbiology approach for thermal inactivation of Hepatitis A virus in acidified red berries without supplemental sugar. This study aims at modelling the behaviour of HAV in acidified berries as a function of thermal treatment and pH. The effects of heat treatments between 65 and 75 °C on survival of HAV were evaluated in experimentally-inoculated fruit purees. This study also aimed at determining if adjustment to more acidic pH values, between 2.5 and 3.5, could promote the thermal inactivation of HAV in these purees.

2. Materials and methods

2.1. Strains and media

HM175/18f strain of HAV (VR-1402) and the foetal rhesus monkey kidney cell line (FRhK-4) were obtained from the American Type Culture Collection. These cells were used throughout the study for the propagation of HAV to prepare inoculums and to measure HAV infectivity. Methods of cultivation, maintenance of cells and preparation of virus pools have been described previously (Flehmig, 1980; Lemon et al., 1985; Cromeans et al., 1987).

2.2. Infectivity assays

Quantitative measurement of the infectivity of the Hepatitis A virus was done by using a titration method by lysis plaque under agar overlayer, described previously (Deboosere et al., 2004). Briefly confluent FRhK-4 monolayers in 6-well cell-culture multiplates were infected with serial ten-fold dilutions of virus samples. Each well was overlaid with a medium containing agar. After 9 to 10 days at 37 °C in humidified 5% CO_2 atmosphere, solid overlayers were removed. The monolayers were fixed and stained with a formalin and crystal violet solution. The average number of plaque-forming units (PFU) was used to determine virus titre of the sampled assayed, expressed in PFU mL^{-1}.

2.3. Fruit products

Raspberries supplied by food industrial partners (Vergers de Boiron; Kerry Ravifruit) were ground to obtain a purée that was used as a reference matrix for the modelling step, and citric acid (anhydrous powder; Arnaud, France) was added to obtain final pH values of 3.3, 3.0, or 2.5. Regarding validation of model predictions, other ground fruits were used with their natural pH: strawberries (pH 3.35), raspberries (pH 3.05) and bilberries (pH 2.87).

2.4. Thermal treatment

Each food matrix was artificially contaminated with HAV to obtain concentrations of 10^6–10^8 PFU mL^{-1} 0.5 g of preparations were then distributed in glass tubes, 100 mm long and 0.5 mm thick (Fisher Bioblock Scientific). Thermal inactivation was performed as described previously (Deboosere et al., 2004), except that samples were left at room temperature for 3 h before heat treatment, as an aggregation step. Heat treatments were performed by simultaneous immersion of the tubes in a glycerol bath set at the desired temperature (65, 70 or 75 °C) for a determined period of time. A thermocouple connected to a data acquisition unit (Agilent Technologies, Actifa, France) was inserted into an uncontaminated aliquot of preparation to monitor the internal temperature throughout the heat treatment. Individual aliquots were removed at periodic time intervals and placed immediately in an ice bath for rapid cooling. The treated media samples were 50-fold diluted in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Invitrogen, France) before virus titration. Each experiment was replicated 3 times.

The desired temperature was reached in approximately 2 min; however, since virus inactivation took place during this step, it was assumed that the target temperature was reached immediately.

2.5. Inactivation model

The experimental data obtained for all heat-inactivation kinetics were processed using the statistical software S-PLUS 2000.

A primary model used to describe viral inactivation kinetics was adapted from a bacterial inactivation model proposed by Albert and Mafart (2005). This model was used to describe bacterial inactivation curves for nonlinear heat treatments and to take into account the residual population observed at the end of heat treatment:

$$\mathrm{Log}_{10}(N) = \mathrm{Log}_{10}[(N_0 - N_{\mathrm{res}})\cdot 10^{-(t/\delta)^p} + N_{\mathrm{res}}] \quad (1)$$

where N is the infectious virus titre, t is time, N_0 represents the initial titre (at time 0), N_{res} is the residual titre at the end of the treatment, δ is the time for first decimal reduction for the population not included in N_{res}, and p is a shape parameter for concavity or convexity of the curve.

The logarithmic reduction, or abatement, in virus titre obtained at the end of the treatment was described using $A = \mathrm{Log}_{10}(N_0) - \mathrm{Log}_{10}(N_{\mathrm{res}})$.

The impacts of temperature and pH on δ and A were described using the following secondary models:

$$\mathrm{Log}_{10}(\delta) = \mathrm{Log}_{10}(\delta^*) - \frac{T - T^*}{Z_T} - \frac{\mathrm{pH} - \mathrm{pH}^*}{Z_{\mathrm{pH}}} \quad (2)$$

$$A = A^* - \frac{T - T^*}{Y_T} - \frac{\mathrm{pH} - \mathrm{pH}^*}{Y_{\mathrm{pH}}} \quad (3)$$

where δ^* (respectively A^*) represents the value of δ (respectively A) in an arbitrary reference condition T^* (65 °C) and pH* (3.3), Z_T (respectively Y_T) is the temperature increase necessary for a 1-unit reduction of $\mathrm{Log}_{10}(\delta)$ (respectively A), and Z_{pH} (respectively Y_{pH}) is

the pH increase necessary for a 1-unit reduction of $\mathrm{Log}_{10}(\delta)$ (respectively A).

It was assumed that the shape parameter p did not depend on temperature and pH, as has been observed previously for bacteria (van Boekel, 2002; Couvert et al., 2005). Likewise, no interaction terms were included in models for bacteria. Based on these results, and for reasons of parsimony, no interaction terms were included here.

A complete model combining the primary and secondary models (Equation (4)) was actually used to evaluate the parameters on the full dataset: the values of δ and N_{res} in Equation (1) were substituted by their expressions from Equations (2) and (3). N_0 values were forced at $N_{(t=0)}$. Titres under the detection threshold were set at the detection threshold (safe assumption). A one-step fitting procedure was thus performed and parameters describing HAV inactivation in raspberries were obtained: p, δ^*, Z_T, Z_{pH}, A^*, Y_T, Y_{pH}.

$$\mathrm{Log}_{10}(N) = \mathrm{Log}_{10}\left[N_0\cdot\left(1-10^{-A^*+\frac{T-T^*}{Y_T}+\frac{\mathrm{pH}-\mathrm{pH}^*}{Y_{pH}}}\right)\cdot 10^{-\left(\frac{t}{10^{\mathrm{Log}(\delta^*)-\frac{T-T^*}{Z_T}-\frac{\mathrm{pH}-\mathrm{pH}^*}{Z_{pH}}}}\right)^p}+N_0\cdot 10^{-A^*+\frac{T-T^*}{Y_T}+\frac{\mathrm{pH}-\mathrm{pH}^*}{Y_{pH}}}\right] \quad (4)$$

3. Results

3.1. Parameters estimation

The heat resistance of HAV in a raspberry puree, acidified to pH 2.5, 3 and 3.3 with citric acid, was analyzed by kinetic evaluation of the loss of infectivity in cell culture. The desired internal temperature was reached after approximately 2 min. The profile of the temperature increase showed: firstly, that the target temperatures were almost reached during the first minute of treatment; and secondly, that the second minute was required to obtain the final 2–3° increase to reach the target temperatures. In some cases, mostly for the 65 °C target temperature, but also for 70 °C, no decrease in viral population was observed until 1 to 4 min after the target temperature was reached, thus resulting in a shoulder. On the other hand, for other experiments, viral inactivation ranging from 1.5 to 4 log units was observed during the temperature increase period, especially for fruits preparation at pH 2.5 heated at 75 °C. Therefore, temperature variations during the heating time were neglected for the data analysis: it was assumed that the temperature was constant at its target value from the beginning of the experiment.

Using the Equation (4), the parameters were estimated from HAV inactivation in acidified raspberry data (Table 1). Inactivation kinetics and model curves are shown in Fig. 1.

Since a global primary and secondary model was used, no individual fit was conducted on any given condition. Therefore, the model describes a global behaviour rather than a sum of individual behaviours, which explains why a "perfect fit" is not observed in Fig. 1.

3.2. Model validation

Predictions with the model were confronted to new experimental data, obtained in another laboratory on strawberries, raspberries and bilberries, when heated at 65, 70 and 75 °C. These comparisons are presented in Fig. 2. Apparent δ values were estimated using the model, with kinetics again including the come-up time to reach the target temperature, but not the temperature variations.

Table 1
Parameters estimates from acidified raspberry data.

Parameter	Estimated value	95% confidence interval
p	3.31	2.56; 4.23
$\mathrm{Log}_{10}(\delta^*)$	0.83	0.80; 0.85
Z_T	24.13	22.19; 26.12
Z_{pH}	−4.67	−5.74; −3.86
A^*	2.25	1.78; 2.75
Y_T	−6.67	−9.78; −5.16
Y_{pH}	0.97	0.70; 1.68

In this work, the model used with parameters estimated on acidified raspberries (Table 1) gave excellent predictions of HAV behaviour in other, non acidified berries. Indeed, predicted heat-inactivation kinetics present a close description of the experimental data obtained in the various fruits for most cases. Failed predictions provided safe results, with higher predicted N_{res} values than what was observed.

4. Discussion

pH of various berries are naturally acidic, with values ranging from 2.5 to 3.3. Sugar contents of berries, without addition, were naturally about 5°Brix (corresponding to 5% (wt/wt) of sugar). Sugar concentration and pH have been previously described as important factors in heat resistance studies of bacteria and viruses, in solutions simulating acidic fruit-based products (Silva et al., 1999; Deboosere et al., 2004). HAV is able to survive for a long time in the environment and extremely stable over a large pH range from 1 to 11 at room temperature (Siegl et al., 1984; Scholz et al., 1989). Moreover, low pH was shown to induce aggregation of virus particles (Volkin et al., 1997; Langlet et al., 2007), which may have a stabilizing effect and so increase viral thermoresistance. Since sugar content corresponded only to natural content of red berries in this study, pH was considered as the most likely factor to interfere in viral thermoresistance. In this study, the effects of heat treatments between 65 and 75 °C on survival of HAV were evaluated firstly in experimentally-inoculated and acidified raspberry purees, and secondly, in strawberries, raspberries and bilberries. The experimental protocol used for measuring thermal inactivation has been adapted to work in a system where the state of virus particles was controlled. Indeed, the observation of a reduction in viral titre may be related to the loss of infectivity, to inactivation, aggregation, or adhesion to the substrate. Phenomena of aggregation and adhesion depend both on environmental characteristics and surface properties of the virus (Langlet et al., 2008). The acknowledgement of the state of the viruses and of their electrostatic and hydrophobic properties is essential in studies on the behaviour of viruses in complex matrices. Langlet et al. (2007) showed that aggregated forms of MS2 phage (a model for enteric viruses), with sizes of few micrometres instead of 30 nm at neutral pH, predominated in suspension at acidic pH, especially as the pH was below the isoelectric point of viral particles (pI(MS2) = 3.9), leading to decrease by 3 log in plaque-forming unit counts. The adsorption/aggregation is not always spontaneously reversible upon return to pH higher than pI. Hydrophobic bonds stabilize the interactions between viruses and the matrix. The different properties of viral capsids could also induce different sensitivities to heat treatments. The MS2 phage is widely used as a model to evaluate survival of enteric viruses in water, because its small size

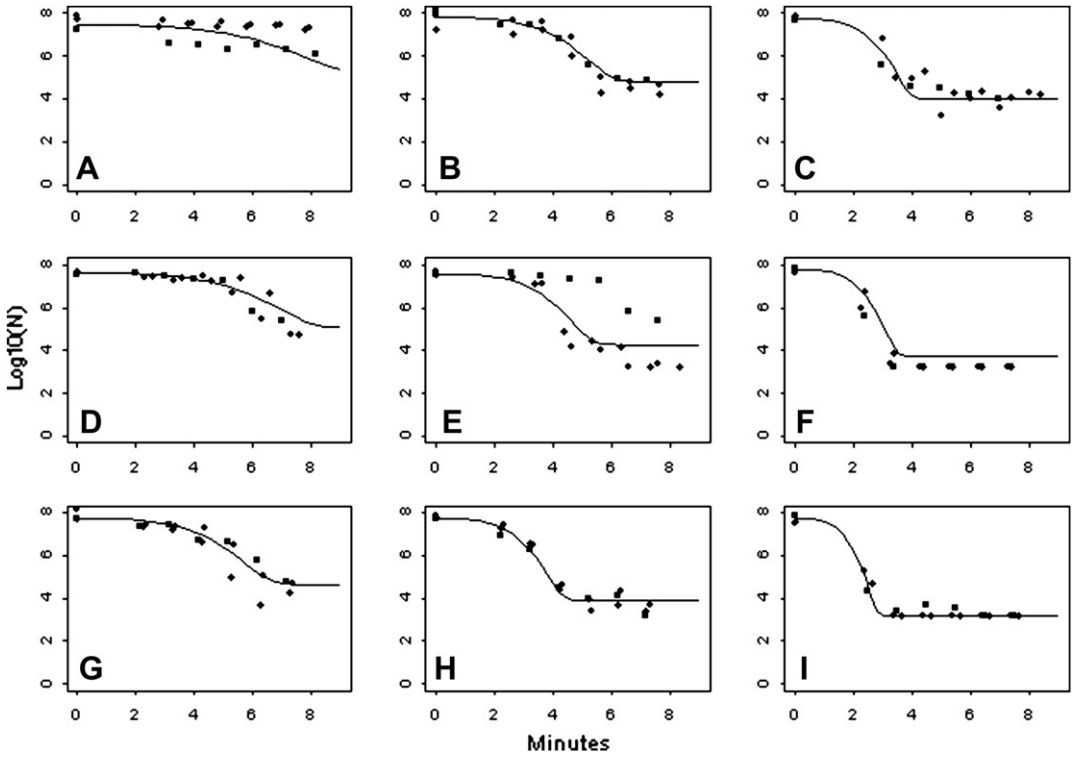

Fig. 1. Inactivation kinetics of HAV in raspberries at 65 °C – pH 3.3 (A), 70 °C – pH 3.3 (B), 75 °C – pH 3.3 (C), 65 °C – pH 3.0 (D), 70 °C – pH 3.0 (E), 75 °C – pH 3.0 (F), 65 °C – pH 2.5 (G), 70 °C – pH 2.5 (H), and 75 °C – pH 2.5 (I); experimental data (symbols, 3 repetitions) and model fit (line).

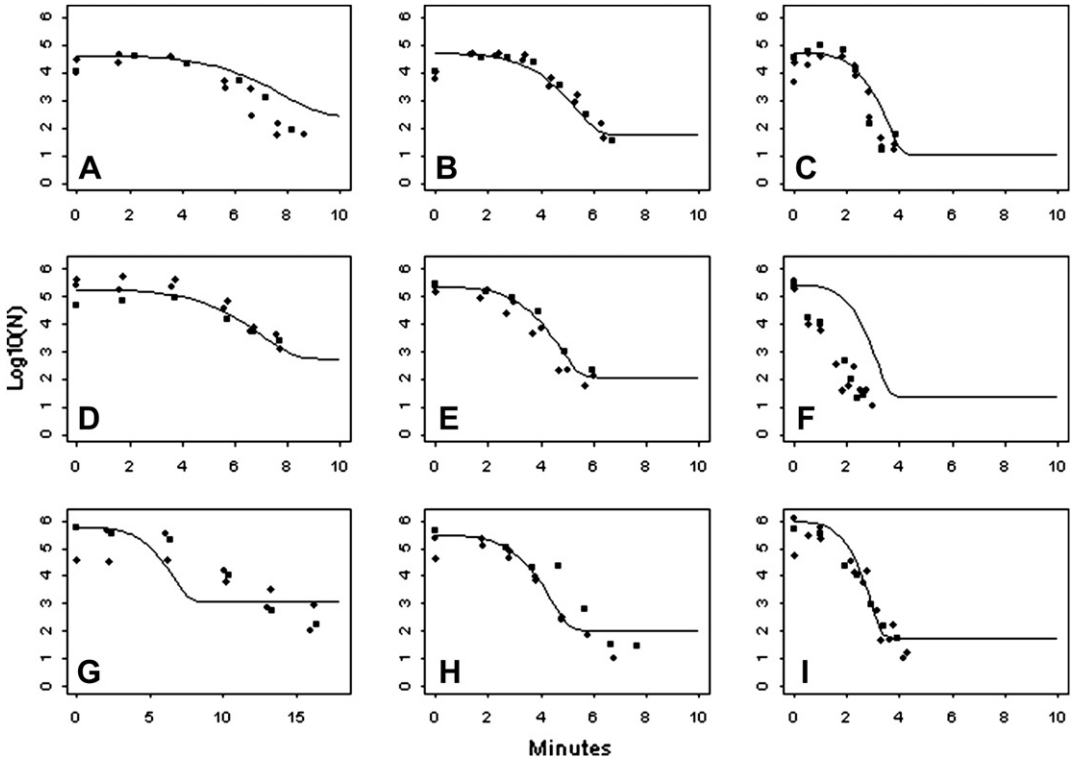

Fig. 2. Inactivation kinetics of HAV in strawberries (pH 3.35) at 65 °C (A), 70 °C (B) and 75 °C (C), in raspberries (pH 3.05) at 65 °C (D), 70 °C (E) and 75 °C (F), and in bilberries (pH 2.87) at 65 °C (G), 70 °C (H) and 75 °C (I); experimental data (symbols, 3 repetitions) and model prediction (line).

and its structure are comparable to that of human pathogenic viruses. Therefore, these results were used to define the conditions of experimental studies carried out for thermo-inactivation, suggesting a preliminary incubation for 3 h at room temperature to aggregate virus particles and subsequently measure only the reduction of infectivity associated with treatment technology.

From these experiments, it appeared that a reduced pH led to a faster thermal inactivation in the tested range. The final reduction in viral titre (parameter A) is greater for higher temperatures and lower pH. Despite the narrow pH range (from 2.5 to 3.3) that was studied, a pH increase has an effect in increasing heat resistance of HAV. Moreover, the acidic pH conditions under which HAV was incubated showed that influence of pH was more pronounced at higher processing temperatures and increase of acidity favoured viral inactivation at higher temperature, as previously observed (Salo and Cliver, 1976; Deboosere et al., 2004).

Experiments on acidified raspberries were performed in triplicate to account for the variability of heat-inactivation kinetics. We obtained nonlinear inactivation curves in acidified raspberries, whereas linear curves had been observed with high sugar concentrations (more than 28%) at 85 °C, which justified the use of linear regression (Deboosere et al., 2004). In this study, three-phase curves with delayed initial decrease and a residual titre at the end of the treatment were observed, mainly at 70 and 75 °C. The chosen model thus took into account the residual population observed at the end of heat treatment, which seemed to be well adapted in order to describe and model experimental inactivation kinetics. The initial shoulder observed in many inactivation curves in this study may be influenced by the fact that experiments were started at room temperature, and that a significant come-up time (around 2 min) was required to reach the target temperature. However, for a number of experiments with target temperatures of 65 °C or even 70 °C, no viral inactivation was observed during this temperature increase period, or for 1 to 4 min after the target temperature was reached, thus indicating that this shoulder could not be considered entirely as an experimental artefact. Yet, the inclusion of the come-up time in inactivation kinetics obviously led to biased estimates of the parameters. Assuming a constant target temperature during the whole kinetics, including the come-up time, is a very simplifying assumption, which will be addressed in future studies.

An integrated model with a single equation nesting secondary models of temperature and pH in the previous model was built to predict the inactivation of HAV in red berries, taking into account heating treatment ranging from 65 to 75 °C and pH range from 2.5 to 3.3. Using this combined approach, the estimated values are expected to be more objective and robust, and the variability in kinetic data is taken into account (Pouillot et al., 2003). This methodology approach is widely used in predictive microbiology studies in the agro-food area. However, no model predictions can be used in confidence unless they were validated on independent data on foods (Delignette-Muller, 1997). Model predictions were then confronted to experimental results obtained in another laboratory on other berries with different pH values. Considering Fig. 2, few differences were observed between predicted and measured infectious virus titre values ($\log_{10}(N)$) in acidic red berries. In some cases, the predicted viral inactivation appeared to be generally safer than the inactivation kinetics experimentally observed for various berry purees (Fig. 2A and F). When predictions failed (Fig. 2G and H), the model predicted higher residual virus titres than what was experimentally observed at the end of experiment and so provided safe results. Consequently the model could be used to predict relatively reliable heat inactivation in soft fruits, acidified or not, in regard to pH variations. While a high sugar content has been shown to increase the heat stability of HAV in fruit-based product (Deboosere et al., 2004), in the present study, pH variation seemed sufficient to explain different heat-inactivation kinetics. Indeed, parameters obtained on raspberries could be applied successfully on strawberries and bilberries, using the measured pH of these fruits: no matrix effect was evidenced in this study. Results confirmed that pH exerted a significant effect on HAV thermoresistance in fruit-based products and that adjustment to acidic pH values, i.e. less than pH 3.3, could promote the thermal inactivation of HAV in red berries-based products without supplemented sugar. In strawberry mashes, although more than 5 min were required at 80 °C with a sucrose concentration above 28% and pH 3.8 to obtain a HAV reduction of 4 log (Deboosere et al., 2004), less than 4 min were required at 75 °C with no added sugar and pH below 3.3 in the present study.

Modelling the thermal inactivation of HAV on berries is very informative to secure the finished berries-based products for consumption. The new modelling approach introduced in this paper is for the second time applied to food virology. A model for thermal inactivation of HAV in red berries, without supplemented sugar, taking into account either heating treatment and acidic pH was successfully developed and validated. In accordance with the objective of predictive microbiology, the use of an equation seems to be suitable to study the virus behaviour of an enteric virus model, i.e. HAV, in a complex food matrix. However, additional studies are clearly needed to take into account heat-inactivation kinetics during the phase of temperature increase to reach the target temperature.

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