GROWTH OF *LISTERIA MONOCYTOGENES* IN THAWED FROZEN FOODS

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Abstract

In February 2008, the FDA released a draft Compliance Policy Guide (CPG) on *Listeria monocytogenes* and proposed that ready-to-eat (RTE) foods that do not support the growth of *L. monocytogenes* may contain up to 100 CFU/g of this pathogen. Frozen foods such as ice cream fall in that category since they are consumed in the frozen state. However, other frozen foods, such as vegetables and seafood that are thawed and served at salad and food bars, may support the growth of *Listeria* and would not be allowed to contain 100 CFU/g according to the draft CPG. In the current study, growth curves were generated for *L. monocytogenes* inoculated onto four thawed frozen foods - corn, green peas, crabmeat, and shrimp - stored at 4, 8, 12, and 20ºC. Growth parameters, lag phase duration (LPD), and exponential growth rate (EGR) were determined using a two-phase linear growth model and the Square Root Model. The results demonstrated that *L. monocytogenes* has a very short LPD on these thawed frozen foods during refrigerated storage and that there would be several orders of magnitude of growth (*i.e.*, more than 1.7 log increase at 4 ºC) of the organism before the product is found to be organoleptically unacceptable. Although it would not be possible to take advantage of any extended lag phase duration caused by freeze injury to the organism, frozen foods containing less than 100 CFU/g of *L. monocytogenes* that are thawed, or thawed and cooked, and then consumed immediately, should not represent a public health hazard.

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Chapter 1. Literature Review – Characteristics of *Listeria monocytogenes*

Listeria monocytogenes is considered a primary pathogen of concern in the area of food safety and public health due to its unique characteristics: 1) *L. monocytogenes* is ubiquitous in the environment; 2) its capacity for growth at refrigeration temperatures (from -1.5 to 4 ºC); 3) the high mortality rate connected with foodborne infection with this organism. There are therefore many publications on *L. monocytogenes* and listeriosis due to the significance of this pathogen. In particular, the third edition of the book, *Listeria, Listeriosis and Food Safety* by Ryser and Marth provides comprehensive information on *L. monocytogenes* and listeriosis (20). There are also many important review papers available such as the inclusive reviews by McLauchlin et al. (16) and by Farber and Peterkin (7), a review of the survival mechanisms of *L. monocytogenes* by Gandhi et al. (9), a review of incidents and issues related to ready-to-eat (RTE) foods in retail environments by Lianou and Sofos (14), and several risk assessments to clarify risks of RTE foods (8, 11, 29). Furthermore, there are numerous research papers published regarding other aspects of *L. monocytogenes* and listeriosis. This chapter reviews the characteristics of *L. monocytogenes* with respect to food safety.

Microbiology and Classification

Listeria monocytogenes is a gram-positive, non-spore-forming, motile, and facultatively anaerobic rod-shaped bacterium. It is catalase-positive, oxidase-negative, and beta-hemolytic. When *L. monocytogenes* grows at 20-25ºC, it expresses motility by flagella, although the organism does not synthesize flagella at a higher temperature such as 37ºC (7). The genus *Listeria* includes six different species: *L. monocytogenes, L. ivanovii, L. innocua, L. welshimeri, L. seeligeri, and L. grayi* (16). *Listeria monocytogenes* is known to cause the illness of listeriosis in humans and animals. *Listeria ivanovii* and *L. seeligeri* are also reported to cause rare illness in humans (16). *Listeria ivanovii* and *L. innocua* are known to cause listeriosis in domestic animals such as sheep, cattle, and goats (16). There are 13 serotypes of *L. monocytogenes* which can cause disease, but the majority of human isolates belong to only three serotypes: 1/2a, 1/2b, and 4b (21).

Natural Reservoirs and Transmission

Listeria monocytogenes is widespread in the environment, being found in plants, soil, wastewater, stagnant water supplies, grazing areas, animal feed, and the intestines of healthy animals and humans (21). The organisim can endure adverse conditions such as freezing, drying, mild heat, and anaerobic conditions, such as conditions can be seen in food processing, longer than most other non-spore forming food pathogens (21). Although *L. monocytogenes* can be found anywhere, contaminated untreated silage/feed is the most probable cause of *Listeria* infections in farm animals (21). Transmission of *Listeria* to humans by raw ingredients such as un-pasteurized raw milk and meat appears to be rare, since *L.monocytogenes* can be killed by pasteurization or cooking (21). Contaminated, untreated manure can be a source of human listeriosis. For example, the coleslaw outbreak in Canada in 1981 was traced back to the sheep manure used for cabbages in the field (21, 22). Once *L. monocytogenes* is introduced into food processing and retail environments by raw ingredients, unsanitary practices, etc., the organism can colonize and contaminate products. Consequently, RTE type of foods are the primary source of human listeriosis (21). The simplified diagram of transmission scheme is shown in Figure 1.1.

Figure 1.1. Transmission of *L. monocytogenes* from farm to human
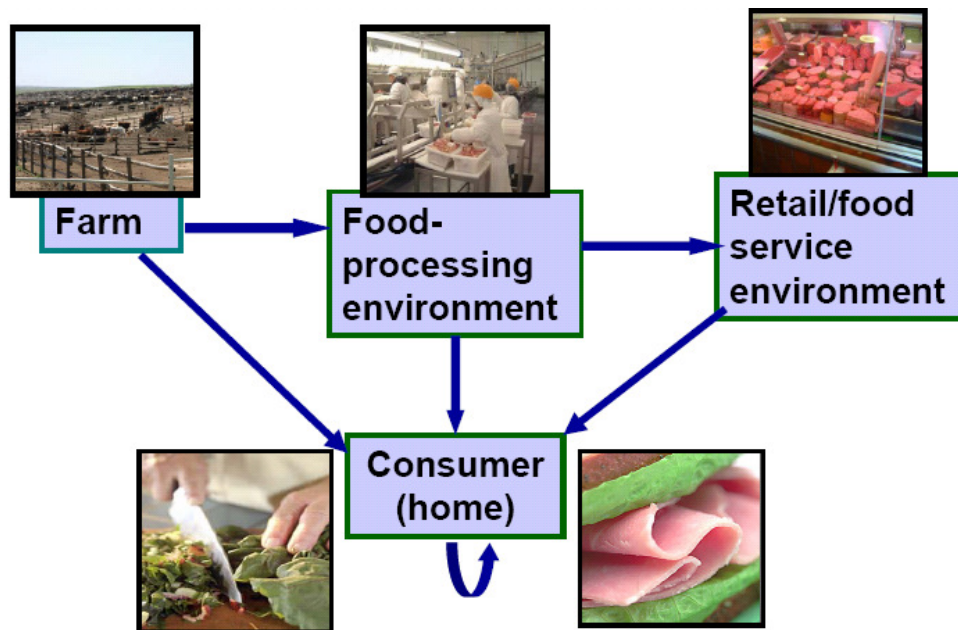
Source: Sofos, 2006 (23)

Growth Characteristics

The details of *L. monocytogenes* growth and survival limits are shown in Table 1.1. As mentioned above, *L. monocytogenes* can survive under adverse conditions although the organism is not heat-resistant and can be inactivated by pasteurization such as 72°C (161°F) for 15 seconds (2). An important aspect of *L. monocytogenes* is that this organism can grow at refrigeration temperatures (-1.5 to 4 ºC), at a pH below 5.0 at the optimum incubation temperatures (30-37 ºC), and in moderate to high salt concentrations (9). Due to these characteristics of the organism it is very difficult to control *L. monocytogenes* for food safety.

Table 1.1. Growth and survival limits of *Listeria monocytogenes*

Parameter	Minimum	Maximum	Optimal	Survival without growth
Temperature (°C)	-1.5 to 3	45	30 to 37	-18
pH	4.2 to 4.3	9.4 to 9.5	7.0	3.3 to 4.2
Water activity	0.90 to 0.93	> 0.99	0.97	<0.90
Salt (%)	<0.5	12 to 16	N/A	≥20

Source: Todd, 2006 (25)

Listeriosis and Mechanism of Infection

Listeriosis is a disease caused by ingesting viable cells of *L. monocytogenes*. The majority of listeriosis occurs by consuming heavily contaminated food (16). Although the number of foodborne outbreaks associated with *L. monocytogenes* is not high, the mortality rates are very high. According to the Center for Disease Control and Prevention, 1600 cases of listeriosis occur annually; 260 cases are fatal (3). The estimated annual number of cases of foodborne salmonellosis, caused by *Salmonella,* is 14 million cases, and 400 cases are fatal (4). In comparison to salmonellosis, the fatality rate of listeriosis is quite high. Also, sporadic cases appear to be more common than outbreak cases (21).

The host's immune system, mainly via cell-mediated immunity (CMI), attacks the pathogen to prevent infection (24). However, if the host's immune system is compromised, some bacteria can survive the host's CMI and invade the intestinal mucosa and spread through intracellular mechanisms, causing serious infections (16). In particular *L. monocytogenes* can penetrate the blood-brain barrier and the placental barrier, causing severe infection of the brain

and fetus, respectively (6). There are two types of listeriosis associated with *L. monocytogenes*, non-invasive and invasive.

Non-invasive Listeriosis

Non-invasive listeriosis demonstrates milder symptoms. It is often referred to as febrile gastroenteritis, and is caused by the ingestion of a high-dose *L. monocytogenes* by immuno-competent people (1). The incubation period is shorter than that for invasive listeriosis, typically 20 hours or so (18). Symptoms include diarrhea, fever, headache, and muscle pain (18).

Invasive Listeriosis

Invasive listeriosis occurs in high-risk people, including immuno-compromised individuals, pregnant women and their fetuses, newborne infants, and the elderly (>65 years old) (11). The incubation period is variable, and ranges from 3 to 70 days (11, 16). In adults, different manifestations can occur depending on the organ system infected; they can include meningitis, pneumonia, septicemia, endocarditis, abscesses, skin lesions, and mild conjunctivitis. Some of these conditions may result in death (16). Infected pregnant women may develop mild flu-like symptoms; furthermore, they may experience premature delivery, miscarriage, spontaneous abortion, stillbirth, or death of a newborn (18). Infants may develop symptoms such as loss of appetite, lethargy, jaundice, vomiting, respiratory distress, pneumonia, skin rash, shock, and meningitis (18).

Infective Dose

The exact infective dose is still unknown (16). The infective dose has been assessed based on animal studies and risk assessments using epidemiological data, and also on prevalence and consumption studies (5, 8, 10, 16, 29). Furthermore, the infective dose appears to vary depending on the strain and on host susceptibility (16). It is assumed that a non-invasive infection may occur with a higher dose ($> 10^5$cells/g). The invasive form of the desease may occur with fewer than 1,000 cells in susceptible populations (6, 15, 16).

Listeria monocytogenes Contamination in Foods

Listeriosis is most commonly caused by consuming contaminated food. *Listeria monocytogenes* has been isolated from many types of foods including raw and pasteurized milk, soft-ripened varieties of cheeses, ice cream, raw fruits and vegetables, fermented raw-meat sausages, raw and cooked poultry, raw meats of all types, and raw and smoked fish (5, 7, 14, 20). In particular, RTE foods that support the growth of *L. monocytogenes* demonstrate the highest risk, since they are usually consumed without the cooking that can inactivate this pathogen (11, 24, 29). Examples of high risk RTE foods that support growth of *L. monocytogenes* include: (28)

- Milk and dairy products, *e.g.*, butter and cream
- Soft unripened cheeses, *e.g.*, queso fresco, cottage and ricotta cheese
- Cooked crustaceans, *e.g.*, shrimp and crab
- Smoked seafood, smoked finfish and mollusks
- Certain vegetables, *e.g.*, cabbage, and non-acidic fruits such as melons
- Some deli-type salad sandwiches, *e.g.*, prepared from non-acidified seafood at retail establishments

Some RTE foods, such as fresh cut fruits and vegetables, may be naturally contaminated with *L. monocytogenes*. Some RTE foods such as seafood and deli meats are susceptible for post-process contamination at the food manufacturer, retail, and at home. If refrigerated RTE foods that do not have any method to control growth of *Listeria* are contaminated with this pathogen, *L. monocytogenes* may grow to unsafe numbers in these RTE foods after prolonged storage, even if the initial contamination level is low.

Control of *Listeria monocytogenes* in Foods

Total elimination of *L. monocytogenes* from some types of foods, such as fruits and vegetables, and from food processing environments is difficult due to the organism's ubiquitous nature (17). Although *L. monocytogenes* can be effectively controlled by pasteurization (2), post-pasteurization contamination or other inadequate processes may still result in contaminated foods. As described earlier, RTE foods are vulnerable to *L. monocytogenes* contamination.

Among them, RTE foods with a long shelf-life and with no preservation method except refrigeration are recognized as high-risk foods (8, 11, 28, 29). Consequently, the following approaches are taken to control *L. monocytogenes* in order to ensure food safety (13, 14, 26, 31);

- Designing products to prevent or suppress the growth of *L. monocytogenes* using pH, water activity, salt, and preservatives.
- Implementing sanitation programs and environmental control programs in food processing facilities and also at retail.
- Using a comprehensive food safety system based on the philosophy of Hazard Analysis and Critical Control Point (HACCP), and a series of prerequisite programs such as Good Manufacturing Practices (GMP), and Sanitation Standard Operating Procedures (SSOP) in food processing facilities.
- Deliverying food safety education and training to retail and food service managers and food handlers and consumers.

Combined efforts to control *L. monocytogenes* in food processing environments are taking place among government agencies, the food industry, and academic institutions (13). The FDA and USDA conduct risk assessments (29), and provide guidance for HACCP, GMP, and SSOP programs and regulations for the monitoring of *L. monocytogenes* in production environments and RTE foods (26, 28), and educational programs to consumers. The food industry continues to improve product food safety by assuring compliance with those guidance materials and regulations. The academic institutions have provided scientific findings to advance our knowledge and technologies to control *L. monocytogenes* in foodstuffs, and have offered educational outreach programs.

Foodborne Disease Outbreaks

The first confirmed foodborne listeriosis outbreak, caused by contaminated coleslaw, occurred in 1981 in Canada (22). The major invasive outbreaks which have occurred in North America are summarized in Table 1.2; they demonstrate that various RTE foods can be vehicles for listeriosis (20).

Table 1.2. Major foodborne outbreaks of invasive listeriosis in North America (1981-2008)

Year	Location	Foods	Number of Cases	Cause
1981	Nova Scotia, Canada	Coleslaw	Resulted in 41 cases, including 34 pregnant women. There were 18 deaths.	Contamination was due to Listeria-infected sheep manure used to fertilize cabbage.
1985	Los Angeles, USA	Mexican-Style soft cheese	Resulted in 145 cases including 93 pregnant women. There were 64 deaths	Contamination was due to inadequate pasteurization and contamination of equipment.
1998-1999	Multi states, USA	Hot dogs and deli meats	Resulted in over 100 cases including 15 deaths and 6 miscarriages	The contamination source was construction dust at the processing plant which contaminated products in the packaging room.
2002	Multi states, USA	RTE turkey deli meat	Resulted in 54 cases including 11 total 3 fetal deaths	Contamination occurred at the plant (post processing contamination)
2007	Massachusetts, USA	Pasteurized milk	Resulted in 5 cases, including 3 deaths	Contamination occurred after pasteurization, i.e., post processing contamination.
219 008	Toronto, Canada	RTE deli meats	Resulted in 57 cases, including 23 deaths	Contamination occurred at the processing plant

Sources: Ryser and Marth, 2007 (20), Public Health Agency of Canada, 2010 (19)

Test Methods

There are three commonly used isolation/detection methods for *L. monocytogenes* in food samples, described below.

i) The Food and Drug Administration (FDA) Method (30)

Enrichment using buffered *Listeria* enrichment broth base containing sodium pyruvate (BLEB) is maintained at 30 ℃ for 4 hours; the selective agents acriflavine, nalidixic acid, and cycloheximide are added and further incubated at 30 ℃ for 44 hours, for a total of 48 hours of incubation. The enrichments are plated at 24 hours and 48 hours onto Oxford (OX) agar, Lithium Chloride-Phenylethanol-Moxalactam (LPM) agar, Polymyxin-acriflavin-lithium chloride-ceftazidime-aesculin-mannitol (PALCAM) agar, or MOX (modified OX) agar. Use of one of the *L. monocytogenes-L. ivanovii* differential selective agars, such as Biosynth Chromogenic Medium (BCM), *Listeria* Ottavani and Agosti (ALOA) agar, RapidL'mono medium, or CHROMagar *Listeria* along with the abovementioned esculin-containing selective agar is recommended.

ii) The U. S. Department of Agriculture (USDA) method (27)

The primary enrichment is with University of Vermont (UVM) broth for 24 hours at 30 ℃, with later plating on MOX agar. The secondary enrichment is done by transferring the UVM to Fraser broth (FB) and then plating it onto MOX agar.

iii) The Netherlands Government Food Inspection Service (NGFIS) method (21)

This method uses enrichment with PALCAM-egg yolk at 30 ℃ for 24 to 48 hours and plating onto PALCAM agar.

Enumeration of *L. monocytogenes* is done by a most-probable-number (MPN) method for a population of less than 100 CFU/g, and by plating methods for a population more than 100 CFU/g (21). For the latter case, the International Organization for Standardization (ISO) method is generally recognized as the reference method and is recommended by the FDA in their draft policy guide for *L. monocytogenes* in RTE foods (28). The ISO method for enumeration is described at ISO 11290-2:1998/Amd. 1:2004(E)" (12).

Summary

This chapter has discussed basic information about *L. monocytogenes* and listeriosis with regard to food safety. *Listeria monocytogenes* is a significant pathogen that causes serious foodborne infection, listeriosis. In particular *L. monocytogenes* causes serious clinical manifestations in immunocompromised populations. It is essential to control this pathogen in food products. Due to its nature and characteristics it is not easy to control *L. monocytogenes* in food and food processing environments. RTE foods pose a high risk for *L. monocytogenes* contamination and foodborne infection. Continuous efforts to control *L. monocytogenes* are necessary in foods and food processing and retail environments in collaboration with government agencies, the food industry, and academic organizations.

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CHAPTER 2. Literature Review – Policies and Regulations Pertaining to *Listeria monocytogenes* in Ready-to-Eat Foods in Different Countries

Currently there is no single policy or regulation on the control of and the tolerable level for *Listeria monocytogenes* in ready-to-eat (RTE) foods among different countries at the international level. Different criteria and recommendations regarding the control of *L. monocytogenes* in RTE foods have been established by different countries over many years. This chapter reviews the policies and regulations regarding *L. monocytogenes* in RTE foods of several representative countries to see current trends in *L. monocytogenes* control at the international level.

United States of America

Currently, the U.S. Food and Drug Administration (FDA) and the U.S. Department of Agriculture (USDA) Food Safety and Inspection Service (FSIS) have a "zero-tolerance" policy with regard to *L. monocytogenese* in RTE foods. This means the absence of the organism in two 25g samples: "zero detection" (18). This policy was issued in late 1980s in response to a major outbreak associated with Mexican-style soft cheese in 1985 (16). This "zero-tolerance" policy was developed because of the pathogenic nature of *L. monocytogenes* and its ability to grow in RTE foods. Important considerations include: 1) *L. monocytogenes* can grow at refrigeration temperatures; 2) *L. monocytogenes* causes the serious foodborne infection of listeriosis which has a high mortality rate; 3) there is uncertainty and variability regarding the infectious dose, host susceptibility, and virulence factors of the organism; 4) RTE foods are susceptible to post-process contamination with *Listeria* and are generally consumed without cooking adding to the lethality of infection (15). In February 2008 the FDA released a draft Compliance Policy Guide (CPG) on *L. monocytogenes* and a draft guidance for industry (22). The CPG proposed that RTE foods that do not support the growth of *L. monocytogenes* may contain up to 100 CFU/g based on information available from risk assessments (10, 25). The zero tolerance policy would still apply to RTE foods that support pathogen growth such as milk, soft cheeses, smoked seafood, and non-acidic fruits. This draft guideline would apply to:

- Foods with a pH less than or equal to pH 4.4, such as acidified deli salads and pickled products.

- Foods with a water activity less than or equal to $a_w 0.92$, such as cereals, crackers, and hard cheeses.
- Frozen foods such as ice cream.

The draft guideline will be similar to the international standards adopted by Europe, Canada, and other nations (15). Furthermore, according to the FDA, there is no epidemiological evidence demonstrating that either a zero or a non-zero tolerance policy leads to better control of *L. monocytogenes* in foods (25). The FDA/FSIS risk assessment (25) categorized RTE foods based upon risk factors such as their ability to allow the growth of *L. monocytogenes*. High risk foods are those which support the growth of *L. monocytogenes*, have a long shelf-life, and are consumed frequently. Classifying RTE foods based on these risk levels will help to clarify the problems and facilitate strategies to ameliorate these issues. The risk assessments demonstrated in addition that foods with low levels of *L. monocytogenes* (*e.g.*, <100 CFU/g) pose very little risk (2, 5, 10, 25). The FDA has not finalized the draft CPG.

According to the FDA and FSIS, the control of *L. monocytogenes* should be achieved by utilizing Good Manufacturing Practices (GMP), Hazard Analysis Critical Control Point (HACCP) systems, an environmental monitoring program, proper product design to suppress *Listeria* growth, listericidal agents, and proper consumer education programs (20, 22, 23, 24).

The FDA's Food Code (21) also serves as a reference document for state and local agencies to ensure food safety at retail and other food service establishments. The Food Code includes recommendations for controlling *L. monocytogenes*, such as employee sanitary practices, sanitization strategies, measures to prevent cross-contamination, and times and temperatures for cooking, cooling, and holding food.

Canada

In contrast to U.S. policy, Canada does not employ a zero tolerance approach for managing L. monocytogenes. The 2004 policy for *L. monocytogenese* in RTE foods provides for the control of *L. monocytogenese*, ensuring tolerable levels through the use of inspection, environmental monitoring, and end-product testing. The tolerable levels in individual food categories are based on the health risks and the capability of the foods to support the growth of *L. monocytogenes*. In response to a major outbreak of listeriosis traced to RTE meat products

which occurred in 2008 (17), Health Canada issued a new policy in November 2010 (14); it became effective in April 2011. The 2011 policy revises the classification of RTE foods from three food categories to two categories; the end product criteria are shown in Table 2.1. The approach of criteria is similar to the Codex Alimentarius Commission (CAC) and the European Commission (EC) (3, 4, 5, 6, 8). Furthermore, the focus is more on environmental verification and control. The new policy also encourages the use of post-lethality treatments and/or *L. monocytogenes* growth inhibitors for products. The scientific base of the tolerance level, 100 CFU/g, is based on risk assessments and epidemiological data (10, 25).

Table 2.1. Sampling methodologies and compliance criteria as outlined in Canadian regulations for controlling *Listeria monocytogenes* in ready-to-eat foods

Food Categories	Sampling	Analysis	Type of analysis	Action level for *L. monocytogenes*	Nature of concern	Level of priority for oversight
1. RTE foods in which growth of *L. monocytogenes* can occur throughout the stated shelf-life, such as deli-meats, soft cheeses, hot dogs, pâté.	5 sample units (min. 100 g or ml each), which are representative of the lot and the production conditions, taken aseptically at random from each lot.	5x25 g analytical units are either analyzed separately or composited	Enrichment only	Detected in 125 g	Health Risk 1	High
2 A) RTE foods in which a limited potential for growth of *L. monocytogenes* to levels not greater than 100 CFU/g can occur throughout the stated shelf-life. Such foods can include refrigerated gravlax/cold-smoked rainbow trout and salmon, fresh-cut produce, etc. **2 B)** RTE foods in which the growth of *L. monocytogenes* cannot occur throughout the stated shelf-life. Examples include ice cream, hard cheese, dry salami, dried-salted fish, varieties of prosciutto ham.	Same as above	5x10 g analytical units	Direct plating only	100 CFU/g	Health Risk 2	Medium to low

Source: Health Canada, 2010 (14)

Australia and New Zealand

In 1996, Australia and New Zealand established the Australia-New Zealand Food Authority (ANZFA) to harmonize their regulations and food safety programs to reduce unnecessary trade issues (19). A joint Australia-New Zealand Food Standards Code (ANZFSC) has been established between the two countries. At present the Food Standards Australia New Zealand (FSANZ) has taken over responsibility for ANZFA and administers the ANZFSC. The Standard 1.6.1 and the user guide include microbiological limits for *L. monocytogenes* in specific foods, as shown in Table 2.2 (11, 13). These limits are similar to those of the CAC and EC. The ANZFA also utilizes risk assessments for developing policy and establishing standards and criteria (1).

Table 2.2. Limits of *Listeria monocytogenes* in foods from Standard 1.6.1 of Australia-New Zealand Food Standards Code

Foods	Level	Action[a]
Unpasteurised milk and butter, raw milk cheese, soft cheeses, cheeses manufactured from thermized milk, packaged heat-treated meat paste and paté, packaged cooked cured/salted meat, and bivalve molluscs that have undergone processing other than depuration.	Absence in 25g (n=5, c=0, m=0)*	Recall
Ready-to-eat processed finfish, other than fully retorted finfish	<100 CFU/g (n=5, c=1, m=10^2)	Recall

*n= the minimum number of sample units which must be examined from a lot of food, c= the maximum allowable number of defective sample units, m= the acceptable microbiological level in a sample unit.
[a]These actions apply to the product sampled at the processing factory or wholesale level, and do not apply to the product at retail level.)
Source: Food Standards Australia New Zealand (11, 12)

The European Community

Historically, different approaches for the control and limitation of *L. monocytogenes* in RTE food have been taken by each country in Europe (19). The microbiological criteria issued by the European Commission (EC) in 2005 brought a harmonization of policy, tolerable levels, and criteria for *L. monocytogenes* in RTE foods for the member countries (8). The criteria include three categories of RTE foods and set a sampling plan and the tolerable levels of *L. monocytogenes* for each food category as shown in Table 2.3. This approach is similar to that of

CAC. The tolerable level, 100 CFU/g, was adapted based upon the opinion of a scientific committee (7).

Table 2.3. Microbiological criteria for *Listeria monocytogenes* in Ready-to-eat (RTE) foods by the European Commission.

Category of Food	Sampling plan	Limits	Criterion
RTE foods intended for infants and special medical purposes.	n=10* c=0	Absence in 25 g	Products in the market
RTE foods able to support the growth of *L. monocytogenes*	n=5 c=0	100 CFU/g during shelf-life or	Products in the market
	n=5 c=0	absence in 25 g while in control of the manufacturer	Before it has left the processor
RTE foods unable to support the growth of *L. monocytogenes*	n=5 c=0	100 CFU/g during shelf-life	Products in the market

*n and c are defined above.
Source: Commission Regulation (EC) No. 2073/2005, 2005 (8)

International Community

Codex Alimentarius Commission

The joint Food and Agriculture Organization and World Health Organization (FAO/WHO) food standards program addresses microbiological criteria for foods in international trade, and the Codex Alimentarius Commission (CAC) implements the program. Within the CAC, the Codex Committee on Food Hygiene (CCFH) provides standards or codes of practices including microbiological criteria related to food hygiene. The "Guidelines on the Application of General Principles of Food Hygiene to the Control of *Listeria monocytogenes* in Foods (CAC/GL-61-2007)" was adopted by the CAC in 2007 (3). The guidelines address recommendations on controlling *L. monocytogenes* during production and transportation, and also on training for RTE manufacturers (Annex I of the guideline). Annex II, proposed draft "Microbiological Criteria for *Listeria monocytogenes* in Ready-to-Eat Foods" (4) addresses criteria for governments within a framework for controlling *L. monocytogenes* in RTE foods. Annex II has been developed based upon the information available from risk assessments (10). Annex III, "Recommendations for the Use of Microbiological Testing for Environmental Monitoring and Process Control Verification by Competent Authorities as a Means of Verifying the Effectiveness of HACCP and Prerequisite Programs for Control Of *Listeria monocytogenes*

in Ready-to-Eat Foods" (5) is for use by competent authorities if they intend to include environmental monitoring and/or process control testing as part of their regulatory activities. In Annex II, RTE foods are divided into three categories: 1) foods for which no criteria are needed; 2) RTE foods in which growth will not occur; 3) RTE foods in which growth can occur. Microbiological criteria have been set for categories 2) and 3) as shown in Table 2.4, and action plans are to be implemented when a criterion is not met. These microbial criteria are similar to those of the European Community for the verification and control of *L. monocytogenes* in RTE foods (6). Canada also takes similar approaches (14). These criteria were developed with a view towards protecting the health of consumers while ensuring fair practices in food trade.

Table 2.4. Microbiological criterion for *Listeria monocytogenes* in ready-to-eat foods (draft Annex II, Codex Alimentarius Commission)

Category of Food	Sampling Plan	Limit	Criterion
RTE foods in which growth of *L. monocytogenes* will not occur	n=5* c=0	≤ 100 CFU/g	throughout the product shelf-life
RTE foods in which growth of *L. monocytogenes* can occur	n=5 c=0	Absence in 25g (< 0.04 CFU/g)	throughout the product shelf-life

*n and c are defined above.
Source: Codex Alimentarius Commission, 2009 (4)

International Commission on Microbiological Specifications for Foods (ICMSF)

The ICMSF is a voluntary advisory organization which sets standards, methods, and criteria regarding the presence of microorganisms in food. The ICMSF states that food sample testing can be a useful tool as part of a verification program in a HACCP system to ensure food safety (9). For *L. monocytogenes*, specific sampling criteria are recommended, as shown in Table 2.5.

Table 2.5. Recommended microbiological criteria for *Listeria monocytogenes* by International Commision on Microbiological Specifications for Foods

Food	Testing/Action
In-pack, heat-treated products	no testing is necessary (documentation for the heat-treatment process)
Raw products and/or products which are to be heat-treated before consumption	no testing is necessary
RTE products, unable to support growth of *L. monocytogenes*	10 samples should be taken and the lot should be rejected if any sample contain > 100 CFU/g
RTE products, able to support growth of *L. monocytogenes*	20 samples should be taken and the lot rejected if any sample contains > 100 CFU/g

Source: from the International Commission on Microbiological Specifications for Foods (ICMSF), 1999 (9)

Summary

Currently, there is no unified policy for tolerance levels for *L. monocytogenes* in RTE foods. Different countries establish different policies and tolerance levels. This may create trade conflicts. However, there is an effort to unify a policy and tolerance level as seen in the Codex Alimentarius Commission (CAC), the European Community (EC), and Canada. The trend is to categorize RTE foods by the attributes of whether or not they support the growth of *L. monocytogenes* and risk factors associated with the food, followed by the setting of limits for *L. monocytogenes* by food category. Also, recommended control measures for *L. monocytogenes* in RTE foods generally utilize systematic food safety programs such as GMPs and HACCP, environmental monitoring and testing programs, appropriate product design for suppressing the growth of *Listeria*, listericidal treatment, and proper consumer education. The development of policies and criteria has been based upon risk assessments to reflect currently available scientific knowledge. This movement has been supported with a view towards protecting the health of consumers while ensuring fair practices in the food business and its trade.

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CHAPTER 3. Literature Review – The Effects of Freezing Treatment and Frozen Storage on *Listeria monocytogenes* in Food

This chapter discusses the effect of freezing and frozen storage on *L. monocytogenes* and food. First, the effect of freezing and frozen storage on food is discussed, then that of the effect of freezing and frozen storage on *L. monocytogenes*. *Listeria monocytogenes* is an important pathogenic microorganism that causes the serious foodborne disease of listeriosis. *Listeria monocytogenes* is not heat-resistant and is easily inactivated by usual thermal processing; however, post-processing contamination is an issue since *L. monocytogenes* exists ubiquitously in the environment, including in food-processing and food-retailing facilities. Therefore, the foods most vulnerable to *L. monocytogenes* contamination are ready-to-eat (RTE) types of foods. Some frozen foods might be consumed without the heating needed to inactivate *L. monocytogenes* before consumption. They might be stored for long periods of time after being thawed so that *L. monocytogenes* could grow to hazardous levels. Therefore, frozen food such as cooked vegetables and cooked meals may be considered RTE foods. Consequently, understanding how *L. monocytogenes* survives in frozen storage is important. Knowing how to select the proper methods to recover *L. monocytogenes* from frozen food samples to evaluate contamination levels is also important. This review contains information gathered from articles published on the effects of freezing on food and *L. monocytogenes*. The information in this review might be used to design methods for preventing *L. monocytogenes* contamination in frozen food.

Effect of Freezing on Food

Freezing is a method commonly used to preserve food. The mechanisms and effects of freezing on food are complex. This section discusses the effects of freezing on food and on microorganisms, with particular reference to *L. monocytogenes*.

Effect of freezing on Food

As the temperature falls to the point where freezing starts, water in food starts to become ice. As the ice crystals form in food, the concentration of the dissolved solids in the still-unfrozen water increases. This leads to a reduction of water available in the food. Thus, the

water activity (a_w) of the food becomes lower. Table 3.1. shows an example of the relationship between subfreezing temperatures and the a_w in meat.   As the water in food becomes progressively crystallized, the solute concentration is increased; therefore, the viscosity, osmotic pressure, and pH of unfrozen parts of food will be changed (23).

Table 3.1. Water activity (a_w) of meat at various subfreezing temperatures

Temperature (^oC)	a_w
25	0.993
-1	0.990
-3	0.971
-5	0.953
-7	0.934
-9	0.916
-11	0.899
-13	0.881
-15	0.864
-17	0.847
-19	0.831
-21	0.815
-25	0.784
-30	0.746

Source: Singhal and Kulkarni 2000 (23)

Freezing Point

Different foods have different freezing points, since the concentrations of substances in water, which may depress freezing point of water, vary. For example, fruit juices have lower freezing points due to their high sugar levels. Compounds such as sugars tend to maintain higher a_w levels and to depress the freezing point of water (16). Therefore, the nature and concentration of compounds in the solute of the food matrix may determine the freezing point of the food (16). Examples of the different freezing points for foods are shown in Figure 3.1.

Figure 3.1. Freezing point of selected foods

Temperature (°F)	Material
32	Water
	Lettuce, cabbage
31	Cauliflower
	Peas
30	Asparagus
	Raspberries
29	Carrots
	Beef, fish
28	Potatoes
27	Lamb
	Veal
26	
	Garlic
25	Bananas
	Coconut
24	
23	
22	
21	
20	Walnuts
19	
18	
17	Peanuts
16	
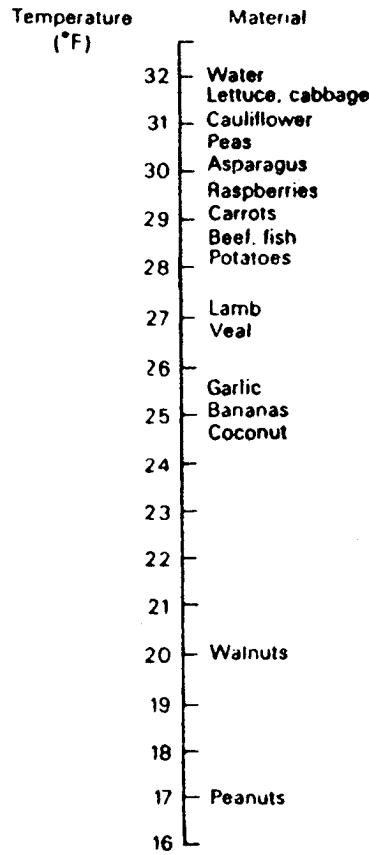
Source: Jay, 2000 (16)

Freezing Rate

Freezing starts from the outside of the food and proceeds towards the center of the food. The freezing rate can therefore be expressed as the rate at which the temperature of the food is lowered to below -20°C. This is the most important factor in frozen food preservation. Slow freezing (2 millimeter [mm]/h) is known to cause large ice crystals in food. Quick/fast freezing (5-30 mm/h) and ultra-rapid freezing (50-1000 mm/h) favor the formation of small ice crystals in food (5). The size of ice crystals affects the quality of frozen food and its shelf life. Bigger ice crystals may cause physical damage to food, disrupting cell membranes, cell walls and internal structures. Commercial freezing is usually quick/fast freezing or ultra-rapid freezing, which conserves food structures better and therefore better preserves the food quality. However, during storage, these ice crystals grow bigger and affect the quality of frozen food deleteriously. Home freezing, on the other hand, is categorized as slow freezing.

During frozen storage, desiccation may take place on the surface of the food due to water evaporation; excessive desiccation causes the removal of ice on the surface of food. This leads to the phenomenon called "freezer burn," involving the oxidation of cellular constituents since food components are exposed to oxygen which induces oxidation. Freezer burn can result in unwanted off-flavors and texture changes in food (23).

While freezing is an excellent technique for preserving food, frozen food may go through the physical and chemical changes stated above during freezing and frozen storage. Fast freezing and a shorter storage period are keys to maintaining the quality of frozen food and thawed frozen food in general. The constituents of food also affect the storage life of the food. Fatty foods such as meat may have shorter storage lives due to their propensity for oxidation whereas foods with abundant carbohydrates such as vegetables have longer storage lives since carbohydrates may act as cryoprotectants.

Effect of Freezing and Frozen Storage on Microorganisms

Freezing techniques are used to preserve microorganisms as well. However, slow freezing and extended frozen storage, especially at higher temperatures such as -2 ℃ may damage microorganisms and lead to the death of some microorganisms. In the food matrix, freezing affects microorganisms in a way similar to the way it affects the food that contains microorganisms. The activities of foodborne microorganisms are slowed around the freezing point and halted altogether at temperatures below freezing (below 0℃).

Damage due to Freezing

Temperature change itself seems to affect and damage microorganisms. This phenomenon, thermal, specifically cold, shock, seems to occur with rapid but not with slower changes in temperature (10). Depending on the microbial species, sudden death appears to happen immediately after freezing; those cells that do survive will die gradually during frozen storage. Mesophilic and thermophilic organisms tend to be more susceptible to death from freezing and frozen storage than psychrotrophs and psychrophiles (16). Also, higher temperatures (around

-2℃) seem to be more lethal than lower temperatures (-20℃) (17).

Environmental changes such as temperature drop and ice formation will affect microorganisms. As stated above, ice formation reduces a_w and increases the solute concentration and pH in the environment surrounding microorganisms. These phenomena cause microorganisms to lose their viability and weaken the integrity of cell membranes, leading to leakage of the intracellular materials, increased sensitivity to surfactants and other compounds, and to the denaturing of proteins within microorganisms. Also, as microorganisms freeze, they go through the same changes as the food matrix and may undergo permanent structural damage including the rupturing of their cell membranes. As with food quality, the freezing rate is also significant. The viability of organisms, in general, is improved when the freezing rate is increased due to the formation of smaller ice crystals (5).

The following effects have been observed when microorganisms freeze: (16)

1) Loss of internal water due to an increase in the osmotic pressure in the surrounding environment.
2) Reduced internal temperature and internal ice crystal formation.
3) Increased concentration of solute in the non-frozen water.
4) pH change (0.3 to 2 pH units) because of the increased solute concentration.
5) Changes in electrolyte concentration in the water due to the reduced internal temperature and internal ice crystal formation, which may affect covalent bindings such as those observed in the lipid-protein bond in membranes (10).
6) Ruptures in the cell membrane, with loss of its functions, including the leakage of cytoplasm (23).
7) Protein denaturing, which causes enzymes to stop functioning.
8) The loss of cytoplasmic gases such as oxygen and carbon dioxide (23).

Resistance and Survival

The ability to resist pH and a_w changes appears to be crucial for enabling microorganisms to survive at lower temperatures. Yeast and mold are more tolerant than bacteria of temperatures below 0ºC. Some yeasts are reported to grow at -34ºC (5). This is because yeast and mold can grow in an environment with a lower a_w. Table 3.2. shows the minimum reported growth

temperatures of food borne microorganisms (5). The growth of microorganisms at lower temperatures may depend upon the food that hosts them. When the freezing point of the food is very low, and the organism is resistant to the low a_w environment, the organism may grow in the food at subfreezing temperatures (16).

Table 3.2. Minimum reported growth temperatures of some foodborne microbial species and strains that grow at or below 7°C

Species/ Strains	°C	Comments
Pink yeast	-34	
Pink yeast (2)	-18	
Unspecified moulds	-12	
Vibrio spp.	-5	True psychrophiles
Yersinia enterocolitica	-2	
Unspecified coliforms	-2	
Brochothrix thermosphacta	-0.8	Within 7 days: 4°C for 10 days
Aeromonas hydrophila	-0.5	
Enterococcus spp.	0	Various species/strains
Leuconostoc carnosum	1.0	
Leuconostoc gelidum	1.0	
Listeria monocytogenes	1.0	
Leuconostoc sp.	2.0	Within 12 days
Lactobacillus sake/ curvatus	2.0	Within 12 days; 4°C in 10 days
Clostridium botulium B, E, F,	3.3	
Pantoea agglomerans	4.0	
Salmonella panama	4.0	In 4 weeks
Serratia liquefaciens	4.0	
Vibrio parahaemolyticus	5.0	
Salmonella heidelberg	5.3	
Pediococcus sp.	6.0	Weak growth in 8 days
Lactobacillus brevis	6.0	In 8 days
Lactobacillus virides	6.0	In 8 days
Salmonella Typhimurium	6.2	
Staphylococcus aureus	6.7	
Klebsiella pneumoniae	7.0	
Bacillus spp.	7.0	165 of 520 species/strains
Salmonella spp.	7.0	65 of 109 strains, within 4 weeks

Source: Chattopadhyay, 2000 (5)

In general, gram-positive bacteria are more resistant to freezing than gram-negative bacteria (5). The mucoprotein complexes and diaminopimelic acid in the cell walls of gram-positive cells prevent the membrane proteins from denaturizing (23).

Protection from Food Materials

The food matrix itself can protect microorganisms from the effects of freezing. Some food components such as proteins, peptides, sugars, and polyhydric alcohols such as glycerol are cryoprotectants. They tend to reduce the translocation of components through damaged membranes and thereby facilitate repair mechanisms enabling cells to function smoothly (23). Researchers have noted that the survival of cells is improved when glucose, sucrose, erythritol, diglycol, or polyethylene glycol exist in the medium in which microorganisms are frozen (5). Proteins and protein-related compounds such as amino acids also display their protective activity at the metabolic level, especially for the hydrogen bonds in protein molecules. Therefore, they prevent proteins from being denatured (5).

Thawing

Thawing can be more injurious to microorganisms than freezing, and repeated freeze-thaw cycles are more lethal than constant frozen storage (5). When it occurs under equivalent temperature differentials, thawing appears to be a slower process than freezing. The temperature approaches near the melting point rapidly and stays there throughout the long process of thawing, therefore allowing an opportunity for chemical reactions and recrystallization (16).

As with freezing, the rate of thawing also influences the number of microorganisms that are able to survive; higher recoveries are observed with faster freezing and thawing (23). This is because faster thawing prevents the formation of bigger ice crystals. In the course of thawing, as the temperature rises, ice crystals grow and affect the integrity of the cell membrane. Therefore, subsequent damage may result, including the leakage of water and materials with low molecular weight, the increased penetrability of certain enzymes, and the increased sensitivity of the cell to certain compounds. Consequently, the freeze-thaw process stresses bacteria and thus results in the increased sensitivity, increased lag phase, decreased generation time, and decreased growth rate of the bacteria (7).

With respect to food spoilage due to microorganisms, thawed frozen food may spoil faster than its fresh counterpart. Textural changes from freezing and thawing may help surface organisms enter into the deeper components of food and facilitate the spoilage process more quickly and thoroughly. Also, water condensation and water-soluble nutrients that appear on the surface of thawed foods may help the spoilage organisms grow faster. Furthermore, the freezing

and frozen storage may eliminate mesophilic and thermophilic organisms in the matrix and create a less competitive environment for psychrotrophs that contribute to the spoilage of food (16).

Repair

Injured cells can be repaired with appropriate recovery conditions, such as being in the presence of necessary nutrients. Injured cells can regain their normal characteristics within several hours if resuscitation occurs properly (23). This recovery occurs during the lag phase of the microorganism's growth. In the laboratory setting, non-selective media such as trypticase soy broth or agar ensure the recovery of both injured cells and uninjured cells (3). Freezing may cause extensive damage to microorganisms, and the food matrix is more complex than laboratory media; therefore, what is needed for the resuscitation of microorganisms in the food matrix is not well known. Cryoprotectants such as sugars, amino acids, peptides and glycerol in food may help microbial cell functions return to their original state (23, 26).

Listeria monocytogenes

Listeria monocytogenes has been isolated from frozen foods (7, 18, 21, 22, 24). Many studies have been performed on the effect of freezing and frozen storage on *L. monocytogenes* in laboratory media and in the food matrix. The most comprehensive review on the effect of freezing and frozen storage on *L. monocytogenes* was done by El-Kest et al. (10). A number of the different studies, including the work done by El-Kest et al., are reviewed in the following sections.

Effects of Freezing and of Frozen Storage

Golden et al. (15) investigated the effect of freezing and frozen storage in tryptose phosphate broth (at -18ºC for 14 days) on four strains of *L. monocytogenes*. They found that death occurred among only 3-6% of the *L. monocytogenes* they tested, whereas 72-80% of the *L. monocytogenes* were injured after the freezing and frozen storage treatment (15).

The effects of freezing, frozen storage, and the freeze-thaw process on *L. monocytogenes* cells in broth, phosphate buffer (PB), and tryptose broth (TB) were studied by El-Kest et al. (7). They showed that slow freezing and frozen storage at -18ºC affected *L. monocytogenes* cells

more than fast freezing and frozen storage at -198ºC. They also found that while the rates of injured and dead cells increased with repeated freeze-thaw cycles, freezing and frozen storage in PB at -198ºC for 6 months resulted in no death or injury to *L. monocytogenes*. On the other hand, freezing and frozen storage in PB at -18ºC for 1 month caused death to 87% and injury to 79% of the remaining cells. Therefore, the number of *L. monocytogenes* might not be reduced in commercially prepared frozen foods, if the pathogen is present in the food before freeze-processing.

Oscroft (19) conducted a survival study on three strains of *L. monocytogenes* in carrot and chicken homogenates at -18ºC for 29-84 days. Oscroft found that freezing and frozen storage did not affect the viable cell counts of *L. monocytogenes*.

Palumbo and Williams (20) studied the effects of freezing and frozen storage on *L. monocytogenes* in ground beef, ground turkey, frankfurters, canned corn, ice-cream mix, and tomato soup at -18ºC. In their study, *L. monocytogenes* was quantitatively recovered on *Listeria*-selective media, except for *L. monocytogenes* from tomato soup which had a lower pH than other food samples. Their study indicated that freezing and frozen storage did not seem to affect *L. monocytogenes* in low-acid food (20).

Gianfranceschi and Aureli (13) examined the effect of freezing and frozen storage on two strains (Scott A and FIL/IDF strains) of *L. monocytogenes* in several food samples–chicken breast, beef hamburger, spinach, mozzarella, and cod fish–at -50ºC for freezing, at -18ºC for frozen storage for 350 days in chicken and beef, and for 250 days in mozzarella, fish, and spinach. They found that chicken breast and hamburger provided *L. monocytogenes* with the most protection, whereas fish provided the least. After freezing, populations of *L. monocytogenes* were reduced with a range of 0.1-1.6 log CFU/g. During frozen storage, the reduction of the *L. monocytogenes* population was only 0.1-1.0 log CFU/g. These results show that the sensitivity to freezing and frozen storage may depend upon the strain type. In the study, one strain appeared to be more sensitive to freezing and frozen storage than the other.

Beauchamp et al. (2) investigated the effect of freezing/thawing on *L. monocytogenes* in frankfurters. Their study found that freezing had little effect on *L. monocytogenes*, regardless of product formulation; only when the bacteria were present in high numbers (3.9 log CFU/cm^2) did freezing result in noticeable ($\leq$ 1 log CFU/cm^2) but not significant reductions. Overall, the thawing treatments in their study did not have a significant effect on the *L. monocytogenes*

populations immediately after thawing and during subsequent aerobic storage at 7ºC (14 d); however, microwave defrosting resulted in slightly lower pathogen populations compared to the other thawing treatments or controls. This was most likely due to the ''hot-spots'' that tend to develop during microwave defrosting cycles.

Damage Mechanisms of Freezing and Frozen Storage

El-Kest et al. (11) suggested that freeze-thaw damage might be manifested by the increased sensitivity of frozen cells to lipase and lysozyme. Using transmission electron microscopy, they observed that freeze-injured cells of three strains of *L. monocytogenes* (Scott A, V7, and California) demonstrated one or more of the following: "(a) retraction of the cytoplasm and folding of plasma membrane to and from mesosomes; (b) extra- and intracellular rupture of the cell wall; (c) formation of 'bubbles' within the cell; and (d) damage to the cell wall and plasma membrane that may have resulted from autolysin activity" (11, p. 687). The results of their study also indicated that the period of frozen storage and strain type appears to determine the degree of the effect on *L. monocytogenes* (11).

Cytoprotectants

El-Kest et al. (8) investigated the protective functions of glycerol, milk fat, lactose, and casein on freeze-injured *L. monocytogenes* cells. Glycerol seemed to be the most effective of the cytoptotectants they tested. The authors further studied the effect that the suspending medium– PB, TB, or milk–had on the freeze-injured *L. monocytogenes* cells. They found that milk had the greatest and PB solution the least protective effect on the *L. monocytogenes* strains they tested (9).

Effect of Growth Temperatures against Freeze-thaw Injury

Azizoglu et al. (1) found that cold acclimation (growth at 4ºC or 25ºC) did not enhance the cryotolerance of *L. monocytogenes* against repeated freeze (-20ºC)-thaw cycles. Cultures grown at 37ºC, especially in liquid media, showed higher survival rates (< 1 log decrease) after the 18 cycles of freeze-thaw treatment. This indicated that a temperature of 37ºC may be required for protection against freeze-thaw stress.

Wemekamp-Kamphuis et al. (25) suggested the possible positive role of the general stress sigma factor (sigma B) in the survival of bacteria grown at 30ºC under repeated freezing and thawing treatment.

Resuscitation

Freeze-injured bacteria may become susceptible to many selective compounds due to damage in their membrane; this may affect the ability to detect *L. monocytogenes* in food or environmental samples. *Listeria monocytogenes* found in food or food-processing environments might be damaged by the processing steps or the harsh environment. Most standard methods utilize selective agents that limit the growth of the background microflora to detect and isolate *L. monocytogenes* from food and environmental samples; however, injured cells may not grow well on selective media (3). This may lead to false negatives that may have negative consequences from a public health standpoint. Injured cells may regain their ability to grow and become functionally normal under favorable conditions (26). With low contamination levels of *L. monocytogenes* in food products, freeze injury may differentiate between a positive and a negative screening test when selective media alone are used (6, 15). Therefore, a suitable resuscitation step must be included when *L. monocytogenes* are isolated from food samples such as frozen food. In general, resuscitation methods involve incubating a sample portion in non-selective broth media or agar plate media for 2-6 hours. The agar plating media might be more advantageous to use than broth media since the former will provide more precise results for enumeration (3).

Golden et al. (14) investigated six direct plating media as recovery media for injured *L. monocytogenes* with low to high population levels. They found that freeze-injured cells demonstrated no detectable differences in physical characteristics, regardless of the test medium examined.

Flanders et al. (12) demonstrated that both trypticase soy broth and *Listeria* repair broth provided good environments for *L. monocytogenes* recovery from freeze-injury. *L. monocytogenes* cultures sustained 44-46% injury in the first 24 hours; however, all of the injury was reversible upon thawing.

Sheridan et al. (22) also demonstrated the importance of the resuscitation method when they isolated *Listeria* from frozen food. With the thin agar layer (TAL) or overlay method, the detection level increased by 2.5 log CFU/g.

Chang et al. (4) investigated three methods for recovering freeze-injured cells. The levels of *L. monocytogenes* recovered from cell suspensions and from pork surfaces using the three methods were not significantly different from the levels of bacteria recovered from non-selective media. On the contrary, the levels of cells on the selective media were significantly reduced compared to the levels for the non-selective media. These results emphasize the importance of researchers performing the recovery steps before trying to detect and isolate *L. monocytogenes*. Of the methods tested, the thin agar layer (TAL) method appeared to be most convenient and demonstrated the best result by comparison to the result from the non-selective media with respect to recovery levels (4).

Summary

Food and microorganisms are subject to damage from freezing and frozen storage, although this damage may be reversible after proper thawing and recovery steps. Freezing and frozen storage are harmful to *L. monocytogenes* and cause injury rather death to them. *Listeria monocytogenes* are most likely to survive with rapid freezing at lower temperatures and at lower temperatures during storage. Repeated freeze-thaw treatment may reduce the population of viable *L. monocytogenes* more severely than a single cycle. However, this is not an ideal way of reducing the contamination level of this organism in food products. Food generally seems to protect *L. monocytogenes* during freezing and frozen storage, more so than does laboratory media. In some conditions, freezing and frozen storage may have only a small impact on *L. monocytogenes* in the food matrix. The degree of the impact of freezing and frozen storage on *L. monocytogenes* depends upon the strain, freezing temperatures, freezing rate, type of suspending media, and storage period. Under the right conditions, *L. monocytogenes* can regain the ability to grow after freezing and frozen storage and become hazardous.

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CHAPTER 4. Literature Review – Predictive Modeling of Microorganisms in Foods

Predictive models for food microorganisms are the techniques used to determine quantitative relationships between microbial behavior (*e.g.,* growth, survival, or death), and the intrinsic and extrinsic factors–pH, concentration of organic acids, temperatures, and gaseous atmospheres–in food (11). Determining these relationships will allow quantitative predictions of the behaviors of microorganisms in foods. Although laboratory experiments such as challenge studies are necessary to assess actual microbial populations or concentrations of toxins produced by microorganisms in food, it is impossible to perform experiments for every possible situation. Therefore, predictions made by mathematical models can be used to evaluate the safety and quality of food, and can reduce some of the burdens of laboratory experiments. Consequently, this area of study has been gaining more interest; predictive models are becoming valuable tools among food microbiologists (12, 52). In particular, many predictive models have been developed for microbial food safety (6, 15, 27, 35, 43, 58). In general, steps to develop a predictive model include: 1) designing experiments; 2) generating experimental data such as microbial growth, survival, or inactivation data; 3) developing a mathematical model to illustrate the effects of intrinsic and extrinsic factors on microbial behaviors; 4) validating the model and 5) utilizing the model. Extensive reviews of predictive modeling for microbiology have been published elsewhere (7, 34, 36, 38, 49, 50, 52). Therefore, this section focuses on a brief overview of predictive modeling for food microbiology, and the introduction of selected models, especially those concerning bacterial growth, and their application to *Listeria monocytogenes*.

Historical Trends

The concept of predictive modeling was introduced in microbiology as early as the late 1920's with thermal death time calculations –D and Z values– to achieve safe products free from the risk of *Clostridium botulinum* (54). These models were used in the fermentation industry to enhance productivity (46). In the 1960's and 70's, food microbiologists started to explore this area of study to prevent bacterial growth. The research on the efficacy of preservatives such as nitrite in sausage meat products provided the foundation for the characterization of interactions of intrinsic and extrinsic factors for predictive modeling in food microbiology (3, 14, 18). The concept and its application were first proposed in food microbiology at the beginning of the

1980's (45). In the 1980's, research efforts were made to predict the probability of formation of botulinum toxin in foods (11, 26, 30). Several predictive models have been developed for the growth of pathogenic bacteria in foods since then (6, 15, 27). One of the reasons that predictive modeling has made great progress in the field of food microbiology over the last 30 years appears to be the improvement of personal computers and software (*i.e.,* DMFit[1], Pathogen Modeling Program[2], and the Seafood Spoilage and Safety Predictor[3]). With this advanced technology mathematical modeling has become much more accessible for non-mathematicians. The trend towards a systematic approach to achieve product safety such as Hazard Analysis and Critical Control Points (HACCP), instead of end product testing, may also have contributed to the increased interest in predictive modeling in food microbiology. Furthermore, the knowledge gap with regard to microbial kinetics, such as lag phase phenomenon or the effect of intrinsic and extrinsic features, has been reduced so that more accurate equations have been developed to better describe bacterial behaviors. Consequently, predictive modeling has been utilized in hazard analysis in HACCP systems as mentioned earlier, and several food safety risk assessments (21, 31, 48, 53, 56). Many models are currently available as mentioned above, and new models are continuously proposed. As consumers demand fresher and more natural and convenient food products, such as refrigerated RTE products, the precise prediction of the behavior of pathogenic bacteria in food has became an important task among food microbiologists to ensure food quality and safety. Food microbiologists have focused especially on the development of models that predict more accurate lag phase duration and growth rate, since food microbiologists' mission is to increase the lag phase and to decrease the growth rate, in order to prevent the growth of spoilage or pathogenic bacteria in foods. The types of topics and methods that can be handled in predictive microbiology are demonstrated in Table 4.1.

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[1] U. K. Institute of Food Research. *DM Fit* v. 2.1. (http://www.ifr.bbsrc.ac.uk/Safety/DMFit/default.html)
[2] U.S. Department of Agriculture-Agricultural Research Service. *Pathogen Modeling Program*, v. 6.1. (http://pmp.arserrc.gov/PMPOnline.aspx)
[3] National Food Institute (DTU Food), Technical University of Denmark. *Seafood Spoilage and Safety Predictor (SSSP)* v. 3.1. (http://sssp.dtuaqua.dk/)

Table 4.1. Diversity of problems addressed by and methods used in predictive microbiology

Problem types	Toxin formation Shelf-life prediction - spoiler growth Pathogen growth Pathogen survival Death or inactivation - pasteurization, canning, irradiation
Model types	Death rate Probability of growth/toxin formation Growth rate Growth limits
Data collection methods	Turbidimetry Metabolite assays Viable counts Impedance/ conductance Luminometry

Source: Ross, et al., 2000 (46)

Types of Models

Nature of Models

Predictive models describe microbial kinetics with mathematical equations using parameters illustrating microbial behaviors. In general, models can be divided into two categories, empirical and mechanistic models (36).

(i) **Empirical Models**: these models are pragmatic and simply describe a set of data in a convenient mathematical relationship without underlying biological phenomena.

(ii) **Mechanistic Models**: these models were developed on theoretical bases of behavior of microorganisms. One of the advantages of this type of model is to be able to apply for further development and expansion of models.

Of late the use of mechanistic elements is more popular in developing predictive models in microbiology, although most existing models do not appear to be purely empirical or purely

mechanistic (46). Nonetheless, both types of models are totally appropriate for the assessment of microbial quality and the safety of food products. It is important to understand a model's characteristics and limitations when selecting and using an existing model.

Classes of Models

A two-step approach, using primary and secondary models, is commonly taken when developing predictive models for describing microbial behavior (12). Primary models illustrate microbial behavior with time. Secondary models describe the effects of intrinsic and extrinsic features, using the value of parameters obtained from a primary model.

1) Primary Models

After obtaining experimental data such as microbial growth, the data are fitted and analyzed by curve-fitting programs to develop a best-fit line to the data. From the curve-fitting analysis, parameters that describe microbial growth characteristics can be obtained as a function of time generally under a constant condition. These parameters characterize bacterial growth kinetics, which are the initial population density (N_0: cfu/ml), the lag phase duration (λ: h), the growth rate (μ: cfu/ml/h), and the maximum population density (n_{max}: cfu/ml) as shown in Figure 4.1. Other additional parameters are used depending upon models. Furthermore, primary models can be subdivided in two types: deterministic population models and stochastic models (9, 52).

Figure 4.1. Typical microbial growth curve–Cells inoculated from \<ENV1\> into \<ENV2\> at constant temperature conditions with indication of the natural logarithm of the initial population density n_0 and the maximum population density n_{max}, the maximum specific growth rate μ_{max} and the lag parameter λ.
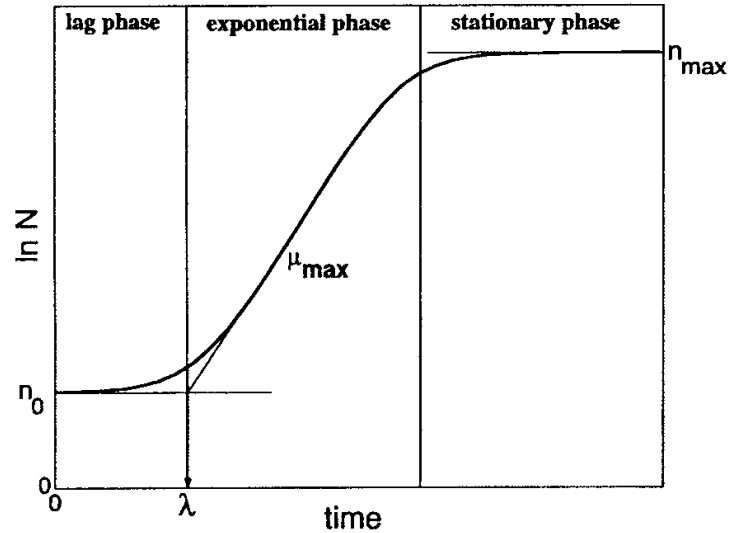
Source: Swinnen et al. 2004. (52)

i) Deterministic Population Models

With this type of model, bacterial behavior can be described by one single (deterministic) set of model parameters, e.g., N_0, λ, μ_{max}, n_{max}. No random parameter is involved. Following are some examples of deterministic population models.

a) **Gompertz (25) and Modified Gompertz (23):** this sigmoidal function has been popular because the function includes four phases corresponding to microbial growth (36). Also, the Gompertz model is used in the USDA's PMP (Pathogen Modeling Program). Its modified version has been more popular and is used in many applications. Examples of works employing this function on *Listeria monocytogenes* are those done by Buchanan and Bagi (13) and Murphy et al. (39). The original model is considered a mechanistic model, whereas the modified model is empirical. The limitations of Gompertz models are the tendencies to underestimate generation time, overestimate the maximum growth rate and lag phase duration, and calculate a negative lag phase duration for some data sets due to the nature of function (5). Gompertz models cannot be used for the growth under dynamic,

varying environmental conditions (28). Also, entire experimental growth data are needed to obtain a good fit. After all, this model was developed based upon ecological considerations not microbiological.

Modified Gompertz: $\log x(t) = A + C \cdot \exp\{-\exp \cdot [-B \cdot (t-M)]\}$ (Eq1.1)

Where $x(t)$ is the number of cells at time t (cfu/ml), A: the asymptotic count as t decreases to zero, C: the difference in value of the upper and lower asymptote, B: the relative growth rate at M, M: is the time at which the absolute growth rate is maximum (h^{-1}) (36).

b) **Barayni and Roberts** (6): This model is mechanistic. The model is developed based on the theory that bacterial cells synthesize an intercellular substance that is important to start the growth during the lag phase (6). The Baranyi-Roberts model is widely used to predict microbial growth due to its ease of adaptation with the user-friendly software, DMFit provided by the Institute of Food Research in the U.K. as mentioned above. Another advantage of the Baranyi model is that predictions can be made for a dynamic environment such as non-isothermal temperature conditions (36). Also, predictions can be obtained without data for the stationary phase. Evidently, this model is applied in many studies. Several publications utilized this model for growth of *L. monocytogenes* (2, 10, 33).

Baranyi and Roberts model: $y(t) = y_0 + \mu_{max} \cdot (t - \dfrac{1}{\mu_{max}} \ln(\dfrac{1+q_0}{e^{-\mu_{max} t} + q_0}))$ (Eq1.2)

$x(t)$ is defined above, x_0 is the initial number of cells (cfu/ml), $y(t)$ is the log of number of cells at time, t (log cfu/ml); y_0 the initial number of cells in ln (log cfu/g); μ_{max} is the maximum specific growth rate (h^{-1}); $q(0)$ represents the physiological state of the inoculum (9).

c) **Hills and Wright** (27)**:** Hills and Wright developed this mechanistic model based on a two-compartment concept, using the phenomena of cell biomass and DNA synthesis (36).

The equations of the Baranyi, Hills, and Wright models appear to be the same, although they are based on different biological hypotheses (9, 52).

Hills and Wright model: $y(t)=y_0+\mu_{max}\cdot(t+\dfrac{1}{\mu_{max}}\cdot\ln(\dfrac{v}{\mu_{max}+v}+\dfrac{\mu_{max}}{\mu_{max}+v}e^{-(\mu_{max}+v)t}))$ (Eq1.3)

$y(t)$, y_0, and μ_{max} are defined above, v is the rate of chromosome replication (9).

d) **Mckellar** (35)**:** this model was developed based upon the concept of heterogeneous populations, growing cells, and non-growing cells during lag time. The equation of this model is equivalent to the equation of the Baranyi and Roberts model, although the two models were developed using two different hypothetical concepts. Therefore, this model has not been utilized as much as the Baranyi and Roberts model (9, 36).

Mckellar model: $y(t)=y_0+\mu_{max}\cdot(t+\dfrac{\ln((1-\alpha_0)e^{-\mu_{max}t}+\alpha_0)}{\mu_{max}})$ (Eq 1.4)

$y(t)$, $y_{(0)}$ and μ_{max} are defined above, α_0 is the proportion of growing cells in the population (9).

ii) Stochastic Models

The stochastic model considers the variability between the individual cells. Therefore, the effect of all influencing factors can be included at cell level including the effect of inoculum size. Model parameters are therefore random variables. Within the stochastic modeling approaches, dynamic and more flexible models are becoming popular (9).

a) **Buchanan et al.** (15): mechanistic three-phase linear model. This model was developed based upon the hypothesis that the variance associated with a bacterial cell's adjustment period and metabolic period are very small. Therefore, the shift from the lag phase to exponential growth is assumed to be sudden. The advantage of this model is that it is very simple and straightforward, therefore easy to use. However, this model appears to give shorter population lag phase duration values (52). Whiting and Bagi (55) used this three-

phase linear model for developing a model for the growth of *L. monocytogenes*. As with the Baranyi-Roberts model, the modified version–the two-phase linear model–can also permit predictions without data from the bacterial stationary phase. Several studies have been published using the two-phase linear model (19, 20, 40, 41).

Buchanan Three-Phase Linear Model:

Lag phase: For $t \leq t_{lag}$, $y(t)=y_0$

Exponential growth phase: For $t_{lag}<t<t_{max}$, $y(t)=y_0+\mu(t-t_{lag})$ \qquad (Eq 1.5)

Stationary phase: For $t \geq t_{max}$, $y(t)=y_{max}$

y_0, $y(t)$ and μ_{max} are defined above, where y_{max}: the log of the maximum population density supported by the environment (log cfu/ml); t: the elapsed time; t_{max}: the time when the maximum population density is reached (h); μ is the specific growth rate (log cfu/ml/h) (52).

b) **Baranyi** (4): This model is empirical. Baranyi developed this model based on the theory that there are the individual cell's lag time (τi) and the population lag time (λ). The theory for this model is actually similar to the one from McKellar' model (9, 35). The equation is identical to Hills and Wright (9, 52).

2) Secondary Models

Secondary models describe the effect of the physicochemical environment including temperature, gaseous atmosphere, salt and/or water activity, pH and organic acids, spices, smoke etc. on microbial behaviors using the parameters obtained from a primary model. Among these influencing factors, temperature is the most important factor for microbial growth. Therefore, many models have been developed to describe the effect of incubation temperature, *e.g.*, the square-root model, Cardinal Parameter models, linear Arrhenius, and non-linear Arrhenius models (47). For a more complex system, polynominal or response surface analysis can be applied. Extensive reviews have been done for the development and application of secondary models on growth rates and lag phase (12, 29, 34, 38, 50, 54). Ross and Dalgaard (47) presented a comprehensive list of secondary models. Therefore, this section touches upon only square-root models as an example of secondary models.

i) Square Root Models

Temperature is the major environmental factor influencing bacterial behavior in food. In particular, refrigerated foods such as RTE foods are becoming popular as consumers demand more convenience. Regarding refrigerated foods, lag duration and growth rate are affected most by the incubating temperature (58). Ratkowsky et al. (44) proposed a simple empirical model to describe the effect of temperature. This simple model and its numerous expansions are termed Square Root models. The Square Root models have been extensively studied and used for especially refrigerated products (47).

a) **Growth rate Models**

Square Root model for growth rate: $\sqrt{\mu_{max}} = b \cdot (T\text{-}T_{min})$ (Eq 2.1)

μ_{max}: the maximum growth rate; b: a constant; T: temperature; T_{min}: a theoretical minimum temperature at which no growth is possible. This is also the intercept between the model and the temperature axis (43).

Based upon the experimental data and the values from a primary model, the values of b and T_{min} in the above equation can be obtained by model-fitting techniques. The equation as shown above can be used to describe the specific growth rate for the temperatures from the minimum temperature at which growth is observed to just below the optimum temperature (43). Ratowsky et al. expanded the above equation to cover the whole biokinetic range of growth temperatures as follows (43). T in above equations is temperature in absolute degrees. However, any temperature scale can be applied since these equations involve the difference between temperatures (42). The simulation of the both equations is shown in Figure 4.2 (47).

Square Root model for growth rate: $\sqrt{\mu_{max}} = b \cdot (T\text{-}T_{min}) \cdot (1\text{-}\exp \cdot (c \cdot (T\text{-}T_{max})))$ (Eq 2.2)

b: a constant as c; T and T_{min}: are defined above. T_{max}: a theoretical maximum temperature beyond which growth is not possible.

Figure 4.2. Simulation of Equation 2.1 (solid line) and Equation 2.2 (dashed line)
$b = 0.025h^{0.5}/°C$, T_{min}=-8 °C, c=0.30 °C^{-1}, and T_{max}=40 °C
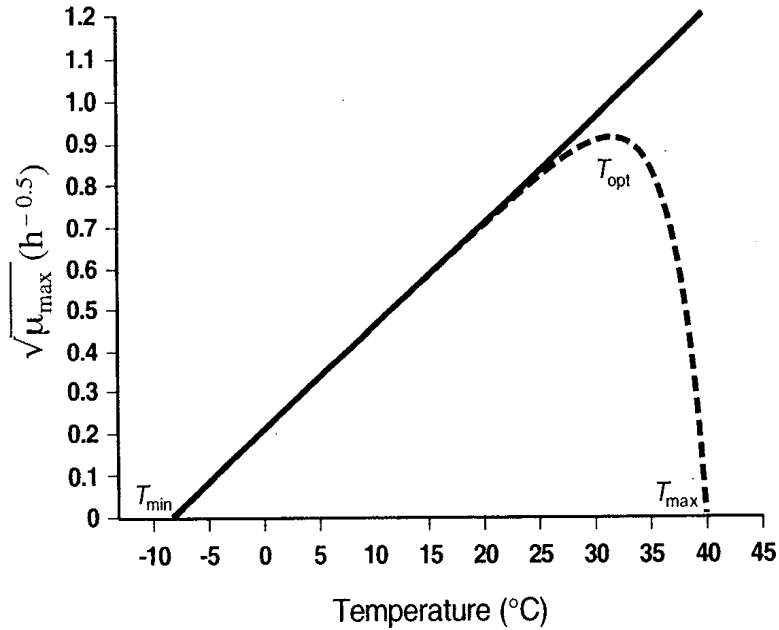
Source: Ross and Dalgaard, 2003 (47)

b) **Examples of Other Square Root Models**

For growth rate, the combined effect of temperature and other factors such as water activity, pH, and carbon dioxide level can be described with the square-root model. Some examples are shown below.

• **Water Activity** (37)

$$\sqrt{\mu_{max}} = b \cdot (T\text{-}T_{min}) \sqrt{a_w - a_{w\,min}}$$
<div align="right">(Eq 2.3)</div>

a_w: water activity, a_{wmin}: the theoretical minimum water activity below which growth is not possible. Other parameters are previously defined.

• **pH** (1)

$$\sqrt{\mu_{max}} = b \cdot (T\text{-}T_{min}) \sqrt{pH_w - pH_{w\,min}}$$
<div align="right">(Eq 2.4)</div>

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pH$_{min}$: the theoretical minimum pH below which growth is not possible; other parameters are previously defined.

ii) Lag Phase Duration

The Square Root model can be used for the prediction of lag phase duration as the growth rate prediction. Smith demonstrated the Square Root of inverted lag time had a linear relation to incubation temperature (51).

Square Root model for lag time: $\sqrt{1/\lambda} = b\cdot(T\text{-}T_{min})$ (Eq 2.5)

Zwietering et al. (58) used the inverted growth rate equations - the same as Eq 2.2 - and further log-transformed the equation before fitting the data.

Square Root model for lag time: $\ln\sqrt{\lambda} = \ln(b\cdot(T\text{-}T_{min})\cdot(1\text{-}\exp\cdot(c\cdot(T\text{-}T_{max})))^{-1})$ (Eq 2.6)

Duh and Schaffner demonstrated the log-transformed inverse of the Square Root model (below) and their new model fit the data best (17).

Square Root model for lag time: $\ln\sqrt{\lambda} = \ln((b\cdot(T\text{-}T_{min}))^{-1})$ (Eq 2.7)

iii) Lag Phase

In the field of predictive food microbiology, attempts have been made to obtain more accurate estimates for lag phase (λ) and maximum growth rate (μ). In comparison to the development of growth rate models, developing lag time models that estimate accurate lag phases are much more difficult since the lag phenomenon is still not clearly understood (9). To create a mathematical equation that can illustrate the lag phenomenon is challenging. Furthermore, there are many factors influencing lag behavior. To incorporate those factors in an equation is complicated. Consequently, accurate predictions of the lag phase are very difficult to achieve. The following are core factors influencing lag time duration (52):

- The new environmental conditions.

- The character and the phenotype of the bacterium.

- The physiological state of cells, their exponential growth stage, stationary stage, and so forth.

- The physiological history of the cells.

- The inoculum size.

- The distribution condition within the food.

Therefore, whatever model is used, it is important to consider the imprecision of lag time predictions (9). It is important to recognize that the model can only describe the simplified form of real phenomena.

Validation of a Model

Errors enter into each step of creating a model. Therefore, predictions from the model will not match actual observed data perfectly. Consequently, it is important to assess the reliability of the models. This step is called validation. Sources of relevant error in developing models are shown in Table 4.2. Among these sources of error, the quality of data and the program for curve-fitting appear to affect the model performance most (9, 36). Another important point is that models should be used to make predictions within the range of conditions that were used in developing the models, termed their interpolation region (8).

Table 4.2. Sources of error in models in predictive microbiology

Error type	Error source
Homogeneity error	Arises because some foods are clearly not homogeneous and/or, at the scale of a microorganism, apparently consistent foods may comprise many different microenvironments. Current predictive models do not account for this inhomogeneity of foods.
Completeness error	Arises because the model is a simplification, *i.e.*, only a limited number of environmental factors can be included in the model in practice.
Model function error	Arises mainly from the compromise made when using empirical models; the model is only an approximation to reality.
Measurement error	Originates from inaccuracy in the raw data used to generate a certain model, i.e., due to limitations in our ability to measure accurately the environment and the microbial response.
Numerical procedure error	Includes all errors that are the consequences of the numerical procedures used for model fitting and evaluation, some of which are methods of approximation only. Generally, these are negligible in comparison with the other types of errors

Source: Ross, et al. 2000 (46)

To assess the performance of a model that has been developed, validation can be done internally by using the data set to develop the model, and externally by using a new data set from additional challenge studies or by using data obtained from other literature. Evaluation of the accuracy of a model that has been developed can be done by a graphical comparison of the observed data against corresponding predictions of the model, or by mathematical and statistical indices, goodness-of-fit, such as mean square error (MSE), the regression coefficient (r^2), the root mean square error (RMSE), the bias factor, and the accuracy factor (24, 42). These mathematical and statistical tools can be used to compare the performance of different models as well. Giffel and Zwietering (24) emphasized the use of a set of criteria when assessing the performance of models, especially the graphical comparison of values.

Comparison of Models

There are many articles that discuss the comparison of performance of different existing models (7, 9, 15, 36, 52, 57). Measures to compare models' performance can be assessed using a goodness-of-fit as mentioned earlier. Also, the t-test and F-test can be used (57). Ultimately, there appears to be no single perfect model which can describe any bacterial behavior precisely. Careful selection should be made when choosing a model based upon the model's characteristics and data condition, environmental factors, and the types and conditions of organisms. Ross and Dalgaard listed some indices when selecting models (47). For example, if simplicity is the main factor in choosing a model and not many environmental factors are involved, Buchanan's three-phase model, involving a primary model and a square-root model for a secondary model are sufficient to develop the predictive models for bacterial growth.

Summary

The predictive modeling of food microbiology is a valuable tool for the assessment of microbial behavior in food, and thus the safety and quality of food. Predictive models can provide the 'first estimate' of microbial responses in food products under defined conditions. Therefore, they can eliminate many extensive laboratory studies and fine-tune the area where the experiment is really necessary. Consequently, in the food industry, predictive models can be used to design new products, to assess the shelf life of products, and to take remedial actions when defective products or deviation occurs. Thus, this area of study has become an important part of systematic approaches such as HACCP systems and risk assessments to accomplish food safety. Government agencies and international organizations have already been utilizing predictive models for their extensive risk assessment studies. User-friendly software to make predictions will contribute to widespread use of predictive modeling among food microbiologists. However, recognizing the limitations of predictive modeling is important. Predictions from models cannot be exactly the same as the data from challenge studies due to uncertainty, errors, and variability associated with the physiology of microorganisms, the nature of food, and the modeling construction process. Most existing models were developed using laboratory media and not real foods. On the other hand, as our technologies to create and utilize new models advance, and as information on the physiological and ecological kinetics of microorganisms in food becomes available, building realistic, mechanistic predictive models that can express

microbial responses in food will become more feasible. Sometimes, a few cells of pathogenic organisms in food can be responsible for their outgrowth and outbreak. Therefore, there has been a trend in predictive microbiology toward a stochastic approach. This involves studies on the behaviors of single cells, growth boundary models, and the use of Monte Carlo simulation software used in risk assessments (16, 22). Additionally, more flexible, dynamic models that can include the effects of changing temperature will be valued since the temperatures of food during processing, storage, and distribution vary and possibly fluctuate. Using predictive modeling as a tool will make it possible for the food industry and government agencies to take proactive steps toward ensuring food safety and quality.

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CHAPTER 5. The Growth of *Listeria monocytogenes* in Thawed Frozen Foods

Introduction

Listeria monocytogenes is a gram-positive rod-shaped bacterium that causes listeriosis in humans. Invasive listeriosis can cause serious diseases such as meningitis, pneumonia, and septicemia and death among susceptible groups such as infants, the elderly and immuno-compromised people (15, 17, 21). Pregnant women may develop flu-like symptoms and further experience miscarriages or stillbirth (15, 17, 21). While outbreaks associated with *L. monocytogenes* are relatively rare, the mortality rate is high. According to the Centers for Disease Control and Prevention (CDC), 1600 cases of listeriosis occur annually of which 260 are fatal (5). Although this pathogen is ubiquitous in the environment (24), it can be readily inactivated in food by pasteurization and cooking (3). Post-process contamination with this pathogen on cooked, refrigerated ready-to-eat (RTE) foods that support the growth of *Listeria* are associated with listeriosis outbreaks if these foods are held for extended periods to allow growth of the listeria to high levels and are then consumed without an additional cook step (11, 15, 32). In the1980s, the Food and Drug Administration (FDA) and U.S. Department of Agriculture (USDA) Food Safety and Inspection Service (FSIS) established a "zero-tolerance" policy for *L. monocytogenes* in RTE foods, defined as the absence of *L. monocytogenes* in a 25g sample of food (26). Since then, several risk assessments of *L. monocytogenes* in RTE foods have been conducted to better understand the risk of consuming *L. monocytogenes* and its effect on public health (11, 32).

In February 2008, FDA released a draft Compliance Policy Guide (CPG) that proposed that RTE foods that do not support the growth of *L. monocytogenes* may contain up to 100 CFU/g of the organism (31). Frozen foods such as ice cream that are consumed while still frozen may fall in that category. However once a frozen food is thawed, held refrigerated and consumed without further cooking, any *L. monocytogenes* present may be able to grow on the thawed food, representing a potential health hazard. A survey on the prevalence of *L. monocytogenes* in Portugal showed that from 14.8% to 22.6% of frozen vegetable samples were positive for this organism (18). Another investigation demonstrated 26% of frozen seafood samples, including frozen cooked shrimp and crabmeat, were positive for *L. monocytogenes*

overall (33). The CPG defines RTE food as food that is customarily eaten without first being cooked by the consumer, even if there are cooking instructions provided on the label. Cooked and frozen shrimp and crabmeat, along with Individually Quick Frozen (IQF) peas and corn, may be considered to be such RTE foods. These types of foods may be thawed and held refrigerated by the consumer or at salad/food bars for extended periods of time. Since *L. monocytogenes* can grow at refrigeration temperatures, holding these foods for extended periods may allow this pathogen (that may have been present at less than 100 CFU/g in a frozen state) to grow to levels that represent a public health concern. The 2009 Food Code allows for the storage of "time/temperature control for safety" (TCS) foods at $\leq$ 5ºC for up to 7 days based on controlling the growth of *L. monocytogenes* to no more than 1 log (30).

There is a gap in the knowledge concerning the growth kinetics of *L. monocytogenes* in thawed frozen foods held at refrigeration temperatures for several days. It is also important to acknowledge that refrigerated foods may be held at what is considered to be abusive temperatures at retail food service outlets and during home storage. EcoSure conducted a survey of product temperatures at retail locations and in consumer home settings (7). According to the survey, 30.7% of products had temperatures higher than 5ºC (41ºF) in retail refrigerators. Many products (9.4%) were found to be stored at greater than 5ºC in retail backroom refrigerators. The same study also showed that 16.8% of products were stored at temperatures exceeding 5ºC in consumers' homes. This indicates that compliance with the 2009 Food Code may be a challenge for some retail food service outlets and that improper storage of refrigerated foods continues to be an issue at the consumer level. Cold salad bar settings may provide more opportunities for temperature abuse and extended storage of food if not properly managed. One investigation revealed that the food surface of potato salads had higher temperatures (13-16ºC) at salad bars although all salad bars investigated seemed to be set to an "ice chilled" condition (28). The same study observed food handling practices that might lead to prolonged display and storage of food items when mixing fresh food and old batches of food that are left over on the salad bar (28). This investigation revealed difficulties in salad/food bar settings in controlling food temperature, and in monitoring how long food products have been exposed to potential temperature abuse. This lack of controls represents a potential food safety risk by exposing consumers to high levels of *L. monocytogenes*.

The current project used mathematical models to determine and predict the Lag Phase Duration (LPD) and the Exponential Growth Rate (EGR) of *L. monocytogenes* on several thawed frozen foods stored in the temperature range of 4-20ºC. Knowledge of the length of the lag phase of this organism could provide more accurate handling guidance for frozen RTE foods that are thawed and subsequently held at refrigeration temperatures.

Materials and Methods

Listeria monocytogenes Strains and Culture Conditions

Twelve strains of *L. monocytogenes* from the Grocery Manufacturers Association (GMA) Culture Collection (Washington, DC) used in this study included 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: BD/Difco, Becton Dickinson and Co.; Sparks, Md., USA) with 0.6% yeast extract (YE: BD/Difco) slants at 4ºC and transferred every six months. Before inoculation, a loopful of each strain was transferred in 10 ml of tryptic soy broth (TSB: BD/Difco) with 0.6% YE (TSB+0.6%YE) and grown aerobically at 35ºC for 24 h (stationary-phase culture).

Inocula and Food Sample Preparation

One hundred µl of each stationary-phase culture, approximately 10^9 CFU/ml, was transferred to an individual 10 ml TSB+0.6%YE tube. Cultures were incubated at 4ºC for 7 days for cells to adapt the cold conditions (25). After a 7-day incubation, each culture had reached approximately 10^8 CFU/ml. All twelve 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 diluted in 0.1% peptone water (PW: pH 7.0, Fisher Scientific, Fair Lawn, New Jersey, USA) to give an initial density of about 10^3 cells per g and inoculated into 25 g of food samples placed in a filtered stomacher bag

(Whirl-Pak®, 24 oz. NASCO, Fort Atkinson, Wis., USA). The initial inouclum level of 10^3/g is quite high in comparison to contamination levels in real food products (14). This level was chosen because it allowed enumeration with plating methods (25). Four kinds of frozen food samples, corn, green peas, cooked snow crabmeat, and cooked salad shrimp without shell, were obtained from a local grocery store and by mail order. Prior to inoculation, the food samples were weighed in a stomacher bag and kept frozen at -18ºC. Crabmeat from frozen cooked snow crab was extracted from the shell as a part of sample preparation before the weighing process. Test samples were inoculated with 100 µl aliquots of the prepared suspension which was distributed randomly over the product surface. The inoculated product was gently shaken to assist inoculum distribution and stored frozen at -18ºC for 7 days. After the 7 day frozen storage, the inoculated food samples were incubated at 4, 8, 12 or 20ºC for up to 20 days.

Enumeration of *Listeria monocytogenes*

The growth of *L. monocytogenes* was measured by plate counts as follows. At predetermined time intervals, samples were removed from the incubators and pulsified with a Pulsifier (Microgen Bioproducts, Ltd. Camberley Surrey, UK) in a 1:10 dilution of buffered peptone water (BPW: 3M, St. Paul Minn., USA) for 30 seconds. From the initial dilutions, further decimal dilutions of samples were made with PW as needed and dilutions were plated onto Polymyxin-acriflavin-lithium chloride-ceftazidime-aesculin-mannitol (PALCAM) agar (BD/Difco) using a spiral plater (model AP 4000, Spiral Biotech, Norwood, Mass, USA). Resuscitation steps for injured cells were not necessary based upon our preliminary experiments. Plates were incubated at 35ºC for 48 h. Cell counts were obtained using a Q count system (model 510, Spiral Biotech). Three independent growth experiments were conducted for each food type at each storage temperature (4, 8, 12, and 20ºC). Prior to each individual growth experiment, random samples from the four types of thawed frozen foods were tested to confirm they were *L. monocytogenes*-negative by using an automated VIDAS analyser (bioMérieux, Marcy-Etoile, France).

Background Microflora and Informal Sensory Analysis

Aerobic plate counts (TSA plates incubated at 35ºC for 48 h) and informal sensory analysis of non-inoculated samples were conducted for all incubation temperatures to observe the relationship between product spoilage and the concurrent growth of *L. monocytogenes* on inoculated foods. The sensory analysis was generally conducted by three or more lab personnel (untrained). Each non-inoculated sample was evaluated for off-odor and acceptability of samples in 5 grades (*off odor*: 1-no off odor, 2-slight off odor, 3-moderate, 4-strong, 5-extremely strong, *acceptability*: 1-acceptable, 2-hesitant, but might eat, 3-not acceptable, 4-reject, 5-absolute reject).

Curve-fitting and Regression Analysis

Each of three replicate experiments of growth curve data were iteratively fit [Excel Solver (worksheet provided by Dr. Richard Whiting (Exponent, Inc.))] to the two-phase linear growth equation (2, 27):

$$N = N_0 + IF [t < LPD, N_0, EGR \times (t - LPD)]$$

Where N = log CFU/g at time t, t = the elapsed time, N_0 = initial log CFU/g, LPD = Lag Phase Duration (hours) and EGR = Exponential Growth Rate [(log CFU/g)/hour]

The average predicted values of three replicate runs and the standard deviation for each food / temperature combination were calculated.

Prediction of Lag Phase Duration by Using Secondary Model

Lag Phase Durations were calculated with the inverted Square Root model (6).

$$\sqrt{1/LPD} = a(T-T_{min})$$

Where LPD = Lag Phase Duration (hours), a = a constant; T = temperature, T_{min} = a theoretical minimum temperature at which no growth is possible.

Prediction of Exponential Growth Rates by Using Secondary Model

Exponential Growth Rates were evaluated with the Square Root model (6, 23).

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

EGR = Exponential Growth Rates [(log CFU/g)/hour)], a, T, and T_{min} are defined above.

Fitting of the models was done by minimizing the residual sum of squares (RSS) with the solver function in MS Excel. Standard deviations were calculated for LPDs and EGRs from two-phase linear model with MS Excel. The goodness of fit of square root models was evaluated by the residual mean squares (R^2) (12).

Results and Discussion

Growth of *Listeria monocytogenes* in Four types of Thawed Frozen Foods

According to the 2008 FDA Draft Compliance Policy Guidance document (31), frozen foods meet the regulatory requirement of not supporting the growth of *Listeria monocytogenes.* It was proposed that consumption of these foods in the frozen state when containing less that 100 CFU/g of *L. monocytogenes* would not represent a public health hazard. However, once the foods are thawed to refrigeration temperatures, they would no longer meet the definition of a RTE food unless it could be shown that the food did not support the growth of this pathogen.

Using twelve strains of *L. monocytogenes* that were grown at 4ºC for 7 days to acclimate them to refrigeration temperatures, frozen samples of corn, peas, shrimp and crabmeat were inoculated and held frozen for an additional 7 days. Upon placing the inoculated samples at 4, 8, 12 or 20ºC, growth was monitored for up to 20 days. Examples of curves represent the growth of *L. monocytogenes* at the four storage temperatures in thawed frozen corn are presented in Figure 5.1 (a-d). As expected, growth occurred much more rapidly as the storage temperature increased. For all of the temperatures and food samples, it was difficult to visually determine a lag phase duration or length of time that growth did not occur during storage at each temperature. For that reason, primary models were created to determine the lag phase duration (LPD) and exponential growth rate (ERG) for *Listeria* on each food type at each storage temperature. Growth studies were carried out to at least the late exponential growth phase or the stationary phase to provide the data necessary to calculate the lag phase using the two-phase linear model (2, 8, 9, 19, 20).

Figure 5.1. Growth of *Listeria monocytogenes* in thawed frozen corn samples stored at 4, 8, 12 and 20ºC (Test 1, 2, 3 represent triplicate replications).
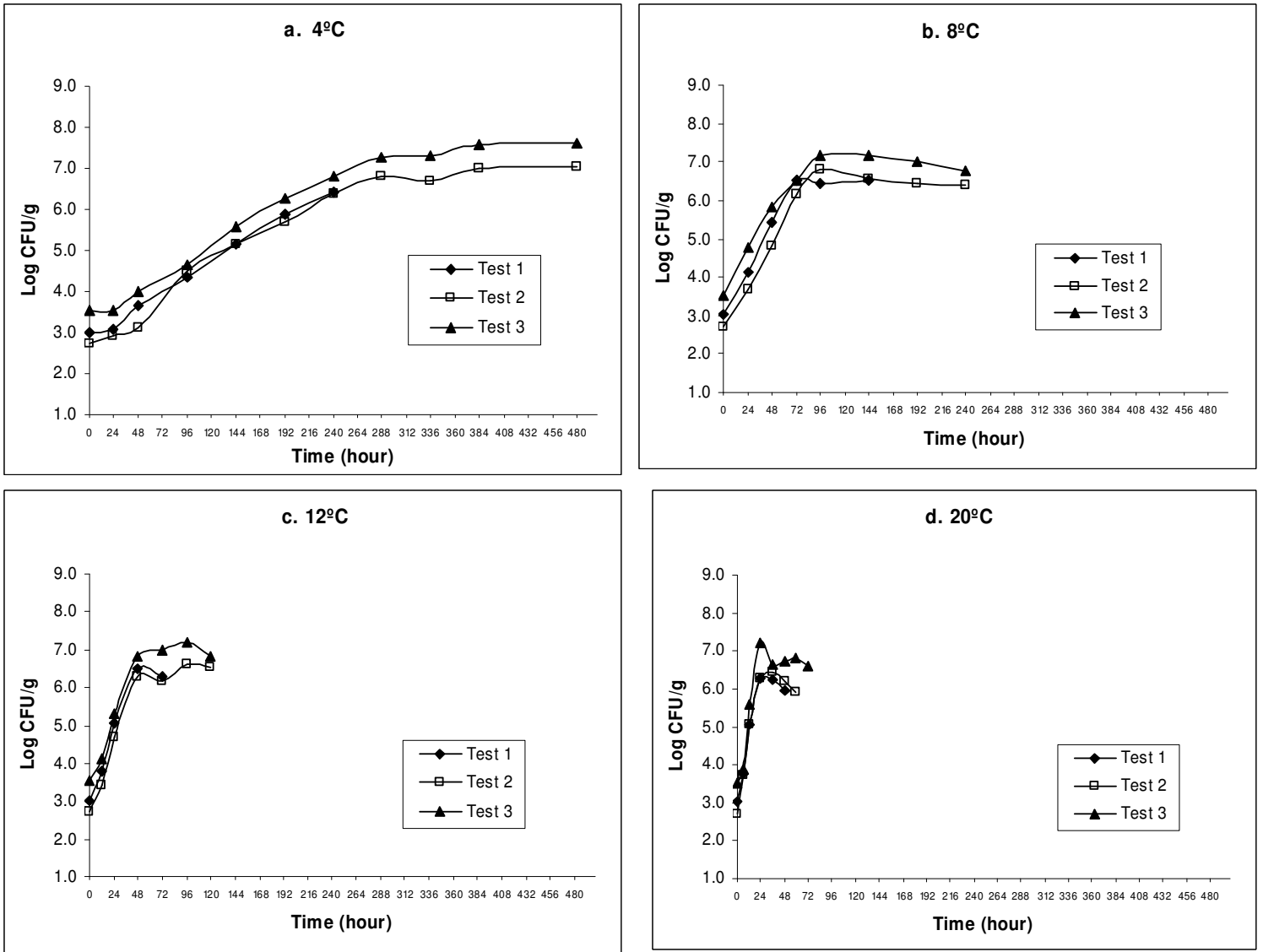
Calculation of LPD and EGR

Data from the three replicate growth curves for each food and storage temperature were fit to the two-phase linear model to determine the lag phase duration (LPD) and exponential growth rate (EGR) values. For the purposes of this study, it was not necessary to determine the prediction for the maximum population.

Mean and standard deviation for the calculated LPD and EGR for each condition are presented in Table 5.1. As expected, the LPDs decreased with increasing storage temperatures, while the EGRs increased with increasing temperature. LPDs in crabmeat and shrimp were longer than in the vegetables; however, the EGRs were similar in all food types. These values were compared to those from the USDA Pathogen Modeling Program (PMP) using salt and pH values similar to those of the food samples (29). For all four temperatures, the PMP predicted a longer LPD and a more rapid EGR than was determined in the current study. We originally hypothesized that freezing of the cultures may have created an extended lag phase for this organism once the foods were thawed and held at refrigeration temperatures (34). However, this was not observed. Differences in predictions for LPD compared to those from the PMP 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; *Listeria* is known to be resistant to injury due to freezing in food and broth systems (10, 22). Beauchamp et al. (1) found that various methods of thawing of frozen hotdogs had little effect on survival and growth of *L. monocytogenes* during refrigerated storage.

Table 5.1. Lag Phase Duration and Exponential Growth Rate of *Listeria monocytogenes* in four food types calculated using a two-phase linear model as compared to a USDA Pathogen Modeling Program (PMP) prediction suggests for same foods

	Predicted Lag Phase Duration ± SD (hour)			
	4ºC	8ºC	12ºC	20ºC
Crabmeat	18.79±14.56	11.79±7.39	6.80±1.77	5.48±1.70
Corn	12.12±5.86	0.32±0.37	1.15±1.10	0.82±1.42
Green peas	4.33±5.43	1.75±2.30	2.80±3.43	3.50±1.88
Shrimp	24.07±20.96	2.51±4.07	5.91±1.33	4.13±1.79
USDA PMP[a]	*62.03*	*31.77*	*17.3*	*6.17*

	Predicted Exponential Growth Rate ± SD [(Log CFU/g)/hour]			
	4ºC	8ºC	12ºC	20ºC
Crabmeat	0.0137±0.0023	0.0469±0.0114	0.0792±0.0036	0.2035±0.0175
Corn	0.0158±0.0006	0.0440±0.0062	0.0752±0.0026	0.1549±0.0199
Green peas	0.0149±0.0002	0.0470±0.0042	0.0851±0.0032	0.2228±0.0257
Shrimp	0.0138±0.0005	0.0438±0.0065	0.0886±0.0081	0.2256±0.0119
USDA PMP[a]	*0.026*	*0.055*	*0.106*	*0.308*

[a] – Assumptions made for the USDA PMP model: Aerobic, broth culture, pH (6.5), NaCl (0.5%), sodium nitrite (0%)

Square Root Model Prediction of LPD and EGR

Using the predictions of the two-phase model for LPD and EGR (Table 5.1.), secondary models were developed by creating linear regressions of the Square Root model results (6, 23). These results are presented in Figures 5.2. and 5.3. and Table 5.2. For the LPD prediction, the square root of the inverse of the LPD prediction from the primary linear model was plotted against temperature to provide the secondary models for each food type. The predicted LPDs were similar for crabmeat and shrimp. The LPD of *L. monocytogenes* on corn was greatly affected by temperature increase. However, there was little effect of temperature on LPD of *L. monocytogenes* on peas. For calculation of EGR values over the temperature range of 4-20ºC, the square root of the EGR values from the primary linear model was plotted against temperature. Linear regressions of these plots created the models for each food type. The predicted EGRs were similar for all of the foods with the exception of growth on corn at higher temperatures, where growth was relatively slower. Predicted lag phase duration values at 4ºC from the Square Root model (Table 5.2.) show that there was a much shorter lag phase of *L. monocytogenes* predicted for the vegetables than was predicted for the seafood. This lag phase represents the time when no growth occurred. Thawed frozen vegetables had a predicted LPD of less than 8 h and thawed frozen seafood had a predicted LPD of less than 24 h at 4ºC. According to the 2008 FDA CPG, foods that do not support the growth of *L. monocytogenes* and which contain less than 100 CFU/g should be safe to consume. It should be noted that the FDA 2009 Food Code allows for the holding of food that is temperature controlled for safety for up to 7 days (168 h) at 5ºC or less (30). The Food Code recommendation is based on the USDA Pathogen Modeling Program estimate for 1 log of growth of *Listeria monocytogenes*. The Food Code does not establish an acceptable number of *L. monocytogenes* in food. Using the predicted values in Table 5.2., the earliest time (including the predicted LPD) for 1 log of growth at 4ºC on the thawed food products used in the current study are as follows: crabmeat (83.2 h), corn (63.0 h), green peas, (70.5 h) and shrimp (91.1 h). These times are much shorter that the 168 h at 5ºC allowed in the FDA 2009 Food Code. These results correspond well with the results of Lianou et al. (16), who demonstrated more than 1 log CFU/cm^2 of *L. monocytogenes* population increase in turkey breast without preservatives when stored at 7ºC for three days. These observations show

that the Food Code guidelines outlined above may need to be re-assessed regarding refrigerated retail storage of foods that support growth of *L. monocytogenes*.

Figure 5.2. Square Root Model predictions of the inverse Lag Phase Duration (hour) of *Listeria monocytogenes* for crabmeat, corn, green peas, and shrimp over the temperature range of 4 – 20ºC
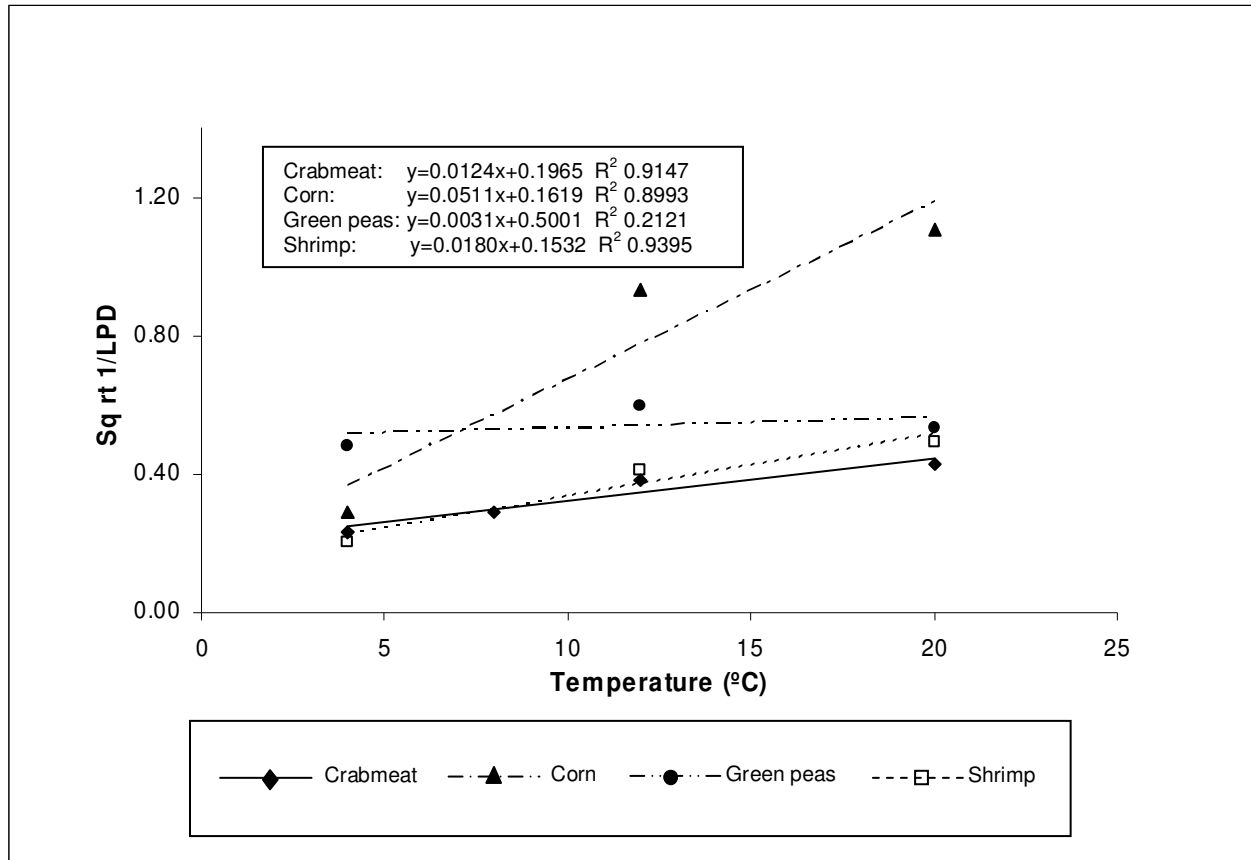
Figure 5.3. Square Root Model predictions of the Exponential Growth Rate [(Log CFU/g)/hour] of *Listeria monocytogenes* for crabmeat, corn, green peas, and shrimp over the temperature range of 4 – 20ºC

The chart legend shows:

Crabmeat: $y=0.0205x+0.0409$ R^2 0.9966
Corn: $y=0.0165x+0.0695$ R^2 0.9936
Green peas: $y=0.0217x+0.0372$ R^2 0.9989
Shrimp: $y=0.0223x+0.0295$ R^2 0.9999

Y-axis: $\sqrt{EGR}$
X-axis: Temperature (ºC)

Legend: Crabmeat — Corn — Green peas — Shrimp

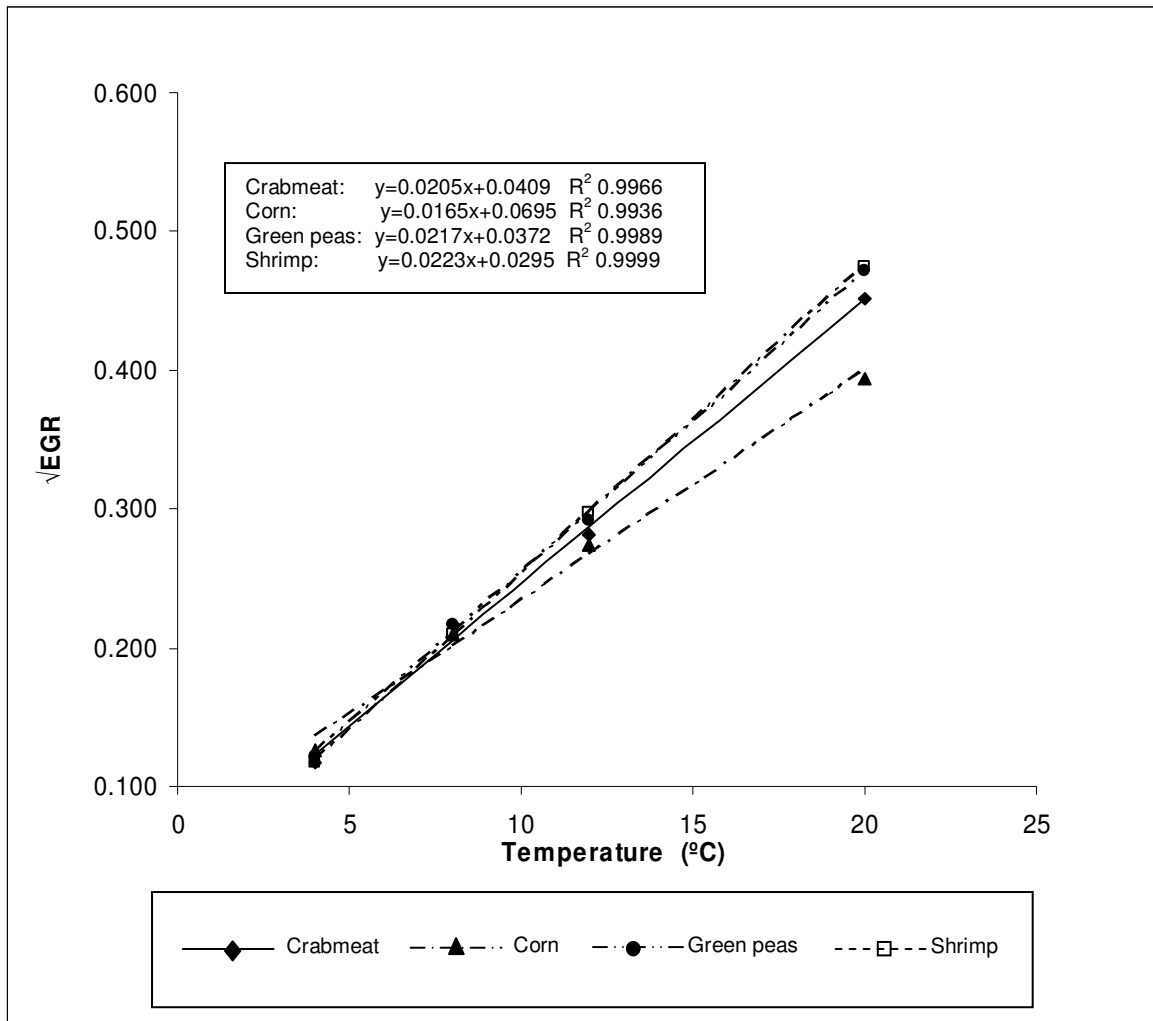**Table 5.2.** Lag Phase Duration and Exponential Growth Rate predictions of *Listeria monocytogenes* for crabmeat, corn, green peas, and shrimp based on the Square Root Model (secondary model).

	Predicted Lag Phase Duration (hours)			
	4°C	8°C	12°C	20°C
Crabmeat	16.50	11.42	8.37	5.05
Corn	7.46	3.07	1.67	0.71
Green peas	3.81	3.63	3.46	3.16
Shrimp	19.72	11.32	7.34	3.80

	Predicted Exponential Growth Rate [(Log CFU/g)/hour]			
	4°C	8°C	12°C	20°C
Crabmeat	0.015	0.042	0.082	0.204
Corn	0.018	0.041	0.071	0.159
Green peas	0.015	0.044	0.088	0.225
Shrimp	0.014	0.043	0.088	0.226

Results of Sensory Analysis

Results of informal sensory panel assessments to determine the earliest time that each food became organoleptically unacceptable are presented in Table 5.3. The period in which a food sample became unacceptable to consume organoreptically was determined when the sample reached to the average score of 3 (as *not acceptable*) for each food type at each storage tempreture. The purpose of the acceptability evaluation in the sensory analysis was to capture a panelist's response based on the sample's overall condition, including appearance and odor. The results from predicted LPD and EGR values (Table 5.2.) were used to predict the population increase (log CFU/g) at the time the food was found to be unacceptable. The earliest times for each food/temperature combination to be deemed unacceptable and the predicted growth (log CFU/g) of *L. monocytogenes* (in parentheses) are shown in Table 5.3. Growth of *L. monocytogenes* was calculated by subtracting the LPD from the time to the earliest unacceptability of the food. This time was multiplied by the predicted EGR for that food/temperature combination. In all cases, *L. monocytogenes* would have grown several orders of magnitude by the time each food was deemed organoleptically unacceptable. At 4ºC, shrimp was found to be unacceptable after 6 days. By that time, it is predicted that *L. monocytogenes* would have increased by 1.7 log CFU/g. Corn was not considered unacceptable until day 14 at which time *L. monocytogenes* was predicted to have increased by 5.9 log CFU/g. These results concur with other research on refrigerated food that demonstrates the growth of *L. monocytogenes* occurs before food is organoleptically unacceptable (4, 13). The absence of spoilage indications during the initial storage period has significant implications for food safety.

Table 5.3. Earliest time in days that thawed frozen food samples (non-inoculated) was found to be organoleptically unacceptable and predicted log CFU/g[a] growth of *Listeria monocytogenes* at that time.

	Days to unacceptability (Predicted log growth CFU/g)			
	4ºC	8ºC	12ºC	20ºC
Crabmeat	10 (3.4)	6 (5.6)	4 (7.2)	1.5 (6.3)
Corn	14 (5.9)	3 (2.8)	2 (3.3)	1.0 (3.7)
Green peas	12 (2.1)	4 (4.1)	3 (6.0)	1.5 (7.3)
Shrimp	6 (1.7)	4 (3.6)	3 (5.7)	1.5 (7.3)

[a] - Log CFU/g = EGR (Time to spoilage - LPD)

Relations among Growth of *L. monocytogenes*, Background Microflora, and Results of Sensory Analysis

Presented in Figure 5.4 (a-d) are 1) counts of *L. monocytogenes* from the triplicate experiments, 2) predicted two-phase linear growth curve up to 240 h for *L. monocytogenes*, 3) mesophilic APC growth curve from the average of three experiments, and 4) time of earliest unacceptability for all four foods at 4ºC. Corn and green peas had higher initial mean APC, 4.05 and 2.87 log CFU/g respectively, while the crabmeat and shrimp had initial APCs of 1.65 and 2.18 log CFU/g, respectively. In all cases, APCs reached to 9 $\log_{10}$ CFU/g in each food type by the end of storage period, whereas the level of *L. monocytogenes* stayed around 6 ~ 8 $\log_{10}$ CFU/g as the maximum count by the end of storage. At the time that each food was found to be organoleptically unacceptable, the average APC log CFU/g was 6.63 (crabmeat), 9.19 (corn), 8.85 (green peas) and 5.69 (shrimp). It is also evident in this figure that at some point during refrigerated storage, the APC exponential growth rate became greater than that of the *L. monocytogenes* strains. The time it took for the spoilage organisms to increase their exponential growth rate may be an indication of the additional recovery time needed for the spoilage organisms after freezing, compared to the cold acclimated *Listeria*. Furthermore, it can be seen in Figure 5.4. (a-d) that the two-phase linear prediction fit well through the triplicate *L. monocytogenes* counts.

Figure 5.4. Predicted growth model of *Listeria monocytogenes* with data from tripricate experiments, including APC (average of three aerobic plate counts of non-inoculated samples)
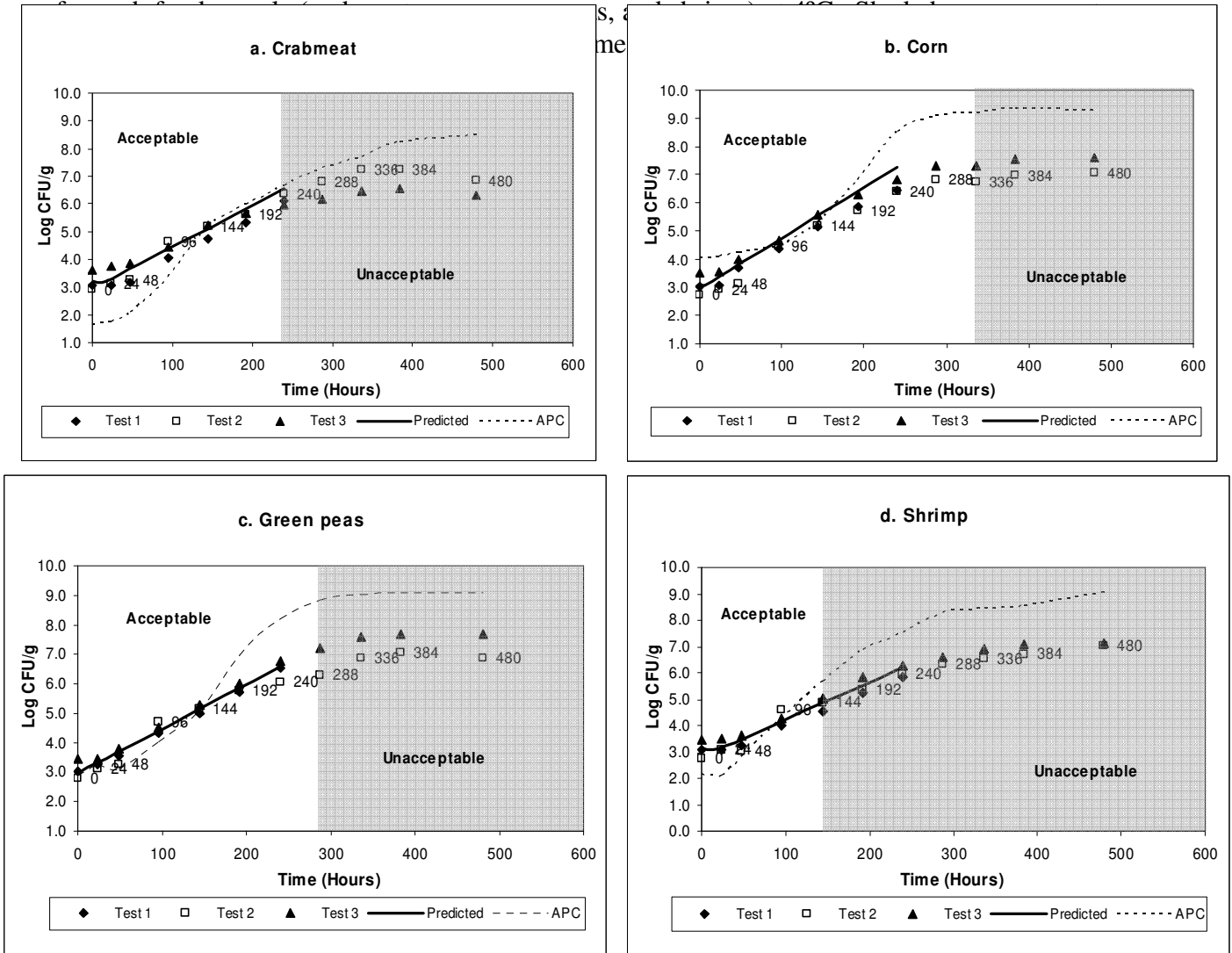
Conclusion

All four of the frozen foods (corn, green peas, cooked crabmeat, and cooked shrimp) used in this study could be considered to be ready-to-eat food (RTE) and could contain up to 100 CFU/g of *L. monocytogenes* as long as they are maintained frozen until consumption according to the FDA 2008 Draft Compliance Policy Guide. Although it was known that *L. monocytogenes* is resistant to freezing, it was originally hypothesized that freezing could possibly create an extended lag phase duration after thawing time, thus allowing these foods to be held refrigerated for more than a few hours prior to consumption. This was not the case in this study, as all of the foods readily supported the growth of *L. monocytogenes* once they were thawed and held at refrigeration temperatures. Individually Quick Frozen (IQF) peas and corn typically are labeled with a recommendation to the consumer that the vegetables must be cooked prior to consumption (making them not ready-to-eat food), although they may be displayed on salad bars and possibly held for extended periods without this treatment. While the shrimp and crabmeat used in this study were fully cooked prior to freezing, such foods may not get additional cooking to destroy any possible post-processing *Listeria* contamination prior to being offered at a food bar.

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/holding guidelines for the foods after thawing. This information could also help the industry develop methods of preventing the growth of low levels of this pathogen during extended refrigeration. Frozen foods containing less than 100 CFU/g of *L. monocytogenes* that are thawed, or thawed and cooked, and consumed immediately, should not represent a public health hazard.

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Appendix A. Sensory Analysis Form

\<Sensory Analysis Form\>

<u>Storage Temperature:</u> _____   <u>Date:</u> _____

Please evaluate samples immediately after opening the sample bags and indicate your opinion by circling the number for both questions (off odor and acceptability) for each sample.

Shrimp

Shrimp①
    <u>Off Odor</u>
      1. None
      2. Slight
      3. Moderate
      4. Strong
      5. Extremely strong

    <u>Acceptability</u>
      1. Acceptable
      2. Hesitant, but might eat
      3. Not Acceptable   (why? _____)
      4. Reject
      5. Absolute reject

Shrimp②
    <u>Off Odor</u>
      1. None
      2. Slight
      3. Moderate
      4. Strong
      5. Extremely strong

    <u>Acceptability</u>
      1. Acceptable
      2. Hesitant, but might eat
      3. Not Acceptable   (why? _____)
      4. Reject
      5. Absolute reject

Crab meat

Crabmeat①
    <u>Off Odor</u>
      1. None
      2. Slight
      3. Moderate
      4. Strong
      5. Extremely strong

    <u>Acceptability</u>
      1. Acceptable
      2. Hesitant, but might eat
      3. Not Acceptable   (why? _____)
      4. Reject
      5. Absolute reject

Crabmeat②
    <u>Off Odor</u>
      1. None
      2. Slight
      3. Moderate
      4. Strong
      5. Extremely strong

    <u>Acceptability</u>
      1. Acceptable
      2. Hesitant, but might eat
      3. Not Acceptable   (why? _____)
      4. Reject
      5. Absolute reject

Corn

Corn①

Off Odor
1. None
2. Slight
3. Moderate
4. Strong
5. Extremely strong

Acceptability
1. Acceptable
2. Hesitant, but might eat
3. Not Acceptable   (why? _____)
4. Reject
5. Absolute reject

Corn②

Off Odor
1. None
2. Slight
3. Moderate
4. Strong
5. Extremely strong

Acceptability
1. Acceptable
2. Hesitant, but might eat
3. Not Acceptable   (why? _____)
4. Reject
5. Absolute reject

Green peas

Green peas①

Off Odor
1. None
2. Slight
3. Moderate
4. Strong
5. Extremely strong

Acceptability
1. Acceptable
2. Hesitant, but might eat
3. Not Acceptable   (why? _____)
4. Reject
5. Absolute reject

Green peas②

Off Odor
1. None
2. Slight
3. Moderate
4. Strong
5. Extremely strong

Acceptability
1. Acceptable
2. Hesitant, but might eat
3. Not Acceptable   (why? _____)
4. Reject
5. Absolute reject

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Appendix B. Additional Figures from Experiments

Figure B.1. Predicted growth model of *Listeria monocytogenes* with data from tripricate experiments, including APC (average of three aerobic plate counts of non-inoculated samples) for each food sample (crabmeat, corn, green peas, and shrimp) at 8ºC. Shaded area represents earliest time when non-inoculated samples became unacceptable organoleptically.
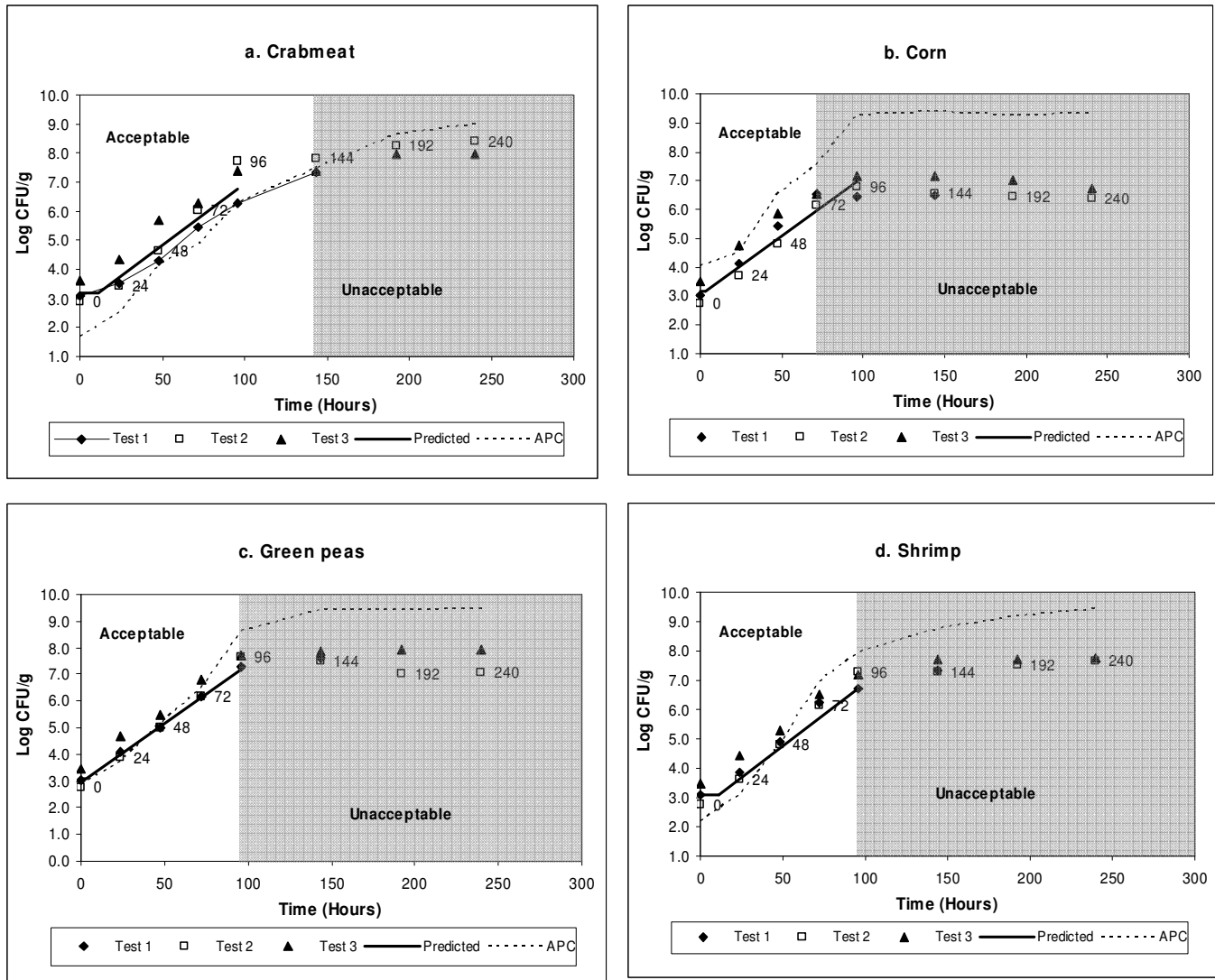
Figure B.2. Predicted growth model of *Listeria monocytogenes* with data from tripricate experiments, including APC (average of three aerobic plate counts of non-inoculated samples) for each food sample (crabmeat, corn, green peas, and shrimp) at 12ºC. Shaded area represents earliest time when non-inoculated samples became unacceptable organoleptically
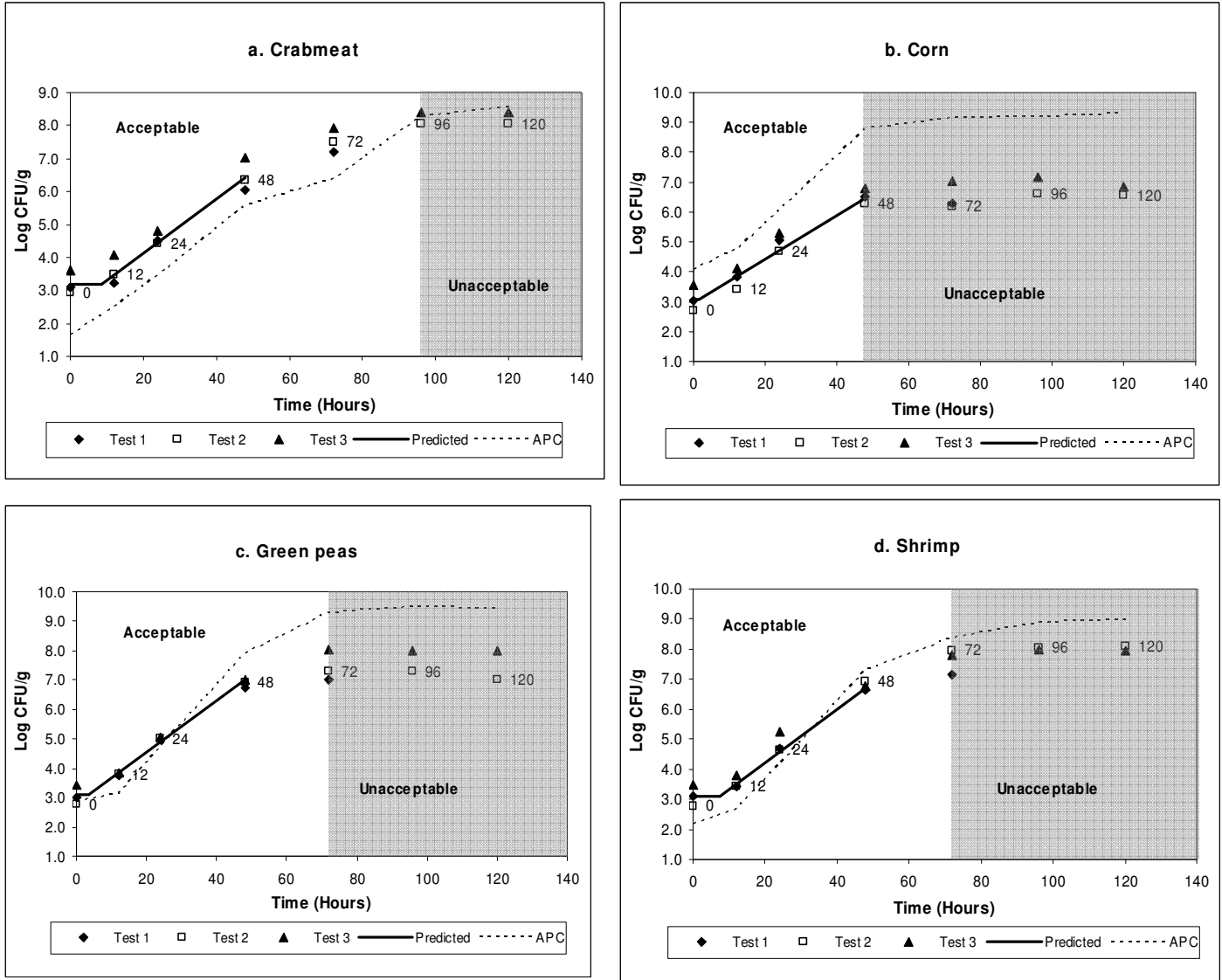
Figure B.3. Predicted growth model of *Listeria monocytogenes* with data from tripricate experiments, including APC (average of three aerobic plate counts of non-inoculated samples) for each food sample (crabmeat, corn, green peas, and shrimp) at 20ºC. Shaded area represents earliest time when non-inoculated samples became unacceptable organoleptically.