Journal of Food Protection, Vol. 73, No. 1, 2010, Pages 140–202

Supplement

Parameters for Determining Inoculated Pack/Challenge Study Protocols†‡

ADOPTED 20 MARCH 2009, WASHINGTON, D.C.
NATIONAL ADVISORY COMMITTEE ON MICROBIOLOGICAL CRITERIA FOR FOODS

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MS 09-287: Received 2 July 2009/Accepted 7 August 2009

ABSTRACT

The National Advisory Committee on Microbiological Criteria for Foods developed guidelines for conducting challenge studies on pathogen inhibition and inactivation studies in a variety of foods. The document is intended for use by the food industry, including food processors, food service operators, and food retailers; federal, state, and local food safety regulators; public health officials; food testing laboratories; and process authorities. The document is focused on and limited to bacterial inactivation and growth inhibition and does not make specific recommendations with respect to public health. The Committee concluded that challenge studies should be designed considering the most current advances in methodologies, current thinking on pathogens of concern, and an understanding of the product preparation, variability, and storage conditions. Studies should be completed and evaluated under the guidance of an expert microbiologist in a qualified laboratory and should include appropriate statistical design and data analyses. This document provides guidelines for choice of microorganisms for studies, inoculum preparation, inoculum level, methods of inoculation, incubation temperatures and times, sampling considerations, and interpreting test results. Examples of appropriately designed growth inhibition and inactivation studies are provided.

SCOPE OF DOCUMENT

This document was prepared at the request of the sponsoring agencies of the National Advisory Committee on Microbiological Criteria for Foods (NACMCF). The document is intended for use by the food industry, including food processors, food service operators, and food retailers; federal, state, and local food safety regulators; public health officials; food testing laboratories; and process authorities. The document is focused on and limited to bacterial inactivation and growth inhibition. The document does not consider toxigenic fungi or the inactivation of viruses.

INTRODUCTION AND STATEMENT OF CHARGE

Statement of Charge

Because of the many questions raised by regulatory and industry users on the definition of potentially hazardous food (PHF) or time/temperature control for safety (TCS) food, the NACMCF is asked for its guidance to clarify these issues.

1. What are the appropriate criteria that must be considered for an inoculated pack/challenge study to determine if a food requires time/temperature control for safety (TCS)? For example, pathogen species/strain selection, use of surrogate organism, number of pathogen strains, inoculation level(s), incubation temperature(s), length of incubation/duration of study, food product physical properties, etc.
2. What are the appropriate uses of mathematical growth and inactivation models? Under what conditions can these models be used as a substitute for inoculated pack/challenge studies? Of the models currently available, which ones are most suitable for use, and what are the limitations of these models?
3. What are the limitations for applying the results of an inoculated pack/challenge study conducted on one food to another similar food?
4. Of the existing inoculated pack/challenge study protocols, e.g., those published by the American Bakers Association, NSF International, and others, which are most suitable for application to a wide variety of foods, and what are the limitations of these protocols? Are there existing protocols that are appropriate for specific food-pathogen pairs?
5. Develop a decision tree to aid in the design of an appropriate inoculated pack/challenge study. Test or ''desk check'' the decision tree using the following five foods: meat-filled puff pastry, (baked) cheese pizza,

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† Sponsored by the U.S. Department of Agriculture, Food Safety and Inspection Service; U.S. Department of Health and Human Services, Food and Drug Administration, and Centers for Disease Control and Prevention; U.S. Department of Commerce, National Marine Fisheries Service; and U.S. Department of Defense, Veterinary Service Activity.

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chopped lettuce, cheese (blocks or slices), and lemon meringue pie.
6. Identify the basic knowledge, skills, education, training, experience, and abilities necessary for a multidisciplinary work group or individual to be qualified to design, conduct, and evaluate an inoculated pack/challenge study and the pursuant results.

Background

The restaurant and retail food store industry, totaling nearly 1.5 million establishments in the United States, and their suppliers routinely use inoculation/challenge testing to determine whether a specific food requires TCS. A food establishment, including restaurants, retail food stores, delis, caterers, and institutions or vending commissaries that provide food directly to the consumer, is defined in the U.S. Food and Drug Administration (FDA) Food Code *(117)*.

When laboratory testing is used to support a change in how the product is handled in a food establishment (e.g., refrigerated to unrefrigerated holding, extending shelf life, increasing ambient temperature storage, or eliminating the need for date marking), the data are submitted to a state or local regulatory agency or directly to the FDA in the form of a variance application for approval. Food establishments or manufacturers submitting laboratory data to support their proposals must ensure the study is appropriate for the food and pathogen of concern and incorporate the necessary elements into the study to yield a valid design and conclusion.

A variance from any provision in the FDA Food Code must also show that no health hazard will result from the modification or waiver and that product handling is under appropriate control using a hazard analysis critical control point (HACCP) plan. Examples of foods in which the need for TCS was questioned include puff pastries with savory meat, cheese, or vegetable fillings; churros (fried dough) batter held unrefrigerated; sliced pasteurized processed cheese held at ambient temperature for more than 4 h; certain cheeses held unrefrigerated. State and local regulators who evaluate a variance application based on this laboratory evidence need criteria to help them determine whether the study was adequately designed and whether the conclusions are valid.

The definition of PHF or TCS food was amended in the 2005 FDA Food Code *(117)*; chap. 1, Definitions to include pH and a_w interaction tables, allowing the hurdle concept to be used in the determination of whether TCS is necessary. The two interaction tables, as well as a decision-making framework, were developed by the Institute of Food Technologists (IFT) and provided to the FDA in the report, ''Evaluation and Definition of Potentially Hazardous Foods'' *(53)* (31 December 2001, IFT/FDA contract no. 223-98-2333, task order no. 4). When the pH and a_w interaction tables and the decision-making framework are insufficient to show that a food does not require TCS, further product assessment using inoculation/challenge testing is likely required.

The IFT report *(53)* with its recommendations to the FDA left a number of unanswered questions regarding the understanding and implementation of a product assessment when based on pH and a_w the producer is unable to determine whether TCS is required. This need for clarification was confirmed in a 2005 survey of stakeholders conducted by the Conference for Food Protection *(18)*.

THE COMMITTEE'S RESPONSE

Use and Limitations of This Document

The primary objective of this document is to provide guidelines for challenge studies necessary to determine whether a variance to TCS may be granted under the Food Code. Secondarily, the guidelines presented in this document may be useful to laboratories conducting pathogen inhibition and pathogen inactivation studies for a variety of foods for evaluation of safety prior to introduction into commerce. It may be useful to review the proposed study with the appropriate regulatory agency to ensure the design and methods are appropriate. Studies should be completed under the supervision of and interpreted by an expert food microbiologist (Table 1). One of the limitations of these studies is the balance of statistical validity with practicality. A certain amount of variability is expected with challenge studies, which can affect the validity and interpretability of results. However, because of resource constraints, variability is generally addressed through the use of worst-case scenarios, which should provide conservative results. Although this document encompasses a variety of sources, individuals who conduct challenge studies must be aware of the most current advances in methodologies and identification of new pathogens or regulatory concerns that may need to be considered as well as pertinent statistical issues. This document does not make specific recommendations with respect to public health.

Types of Challenge Studies

There are several types of challenge studies that deal with validation of food safety processing procedures, product storage conditions, and shelf life. Shelf-life studies focusing on product quality are not addressed in this report because they are generally not related to food safety. Nevertheless, many of the principles of food safety–related challenge studies are applicable to quality shelf-life studies. Food safety–related challenge studies differ according to the objective of the study, such as a pathogen growth inhibition study or a pathogen inactivation study or a combination of the two, and depend on the type of product, production process, and hazard analysis of the product.

Food safety–related challenge studies include the following:

Pathogen growth inhibition studies. These studies are used to evaluate the ability of a particular food product formulation with a specific type of processing and packaging to inhibit the growth of certain bacterial pathogens when held under specific storage conditions (time and temperature).

TABLE 1. *Recommended minimum expertise needed for designing, conducting, and evaluating microbiological studies*[a]

Category	Design	Conduct[b]	Evaluate
Knowledge and skills	Knowledge of food products and pathogens likely to be encountered in different foods. Knowledge of the fundamental microbial ecology of foods, factors that influence microbial behavior in foods, and quantitative aspects of microbiology. Knowledge of processing conditions and parameters. Knowledge of statistical design of experiments.[c]	Knowledge of basic microbiological techniques. Ability to work using aseptic technique, to perform serial dilutions, and to work at biosafety level 2 *(114)*.	Knowledge of food products and pathogens likely to be encountered in different foods. Knowledge of the fundamental microbial ecology of foods, factors that influence microbial behavior in foods, and quantitative aspects of microbiology. Knowledge of statistical analysis.[c]
Education and training	Ph.D. in food science or microbiology or a related field or an equivalent combination of education and experience.	B.S. in food science, microbiology, or a related field or an equivalent combination of education and experience. Appropriate hands-on experience in food microbiology is also recommended.	Ph.D. in food science, microbiology, or a related field or an equivalent combination of education and experience.
Experience	Two years of experience conducting challenge studies independently and experience in design of challenge studies under the guidance of an expert food microbiologist.	Two years of experience conducting challenge studies is useful; however, close supervision by an expert food microbiologist may substitute.	Two years of experience conducting challenge studies independently and experience in evaluation of challenge studies under the guidance of an expert food microbiologist.
Abilities	Ability to conduct literature searches. Ability to write an experimental protocol.	Ability to read and carry out an experimental protocol. Ability to perform microbiological techniques safely and aseptically.	Ability to analyze and interpret microbiological data.

[a] State or local regulatory food programs that are presented an inoculation study in support of a variance request may not have expert food microbiologists on staff to confirm the validity of the study. Options available to them include consulting with expert food microbiologists in their state or local food laboratories or requesting assistance from FDA food microbiologists through their regional retail food specialist.

[b] Working independently under the supervision of an expert food microbiologist.

[c] It may be appropriate to consult with a statistician with applicable experience in biological systems.

Pathogen inactivation studies. These studies are used to evaluate the ability of a particular food product formulation, a specific food manufacturing practice, or their combination to cause the inactivation of certain bacterial pathogens. These studies may also be impacted by food storage and packaging conditions and must account for these variables.

Combined growth and inactivation studies. Combined studies may be used to evaluate the ability of a particular food or process to inactivate certain bacterial pathogens and to inhibit the growth of certain other pathogenic bacteria or to achieve a level of inactivation followed by inhibition of the growth of survivors or contaminants introduced after processing.

Determining When a Challenge Study Is Needed

The first step in determining whether a challenge study is needed is to describe the product and process, conduct a hazard analysis to determine the significant biological hazards, and assess what is known about the growth or inactivation of these in the product *(80)*. Consideration should be given to potential routes of contamination, intrinsic factors such as water activity (a_w) and pH that affect the likelihood of the product to support growth, the use of processing technologies that destroy pathogens of concern, and the historical record of safe use of the product *(53, 80)*. In 2000, the FDA requested the IFT to assemble a scientific panel to examine the issue of determining when foods required refrigeration for safety. In addressing their charge, the panel defined these foods as TCS foods and developed a framework for determining whether time and temperature control is required for safety. This framework included two tables (one for control of spores and one for control of spores and vegetative cells) with a_w and pH value combinations that indicate when product assessment (e.g., a microbiological challenge study) is needed *(53)*. This concept was subsequently adopted as the basis for defining when foods need refrigeration or some other form of time-temperature control in the 2005 FDA model Food Code *(117)*. These a_w and pH combinations are not specific to individual pathogens; therefore, for specific foods where the pathogen of concern is established, other pH and a_w values may define the need for refrigeration. Information on parameters associated with control of growth of various pathogens can be found in the literature, e.g., the

International Commission on Microbiological Specifications for Foods publication *Microorganisms in Foods 5. Characteristics of Microbial Pathogens (54)*. When the intrinsic factors of a food are consistent with parameters that are well recognized as controlling the growth of a pathogen, microbiological challenge studies are not needed *(91)*. For example, there would be no need to assess whether a product with a pH of 3.5 supported growth of *Salmonella,* because this organism will not grow at pH values this low. However, studies to determine whether *Salmonella* survives at this pH or whether it is inactivated over time may be warranted under some circumstances. It is important to use expert food microbiologists and technologists to assess the need for challenge testing (Table 1).

A challenge study may be needed to assess whether the pathogen can grow in the product if properties such as pH, a_w, or their combination do not ensure pathogen control. For more details on the use of pH and a_w to control the growth of bacterial pathogens, consult the *Compendium of Methods for the Microbiological Examination of Foods (90)*. Determination of the need for a challenge study is referred to as product assessment in the IFT and Food Code tables *(53, 117)*.

When growth inhibition occurs due to factors other than or in addition to pH and a_w, such as the addition of preservatives including lactate and diacetate, the literature may provide information relevant to the pathogen and food product. However, it is necessary to ensure that the data are applicable to the specific product and conditions of use. The efficacy of an antimicrobial agent may be dependent on the formulation of the product. For example, factors such as fat content can decrease the efficacy of antimicrobial agents such as nisin *(36, 58)* and sorbate *(82, 98)*. Conversely, a low pH may potentiate the activity of antimicrobials such as sorbate and benzoate *(39)*. These evaluations should be done by expert microbiologists and food technologists with knowledge of the characteristics and the mechanism of action of microbial inhibitors.

It is not reasonable to expect that every individual food product would need a microbiological challenge study. Many food products for which the assessment tables indicate that product assessment is needed have a long history of safe use. However, safe history of a food product is relevant only when all conditions remain the same. Even apparently minor changes to a food product, process, or packaging method may have a large impact on the safety of the product. Moreover, changes in the ecology, physiology, or genetic makeup of a pathogen may result in food safety issues in products with a history of safety *(31, 73, 84)*.

RESPONSE TO QUESTIONS

The Committee was asked by the supporting federal agencies to answer six questions. The responses are provided in order below.

1. What are the appropriate criteria that must be considered for an inoculated pack/challenge study to determine if a food requires time/temperature control for safety (TCS)? For example, pathogen species/strain selection, use of surrogate organism, number of pathogen strains, inoculation level(s), incubation temperature(s), length of incubation/duration of study, food product physical properties, etc.

General Factors to Consider When Designing a Challenge Study

Standardization of methods is beneficial for comparing results among different studies, but it is not possible to develop a single protocol that is broadly applicable to a wide variety of food types or even to one category such as fruits and vegetables *(12)*. Parameters that should be considered when designing a microbial challenge study are outlined below *(12, 53, 80, 91, 122)*.

1.0. Obtaining expert advice and identifying a laboratory
2.0. Type of study
  2.1. Growth inhibition studies
  2.2. Inactivation studies
  2.3. Combination studies
3.0. Factors related to the test product
  3.1. Product preparation
  3.2. Product variability
  3.3. Competitive microflora
4.0. Target organism(s)
  4.1. Identifying the pathogen(s) of concern
  4.2. Use of surrogate organisms
  4.3. Type and number of strains
5.0. Inoculum levels
  5.1. Growth studies
  5.2. Inactivation studies
6.0. Inoculum preparation
7.0. Method of inoculation
8.0. Storage conditions
  8.1. Packaging
  8.2. Storage and shipping
9.0. Sample considerations
  9.1. Sampling
  9.2. Sample analysis for target pathogens or toxins
  9.3. Enumeration of indigenous microbial flora
  9.4. Determination of physical parameters
10.0. Duration of study and sampling intervals
11.0. Interpreting test results
12.0. Elements to include in the report

1.0. Obtaining expert advice and identifying a laboratory

Challenge studies must be designed and evaluated by an expert food microbiologist. This expertise may or may not reside within the staff of a testing laboratory. When it does not, it is important to choose an advisor who can work with the laboratory to conduct a proper study. Potential sources of expertise include in-house experts, university faculty, testing laboratories, and independent consultants. Once a study design has been developed it may be appropriate to consult with a statistician with applicable experience in biological systems and have the design reviewed by the regulatory body or intended recipient of

TABLE 2. *Potential pathogens*[a] *of concern for growth studies based on interaction of product pH and* a_w[b]

a_w values	pH values: <3.9	3.9 to <4.2	4.2–4.6	>4.6–5.0	>5.0–5.4	>5.4
<0.88	NG[c]	NG	NG	NG	NG	NG
0.88–0.90	NG	NG	NG	NG	*Staphylococcus aureus*	*S. aureus*
>0.90–0.92	NG	NG	NG	*S. aureus*	*S. aureus*	*L. monocytogenes* *S. aureus*
>0.92–0.94	NG	NG	*L. monocytogenes* *Salmonella*	*Bacillus cereus* *Clostridium botulinum* *L. monocytogenes* *Salmonella* *S. aureus*	*B. cereus* *C. botulinum* *L. monocytogenes* *Salmonella* *S. aureus*	*B. cereus* *C. botulinum* *L. monocytogenes* *Salmonella* *S. aureus*
>0.94–0.96	NG	NG	*L. monocytogenes* Pathogenic *E. coli* *Salmonella* *S. aureus*	*B. cereus* *C. botulinum* *L. monocytogenes* Pathogenic *E. coli* *Salmonella* *S. aureus* *Vibrio parahaemolyticus*	*B. cereus* *C. botulinum* *L. monocytogenes* Pathogenic *E. coli* *Salmonella* *S. aureus* *V. parahaemolyticus*	*B. cereus* *C. botulinum* *C. perfringens* *L. monocytogenes* Pathogenic *E. coli* *Salmonella* *S. aureus* *V. parahaemolyticus*
>0.96	NG	*Salmonella*	Pathogenic *E. coli* *Salmonella* *S. aureus*	*B. cereus* *C. botulinum* *L. monocytogenes* Pathogenic *E. coli* *Salmonella* *S. aureus* *V. parahaemolyticus*	*B. cereus* *C. botulinum* *L. monocytogenes* Pathogenic *E. coli* *Salmonella* *S. aureus* *V. parahaemolyticus* *V. vulnificus*	*B. cereus* *C. botulinum* *C. perfringens* *L. monocytogenes* Pathogenic *E. coli* *Salmonella* *S. aureus* *V. parahaemolyticus* *V. vulnificus*

[a] *Campylobacter* spp., *Shigella,* and *Yersinia enterocolitica* do not appear here because they are typically controlled when the pathogens listed are addressed.
[b] Data are based on the PMP *(106),* ComBase predictor *(50),* ComBase database *(49),* or peer-reviewed publications *(11, 17, 45).*
[c] NG, no growth; when no pathogen growth is expected, but formulation or process inactivation studies may still be needed.

the study. Suggested modifications can then be incorporated before the study is executed.

Choosing a laboratory requires careful consideration because not all laboratories have the expertise to design challenge studies and the quality control procedures necessary to produce valid results that will be accepted by the regulatory authority or other reviewer. Laboratories may be certified by various organizations and state or federal agencies for various types of testing, e.g., water and waste water testing, ISO 17025, and Grade A dairy testing. However, these certifications do not necessarily qualify a laboratory to design and conduct microbiological challenge studies. A laboratory selected for challenge testing must be able to demonstrate prior experience in conducting challenge studies. It is necessary to ensure that personnel are experienced and qualified (Table 1) to conduct the types of analyses needed for the challenge studies and that these workers will follow generally accepted good laboratory practices. Laboratories conducting microbial challenge studies should use test methods validated for the intended use. Some examples of generally accepted methods are available in the most recent editions of the references listed in Appendix A. In situations in which approved methods are not available or applicable, laboratories may consider using other widely accepted methods, such as those that have been cited in peer-reviewed journals. Failure to properly design the study and use valid methods and appropriate controls may render the challenge study unacceptable and require additional time and resources to repeat the study. See the questions in Appendix B for assistance in selecting a laboratory.

2.0. Type of study

Challenge studies are conducted for a variety of reasons. The specific purpose of the study drives selection of bacterial strains and inoculum level, choice of parameters tested, types of analysis, and duration of the study as described below. For example, studies evaluating growth inhibition should consider bacterial species listed in Table 2, whereas the choice of species for lethality or survival studies depends on the selection of resistant strains relative to the process and technology as well as compliance with regulations for specific foods (e.g., FDA, U.S. Department of Agriculture, Food Safety and Inspection Service [USDA-FSIS], and state laws based on the Pasteurized Milk Ordinance).

2.1. Growth inhibition studies

The objective of a growth study may be to request exemption (variance) from TCS or other requirements defined by the Food Code, Pasteurized Milk Ordinance, or

FDA, USDA-FSIS, national, state, provincial, or local regulations. Other objectives may be to demonstrate safety of a current formulation during extended shelf life under normal refrigerated or ambient temperatures, to determine whether formulation or processing changes are required if the product is subjected to temperature abuse, or to determine the effect of a modified formulation, process, or packaging technology.

2.2. Inactivation studies

Inactivation studies may be used to determine whether thermal processes provide adequate log reduction of a target pathogen as defined by regulations or government policy (e.g., FSIS requirement for a 5-log kill of *Escherichia coli* O157:H7 in fermented dry sausage) *(110)*. Inactivation studies may also be used to determine whether nonthermal technologies or combinations of pH, a_w, preservatives, and holding for specified times at specific temperatures prior to release of product will provide sufficient lethality to render a food product safe (e.g., 2-year aging of raw milk Parmesan cheese or 3-day holding at room temperature to inactivate *Salmonella* in mayonnaise).

2.3. Combination studies

Other studies involving both verification of inactivation and evaluation of changes in the number of microorganisms during extended storage combine concepts from both study types above. For example, a manufacturer of processed meat wishing to have a product line classified as Alternative 1 for control of *Listeria monocytogenes* by FSIS regulation (9 CFR 430) *(113)* may undertake a study to demonstrate a 2-log postlethality kill step of *L. monocytogenes* on ready-to-eat meats by high pressure processing followed by growth inhibition by product formulation during extended refrigerated storage. A producer of a cold-filled hot sauce with pH 3.5 may wish to demonstrate a 5-log kill of acid-tolerant *Salmonella* when held at 20°C (68°F) for 3 days as well as no recovery or growth of the pathogen during ambient-temperature storage for 1 year.

3.0. Factors related to the test product

3.1. Product preparation

The product should be prepared under conditions most conducive to growth or survival based on the intended conditions of use and expected product variability. Consideration should be given to the physical properties (pH, a_w, etc.) of the prepared product and the impact that these properties can have on the results of a challenge or inactivation study. The product should be prepared so that the critical physical properties are at the appropriate minimum or maximum control limits intended for the finished product (see section 3.2 below on product variability).

Multicomponent products may take days to equilibrate moisture, a_w, or pH. Such products should generally be inoculated prior to equilibration in regions of the product that are considered the most permissive to growth, provided these are areas reasonably likely to be contaminated. In general, larger particles take longer to equilibrate. Studies to determine growth, inactivation, or survival of a pathogen present due to recontamination would involve inoculation of product after equilibration.

3.2. Product variability

Knowledge of the manufacturing or production variability is needed to determine the appropriate test parameters for a challenge study. Variability within and among lots should be determined by measuring formulation factors such as pH, a_w, etc. The greater the variability, the more samples of product need to be evaluated, e.g., more measurements need to be made to determine the upper or lower control limits. When choosing an attribute such as pH during the challenge test, that pH (including the uncertainty in the measurement or manufacturing capability) becomes the upper limit of the pH specification range for the product subsequently manufactured.

Whenever possible, food from a commercial production facility (manufacturing or food service kitchen or commissary) or manufactured in a laboratory that has pilot food processing facilities should be used for the study. The food produced in a pilot facility should be processed to mimic conditions used during commercial operations (cooking temperature and time, homogenization, hot fill, slicing, etc.). The product lots used for the challenge study should be representative of normal production with the exception of necessary adjustments to acidity, moisture, salt, a_w, etc., to yield the conditions most permissive to pathogen growth or survival at each formulation control limit (worst-case scenario based on knowledge of manufacturing variability). Percent salt and moisture may be easier than a_w to measure and control by the producer for some products such as processed meats, cheeses, and smoked seafood and, therefore, may be used for control parameters in the challenge study.

The target limits for moisture or a_w will vary depending on whether the objective of the study is to verify inactivation or growth inhibition. For thermal inactivation studies, lower moisture or a_w levels should be used because pathogens may have increased heat resistance under these conditions *(10, 24, 25, 38, 102)*. Similarly, increased solute content has been shown to protect *L. monocytogenes* against high hydrostatic pressure *(43, 63)*. In contrast, for growth challenge studies, targeting the upper limit of moisture or a_w is appropriate. For example, if the typical moisture range is 56 to 58%, a thermal inactivation study should be conducted at no more than 56% moisture, but a growth challenge study should be conducted at no less than 58% moisture.

When pH is one of the controlling factors, the food should be prepared with the lowest amount of acid allowed in the formulation so that the pH is at the upper range and adjustment in the laboratory is not necessary. If the target pH is 4.8 but the maximum pH observed in multiple production batches is 5.0, a growth inhibition study or an inactivation study should be conducted at a pH no lower than 5.0. If pH adjustment is necessary and pH is adjusted upward using sodium hydroxide, the titratable acidity prior to pH adjustment should be measured and reported so it can

be compared with that of the adjusted food product. If the pH of the product needs to be reduced, it is important to use the same acids that are predominant in the product.

Acidulants exert different degrees of antimicrobial activity at the same pH. For example, acetic acid is the most inhibitory for many microorganisms, followed by lactic acid, with citric acid the least inhibitory *(2, 3, 28, 30, 83)*. As a result, if the challenge study were conducted on a product formulated with acetic acid (vinegar), the study may not be valid for a reformulated product containing citric acid (lemon juice) even if the final pH were the same. In some cases, the number of challenge tests can be reduced for multiple formulations having similar proximate analysis, acidity, and a_w, provided the formulation most permissive to growth or survival is tested.

3.3. Competitive microflora

Competitive flora can affect the outcome of a challenge study, particularly one determining growth of pathogens in a food product. Inoculated product should contain typical levels of competitive microflora, including starter cultures, which may interfere with consistent growth of pathogens during the study. The freshest product possible, i.e., within the first 10% of its shelf life, should be used. For example, if the shelf life is $<$1 month, product should be used within 1 to 3 days of production. (For purposes of this document, shelf life is defined as the time at a specified storage temperature during which product quality is considered acceptable for consumption. This assessment includes acceptable flavor, appearance, and functionality based on chemical changes or growth of spoilage microorganisms but does not necessarily infer product safety by accepted definitions in all countries.) Care should be taken during the inoculation step to not introduce atypical spoilage microorganisms that may inhibit pathogen growth. In rare cases, naturally occurring bacteria can enhance growth or survival of pathogens, potentially reducing the safety of the product *(19)*.

4.0. Target organism(s)

4.1. Identifying the pathogen(s) of concern

An expert food microbiologist should determine the appropriate organisms for challenge testing. There are a number of issues the microbiologist must consider, including the specific product, the process used to prepare it, and any pathogens that are epidemiologically or ecologically relevant. There are a number of resources available to assist in determining appropriate pathogen(s) for a given food. Examples of assessments of the appropriate challenge organism for specific food products can be found in the IFT report on evaluation and definition of potentially hazardous foods *(53)* (specifically, see Table 1, Table A, Table B, Table 4-1, and Table 6-1 in that report). For easy reference, please refer to Appendix C.

Table 2 provides combinations of pH and a_w values that may allow growth of pathogens of concern based on model predictions and published literature. This table may be useful in selecting organisms for use in studies to assess growth or inactivation by formulation. Although many pathogens are listed for some pH and a_w combinations, it may not be necessary to evaluate each pathogen for a specific food, because epidemiological attribution or product characteristics may narrow the choice of appropriate challenge organisms. For example, a seafood product might be challenged with *Vibrio* or *Salmonella* because of epidemiological attribution, whereas a pasteurized product in which vegetative cells of pathogens have been eliminated might be challenged with pathogenic sporeformers. *L. monocytogenes* might be used if the study is designed to determine growth or inactivation due to recontamination with this organism in a ready-to-eat product.

The organism used for a challenge study to determine inactivation due to product formulation may need to be selected based on the resistance of the pathogen to the bactericidal properties. For example, enterohemorrhagic *E. coli* may be selected over *Salmonella* or *Staphylococcus aureus* for a food with a pH of 4.3 and an a_w of 0.98 because it is generally considered to be more resistant to acid.

Ideally, when conducting a study to determine pathogen growth in a food formulation, the fastest growing pathogen(s) likely to be present would be used. Predictive models can be useful for determining which pathogen may grow fastest under the conditions of the study. For example, if predictive modeling demonstrates that *Salmonella* grows better at a given pH and a_w combination, then this pathogen may be considered the best choice among the organisms of concern for that product for use in a challenge study.

Although Table 2 is similar to Table B in the Food Code (see Appendix D) and the IFT report *(53)*, it is not identical, and some explanation is required. First, Table 2 is more extensive than Table B and includes both higher and lower pH values and more defined categories for higher a_w values. Second, the IFT report *(53)* and the Food Code *(117)* are specifically focused on foods that require temperature control for safety, whereas the focus of this Table 2 is broader. Finally, Table 2 considers time scales that may be considerably longer than those typically of concern in retail food safety. The table should not be interpreted to suggest that a food falling within a particular pH and a_w range needs to be challenged with a pathogen, e.g., that high a_w foods with a pH of 3.9 need to be challenged with *Salmonella*. Although *Salmonella* has been shown to grow at pH values as low as 3.9, these studies have been done in laboratory media under conditions ideal for growth, other than the pH value. In foods, many factors interact to support or inhibit pathogen growth. An expert microbiologist should use Table 2 as a guideline to assess whether a challenge study on a particular food with a specific pathogen is warranted.

Table 2 is useful for identifying appropriate pathogens of concern for particular pH and a_w combinations. However, this information should not typically be used for the selection of organisms in process inactivation (e.g., thermal inactivation) studies. The choice of organism for these types of studies should be based on the likelihood of pathogen association with the specific food and pathogen resistance to inactivation, as well as the public health objective of the process and the intended use of the product. For example,

nonproteolytic strains of *Clostridium botulinum* might be selected as the appropriate target organism for some refrigerated foods and *L. monocytogenes* for others, depending on how likely it is that nonproteolytic *C. botulinum* will be present, how long the product will be held refrigerated, whether the product is ready-to-eat or will be cooked prior to consumption, and other factors.

4.2. Use of surrogate organisms

Inoculation of foods with bacterial pathogens requires adequate biological containment facilities and may require governmental approval in the case of certain pathogens such as *C. botulinum*. Therefore, in limited cases, nonpathogenic surrogate organisms are especially useful for testing specialized processing equipment in the plant, where the introduction of the pathogen would pose an unacceptable risk. Surrogates may also be useful for selecting the study parameters before conducting the full study with the pathogen. Care should be taken when using surrogates for in-plant challenge studies because these organisms may have adverse sanitary or regulatory implications should they survive and contaminate the plant environment.

Surrogates are typically nonpathogenic proxies for the pathogen of concern that have similar or more robust survival capabilities under the conditions being studied. Such proxies may include avirulent strains of pathogens, where appropriate. The ideal surrogate should have the following characteristics: nonpathogenic, inactivation characteristics and kinetics that can be used to predict those of the target pathogen, similar susceptibility to injury, reproducible growth, easy preparation of high-density populations that are stable until used, easily enumerated and differentiated, similar attachment capabilities, and genetically stable *(52)*.

Clostridium sporogenes PA3679 has proven to be an excellent surrogate for *C. botulinum* when used in inoculated pack studies to validate thermal processes for low-acid canned foods. In certain cases, *C. sporogenes* may be suitable for reducing the number of formulations to be verified using *C. botulinum* because these organisms are culturally similar. Formulations that support growth of *C. sporogenes* can be excluded from further validation studies with *C. botulinum*. However, *C. sporogenes* cannot be used as a direct substitute to validate a product for inhibition of botulinum toxin production *(64)*. Other examples of surrogate-pathogen pairs include *Listeria innocua* and *L. monocytogenes (99)* and nonpathogenic *E. coli* and *E. coli* O157:H7 *(26)*.

A surrogate that works well to predict the target response for one type of process may not be an appropriate surrogate in a different type of process. For example, the heat resistance of various strains of *C. botulinum* spores did not correlate with their resistance to high hydrostatic pressure *(71)*, so although *C. sporogenes* may be the preferred surrogate for *C. botulinum* for canning processes, another organism, such as *Bacillus amyloliquefaciens* may be appropriate as a surrogate for *C. botulinum* for high hydrostatic pressure studies *(71, 86)*.

The choice of the surrogate needs to be justified, and supporting documentation for its appropriate use for the pathogen, food, and treatment being evaluated should be incorporated into the final report. If no directly relevant published comparison data are available, studies need to be conducted to establish the validity of using a particular surrogate-pathogen-process combination.

4.3. Type and number of strains

In order to account for variations in growth and survival among strains, challenge studies should generally be conducted using three to five bacterial strains either individually or in combination *(53, 75, 91)*. Where there is considerable variability among strains or there is little known about the growth of the organism in a particular food product, as many as 10 strains may be used (e.g., some *C. botulinum* or *L. monocytogenes* studies).

Generally, using an inoculum composed of multiple strains (i.e., a cocktail) of a given pathogen is preferred because it will help to encompass the variability among organisms and may reduce the number of required tests. Prior to use in the study, the strains selected should be screened for antagonism that can be caused by production of bacteriocins or other antimicrobial factors *(53)*. Another approach is to screen several strains in the food matrix under investigation and determine which strain has the greatest resistance, grows fastest, etc., and then to conduct the challenge studies using that single strain *(12, 91)*. Screening parameters depend on the purpose of the challenge study, e.g., to determine inactivation or growth characteristics in a product. However, there are strains with atypical resistance, e.g., the extremely high moist heat resistance of *Salmonella* Senftenberg 775W *(79)*. These strains may not be appropriate for use in some studies because they are not representative of strains reasonably expected to be present in the applicable foods. Whether to use an individual strain or cocktails of strains should be determined by an expert microbiologist knowledgeable in food microbiology and pathogen control.

Strains carrying markers such as antibiotic resistance or green fluorescent protein may be useful for confirming that the organisms recovered are the test organisms. When such strains are used it is important to determine that they possess the same characteristics as the parent strain without the marker with respect to factors critical to the challenge study. Furthermore, carriage of the resistance marker should be verified to be stable under stressful conditions that may be encountered during the challenge study.

Isolates should be appropriate for the food product being challenged *(53, 80, 91)*. This approach includes using isolates from the food type, the food processing environment, and clinical specimens, as appropriate. Inactivation studies should use strains that demonstrate tolerance to the specific process for the product under consideration, such as heat or high pressure processing *(16, 24, 25, 71)*. Biochemical characteristics, serology, genetic profile, virulence, or toxicity should be periodically reconfirmed as appropriate. The test strains for growth challenge studies should demonstrate robust growth in laboratory media or a similar food without inhibitors under the conditions of the study (e.g., temperature and atmosphere).

5.0. Inoculum levels

The inoculum level used in the challenge study depends on whether the objective of the study is to determine growth or inactivation of a pathogen. It may be desirable to conduct challenge studies using multiple inoculum levels to determine the margin of safety in the process/formulation *(91)*.

5.1. Growth studies

When conducting studies to determine whether a pathogen grows in a product, ideally the number of organisms used should reflect the numbers normally expected in the product. Typically, an inoculum level of between 2 and 3 log CFU/g is used, even when this exceeds expected numbers, because this level allows enumeration by direct plating *(53, 91)*. Lower levels may be used if documentation of low levels of natural contamination exists, because these lower levels will more accurately represent the product's ability to support growth *(91)*. When very low seeded populations (e.g., less than 100 cells per sampling unit) are most appropriate, consistent inoculation among individual samples may be difficult to achieve. Calculating the level of organisms in the product from the initial inoculum suspension, increasing sample size (e.g., from 25 to 250 g) and the number of replicate samples analyzed (e.g., from three to six samples), and/or using enumeration methods such as the most probable number (MPN) method will increase confidence in the number of organisms in the inoculum.

The inoculum level may affect the apparent efficacy of an antimicrobial or formulation combination to inhibit microbial growth. If the inoculum populations are too high, the factors inhibiting growth may be overwhelmed by the inappropriate inoculum level, leading to the incorrect conclusion that the formulation does not inhibit growth *(53, 80)*. In the case of sporeformers, germination and time to observable growth or toxin production may be significantly reduced if high initial spore loads are used *(69, 126)*. In contrast, a high inoculum level of vegetative cells (e.g., 5 to 7 log CFU/g) in a growth study may also mimic the population nearing stationary phase, which may result in an apparent no-growth or low-growth observation.

5.2. Inactivation studies

When conducting inactivation studies, high numbers of organisms are typically used, e.g., 6 to 7 log CFU/g *(53, 91)*, in order to quantify survivors and/or to document high levels of inactivation. The target level of reduction, which influences the inoculum level used, may depend on regulations for specific food types, e.g., a 5-log reduction of the appropriate pathogen in juice (21 CFR 120.24) *(120)*, 4-log reduction for treatment of almonds (7 CFR 981) *(105)* to inactivate *Salmonella,* and a 7-log reduction of *Salmonella* in poultry products (9 CFR 381.150) *(112)*. Laboratories conducting inactivation studies in products that are subject to regulations should be aware of the most current requirements.

Inactivation studies may be conducted to assess the lethality delivered by a specific process, e.g., the ability of UV light to achieve a 5-log reduction of *E. coli* O157:H7 in apple cider, or to determine inactivation of a pathogen over time, e.g., the effect of preservatives on pathogen inactivation during storage of a food product. In the former case, relatively high inoculum levels are generally used, as noted above. However, in the latter case, lower inoculum levels consistent with expected pathogen contamination levels might be used because preservatives would generally not be expected to inactivate large numbers of pathogens, depending upon the pH and other conditions. Studies might also be important to determine survival or inactivation of a pathogen in a product that is recontaminated.

Initial inoculum levels may affect the rate of die-off in some foods *(32, 95, 125)*, and this phenomenon needs to be taken into consideration.

6.0. Inoculum preparation

Ideally, isolates from foods should be stored in a manner to preserve the strain characteristics with respect to survival, growth, resistance, etc. (e.g., frozen in glycerol or freeze-dried). When reviving strains from the frozen or lyophilized state, there should be one to two successive transfers in a nonselective growth medium. Working cultures, e.g., refrigerated slants, may be prepared and used for a period of time (e.g., 7 to 30 days). The number of times a culture is transferred to produce new working stock cultures should be minimized to avoid genetic changes that affect the phenotypic properties of the organism *(91)*. AOAC International guidelines for laboratories *(8)* indicate there should be not more than five passages from the primary reference material. In some instances even fewer transfers may be appropriate, because organisms may readily lose extrachromosomal elements such as plasmids or other genetic markers and phage.

For challenge studies using vegetative cells, stationary phase cells (18 to 24 h) grown on nonselective media under conditions suitable for optimal growth of the specific challenge culture should generally be used *(53)*. However, in certain instances it may be desirable to precondition or adapt the culture to specific conditions that may be applicable to the specific characteristics of the food product. For example, for low pH foods it may be appropriate to acid adapt cultures *(34, 46, 65, 66)*, which can often be accomplished by growing the culture in tryptic soy broth with 1% glucose *(14, 27)*. Cold adaptation at 7 to 8°C (44.6 to 46.4°F) for 7 days may reduce the lag phase for pathogens *(121)*, which may be important for assessing the shelf life of refrigerated ready-to-eat products. Cold adaptation may be more important for challenge tests of foods with a short refrigerated shelf life, e.g., less than 7 days. Care should be taken to avoid habituation procedures that cause cells to be more sensitive to the adverse environment, e.g., simultaneous adaptation to cold and acid conditions *(95)* or acid stressing cells prior to a heat treatment *(87)*.

For inactivation studies, cells that are grown at higher than optimum temperatures may become more resistant to heat than are cells grown at optimum temperatures *(79, 96)*. Increased heat resistance can also be observed with brief

exposure to sublethal temperatures (heat shock) *(15, 94, 123)*. For either inactivation or growth studies, adaptation of cells should attempt to mimic the likely physiological state of the organism at the time it contaminates the food.

Prior to use, cells should be washed (e.g., in buffer or carrier medium) to remove nutrients or metabolites in the spent medium that could have an impact on growth in the test product. Cells should then be suspended in a carrier (buffer or homogenized portion of the food) to inoculate the food.

Composites containing multiple strains should have approximately equal numbers of the individual strains. This can be accomplished by previous experience enumerating the strains under specific growth conditions or by using turbidity measurements (e.g., optical density or McFarland standards).

Spores of pathogens such as *C. botulinum, Clostridium perfringens,* and *Bacillus cereus* can be prepared, washed, and suspended in sterile water and frozen, preferably at −20°C (−4°F) or below. As with vegetative cells, composites should contain approximately equal numbers of each strain. Spore suspensions can be enumerated to determine the number of spores and then appropriate volumes can be combined to prepare the inoculum.

Spore inocula are often heat shocked prior to use, unless they are inoculated into the product immediately prior to heating or processing. The decision on whether or not to heat shock a spore inoculum will depend on the expected state of the naturally occurring spores in the food product and the conditions of use of that product. For example, spores would not be heat shocked if the challenge study is being conducted in a raw commodity that will not be heated (e.g., raw reduced-oxygen-packaged fish). When it is desirable to have a mixture of vegetative cells and spores, the suspension should not be heat shocked.

It is important to verify the numbers of viable organisms in the inoculum used. In addition to enumerating the inoculum suspension itself, the inoculated food should be enumerated to obtain a zero-time count. If the inoculum level is low, an increased number of replicates of the inoculum and/or product may be necessary. Rapid and significant reductions in microbial populations are frequently observed when the food includes bactericidal ingredients such as nisin or other commercial fermentation by-products used for shelf-life extension. For example, a 0.5- to 2.5-log reduction in *L. monocytogenes* was observed immediately after inoculation in fresh soft cheese and in bologna and ham containing lactic acid bacteria (LAB) fermentate or nisin *(35, 37)*.

A dry inoculum may be required for studies in low-moisture foods or when added moisture needs to be avoided. Inoculum can be prepared by freeze-drying *(53, 80)* or dried on a product similar to the challenge food *(53)*. For preparation of a dehydrated inoculum, the organism may require several days to months to stabilize (e.g., *Salmonella* in skim milk powder) *(59)*. As a result, viable populations of the stabilized dried inoculum should be determined prior to use.

7.0. Method of inoculation

Inoculation procedures for challenge studies are described in the IFT report *(53)*. As that report notes, several critical considerations for the delivery of the inoculum to the product include maintaining the intrinsic or extrinsic characteristics of the product; simulating contamination that could realistically occur under manufacturing or storage conditions; and ensuring that, where appropriate, each of the unique interfaces of the product components receive the inoculum.

Two factors important to maintaining the intrinsic characteristics of the challenged product are minimizing inoculum volume and matching the critical factors of the food, such as pH and a_w. Typically the inoculum volume should be no more than 1% of the volume of the food and when possible, less. Some methods that have been used to minimize the inoculum volume include growing the pathogen to high populations and concentrating by centrifugation or growing the pathogen on a solid growth medium and then harvesting a paste for use as the inoculum. When challenging food products with reduced a_w or pH, the a_w or pH of the diluent can be adjusted using a humectant or acidulant similar to that contained in the food *(53)*. However, preliminary analysis should verify that modified pH or a_w of the buffer does not adversely affect viability of the pathogens.

An important extrinsic factor is the package atmosphere (see section 8.1. below: storage conditions, packaging). Ideally, product should be first inoculated and then packaged under an appropriate atmosphere that closely duplicates the packaging system to be used during commercial production. Alternatively, a common practice is to use a needle to inoculate through the packaging using some type of self-sealing rubber or silicon septum. Two disadvantages of using the latter type of inoculation method are long-term package integrity and inoculum distribution. Also, when inoculating with a needle, culture should be distributed over as large an area as possible to reduce the concentration of cells, moisture, and/or nutrients in limited areas. Package atmosphere (e.g., oxygen and carbon dioxide in the headspace) should be monitored during the duration of the study to assess the integrity of the package and to ensure that the effect of changes in gas composition is considered.

In general, the method of inoculation should place the inoculum on or within the product in a manner that realistically simulates potential contamination that might occur during manufacture, preparation, shipment, or display of the product. Liquid foods are inoculated by mixing the inoculum throughout the product with agitation. In solid foods, the inoculum may be mixed throughout a ground product or applied on the surface by dipping, aerosolizing, or spreading on the entire surface or on selected spots. Dipping the product in a liquid inoculum or using an aerosolized inoculum will allow organisms to be spread over the entire surface of the product, including cracks and crevices. However, if an aerosolized inoculum is used, inoculation should be conducted in a biological safety

cabinet to protect employees from the challenge organism. Preliminary studies should be conducted to standardize the amount of inoculum that contacts the product.

Many challenge products have multiple components or layers. If contamination during assembly is possible, the challenge inoculum should be applied to the various layers or components. Unique growth conditions can exist at the interfaces between components, such as the microenvironment between a pie crust and a pie filling. This area might have a unique combination of factors that will allow growth, so these areas should receive a portion of the inoculum. For this reason, the food should not simply be homogenized and inoculated. Other conditions of the microenvironment should also be considered, such as fat-water emulsions, microdroplets, or partitioning.

Inoculating a large batch prior to packaging or inoculating individual samples can be valid depending on the likely route of contamination, packaging considerations, and practicality. Inoculating a single batch of product will minimize the variability of the starting concentration as well as create a less heterogeneous distribution of the pathogen if the food can be mixed without destroying the product integrity. This issue is particularly critical in growth or inactivation studies in which documentation is needed to meet a specific regulatory requirement (e.g., no more than a 1-log increase as evidence of growth inhibition of *L. monocytogenes* in a deli salad or a 5-log reduction of *E. coli* O157:H7 in juice). Dividing a large inoculated batch into discrete portions for testing at each sampling interval reduces the risk of contamination caused by repeatedly resampling a large batch. Inoculating individual samples may be more appropriate for studies representing postprocess contamination by contact (e.g., cooked frankfurters or slices of cheese made with pasteurized milk) or when production cannot be readily replicated in the laboratory (e.g., filled pastries or individual packages with unique atmosphere and packaging materials). Inoculation methods that result in highly variable inoculum levels or uneven distribution require a greater number of samples at each sampling interval and potentially additional replicate batches to be analyzed.

8.0. Storage conditions

8.1. Packaging

Product packaging for the challenge study should be representative of typical commercial production. If the commercial product is to be packaged under vacuum or a modified atmosphere, the challenge study sample should be packaged under the same conditions, including the use of the exact gas mix used for modified atmosphere packaging, use of packaging material of the same gas permeability, and use of similar vacuum levels for vacuum-packaged product. Specific modified atmospheres or vacuum packaging may be inhibitory to some microorganisms but may stimulate growth or toxin production by other microorganisms *(53)*. Care should be taken to ensure that headspace volume and gas composition of the challenge study samples mimics the commercial food product as closely as possible.

8.2. Storage and shipping

Storage temperatures used in the challenge study should be representative of the expected temperature range that the product will be exposed to during commercial distribution and storage. For refrigerated foods, NACMCF recommends that the studies be conducted at 7°C (44.6°F) to account for expected consumer storage temperature in the United States *(75)*. Refrigeration studies may incorporate additional temperatures (e.g., 4 to 6 or 10 to 12°C [39.2 to 42.8 or 50 to 53.6°F]) when a better understanding of the behavior of the challenge organism is desired, such as with some antimicrobial compounds whose inhibition of microbial growth is temperature dependent *(21, 91)*.

Temperature changes may be incorporated into a challenge study protocol if, for example, a manufacturer distributes a refrigerated product under well-controlled conditions for a portion of its shelf life, after which the product may be subjected to elevated temperatures immediately prior to and during use *(53)*. For shelf-stable products, typical temperatures range from 24 to 35°C (75.2 to 95°F) depending on expected storage room temperatures *(21)*. Humidity should also be considered as a factor in storage conditions; for those products where the moisture content can change in response to ambient humidity conditions, the challenge study should be designed to incorporate representative environmental humidity variation *(80)*.

It is necessary to ensure that appropriate storage space is available and that proper temperatures are maintained and recorded throughout the study. Temperatures during storage and transportation of commercially made products to the laboratory should be monitored with continuous temperature recorders, data loggers, or periodic manual temperature verification. Samples inoculated with pathogens should be segregated and clearly labeled to prevent inadvertent human consumption.

9.0. Sample considerations

9.1. Sampling

Sampling schemes for food microbiology experiments are often dictated by common practice not solely based on statistical design. The suggestions below reflect this convention. The number of samples to be analyzed initially and at each time interval during processing and/or storage should be at a minimum two; however, analysis of three or more samples is preferred. Replicates should be independent trials using different batches of product and inoculum to account for variations in product, inoculum, and other factors. Generally, the number of samples and replicates should be increased in situations of higher variability or uncertainty. When the number of samples analyzed at each time interval is only two, it is better for the study to be repeated (replicated) more than two times. In studies with three or more samples tested at each time interval, two replicates are usually adequate. When analyzing samples for botulinum toxin it is appropriate to select a greater number of samples (e.g., five or more) per time point because of the potential variability in toxin production among samples

(74). For end-point lethality determination, 5 to 10 samples per time interval may be appropriate. If supporting data from other studies exist, the need for replication may be reduced *(91)*. Appropriate statistical experimental design can improve the validity of the study. There are quantitative methods for assessing the statistical quality of a study, e.g., power analysis. The study design may benefit from consultation with a statistician familiar with food microbiology studies.

The sample preparation method should be selected based on the type and properties of the food and the method of inoculation, which depends on the food product and the inoculation procedure *(53, 91)*. In cases of solid foods inoculated on their surface and in products where the contamination is expected to be localized on their surface, samples may be swabbed or sponged, washed or rinsed, and/or agitated in a liquid buffer or diluent of known volume. After thorough mixing, the rinsate is analyzed by direct plating of appropriate dilutions onto appropriate culture media (see section 9.2 below). The results can be expressed per unit of surface area or per sample, especially for items of irregular conformation. For example, surface-inoculated frankfurters may be prepared for detection of *L. monocytogenes* as whole links washed or rinsed with diluents, and the results may be expressed per unit surface area or whole link, if the links are of uniform size.

Alternatively, surface samples may be excised and homogenized in diluent. The results may be expressed per unit of surface area or per gram. For example, a spot-inoculated leafy green may be sampled by cutting a surface area surrounding and greater than that inoculated and the sample can be homogenized or macerated to release bacterial cells. Some foods, e.g., surface-inoculated whole tomatoes or melons, may be sampled with a sterile cork borer, extracting a defined section from an area of the surface that was inoculated or treated.

Caution should be exercised when considering analysis of composited samples in challenge studies. Compositing multiple samples for pathogen enumeration eliminates detection of variability among discrete samples and may reduce sensitivity of the analysis. Furthermore, composited samples may dilute toxins to less than detectable levels if these toxins are present in only one of the multiple samples. However, compositing samples before or pooling samples after an enrichment procedure may be appropriate to confirm absence of survivors in an inactivation study. Pooling after enrichment can be used as a screening procedure that will later allow determination of how many original samples were positive. Compositing or pooling approaches must be validated to assure sensitivity is not lost.

9.2. Sample analysis for target pathogens or toxins

The objective of sample preparation for microbial analysis is to retrieve all microbial spores or cells of interest (or toxin, where appropriate). Sample preparation should provide conditions that will allow the metabolic activity to lead to detectable colonies or other measurements indicating activity and allow a measurement of survival or growth levels. It is common to use a 1:10 initial dilution in Butterfield's phosphate buffer or buffered peptone water for vegetative pathogens or spores. However, if the product has a high salt or sugar content it may be necessary to modify the dilution buffer to avoid shocking the cells. Enrichment procedures for the target pathogen should be considered at time points when levels of survivors are expected, or previously determined, to be below the experimental limit of detection by direct plating. Rapid detection methods that have been validated (see Appendix A) are appropriate when enumeration is not necessary.

Sample analysis must be done using methods that permit the accurate and reproducible recovery of microorganisms. In all cases the amount of buffer or diluent used must be defined and constant among samples, and this level should be selected based on sample size, level of contamination expected, and minimum level of detection desired. The sample preparation protocol and washing, rinsing, or blending time should be consistent, and the time between sample processing and plating should be short and constant for all samples. Sample preparation temperature and time and conditions and variables involved in sample preparation should be maintained constant to the extent possible; these variables include volume or weight, surface area, composition, and properties (e.g., pH) *(12)*.

For growth studies, pathogens should be enumerated on appropriate selective agar (see Appendix A). Inactivation studies may result in injured cells, and direct plating of these cells onto selective agar can overestimate the extent of death. In such cases, samples should be prepared and tested in ways that allow repair and recovery of injured organisms. Recovery of injured cells can be enhanced by using nonselective media such as tryptic soy agar (TSA) or plate count agar overlaid with selective agar after 2 to 4 h of incubation at optimum temperature *(20, 40),* by using selective agar overlaid with nonselective agar *(124),* by using agar underlay techniques *(60, 61),* or by replica plating from a nonselective agar such as TSA to a selective agar *(100)*. Standard methods for extraction of *C. botulinum* neurotoxins and *S. aureus* enterotoxins from foods can be found in the references provided in Appendix A.

9.3. Enumeration of indigenous microbial flora

In addition to inoculated product, sometimes it is also useful to test corresponding uninoculated control samples to determine levels of background microflora surviving the process or changes that occur during product shelf life *(53, 91)*. Moreover, protocols for challenge studies to determine growth inhibition or inactivation based on product formulation should consider and address potential effects of naturally occurring microflora on the pathogens of concern. In addition, spoilage and the end of shelf life are usually associated with an increase in microbial populations. Thus it is recommended that microbiological numbers such as aerobic plate counts and number of spoilage organisms typical for the product (e.g., LAB or yeasts and molds) be obtained. Testing for these or other indicator microorganisms cannot substitute for pathogen testing. In addition, the presence or absence of spoilage bacteria cannot be used as an indicator of safety.

LAB are expected in fermented or cultured food products at relatively high populations (e.g., 6 log CFU/g), but indigenous populations are low in most processed foods. This group of bacteria is known to compete well with low levels of pathogens for nutrients, can grow over a wide range of temperatures, and can reduce the pH of the food through acid production, and some strains can produce bacteriocins that may inhibit some pathogens. Relying on the presence of naturally occurring background levels of LAB in foods is an unreliable method to control pathogens. Conversely, competitive microflora may inhibit growth of specific pathogens, and failure to account for this interaction could lead to erroneous conclusions. Thus, it may be important in some circumstances to monitor LAB growth during the challenge study to determine if competition may contribute to inhibition of pathogens during the trial.

Although they may be present, molds and yeasts may not be initially visible on the food. Deamination of food proteins by molds can produce ammonia and a localized increase in pH that can increase the potential for pathogen growth in that microenvironment *(81)*. Populations of molds and yeasts can be enumerated by using a variety of selective plating media or by other validated procedures.

9.4. Determination of physical parameters

Food properties such as proximate composition (protein, fat, and moisture), pH, titratable acidity, a_w, salt content, and residual nitrite can influence the behavior of pathogens. It may be important to measure these factors as part of the challenge study. Some parameters, such as pH, that may change during the study may need to be monitored at appropriate points throughout the study in parallel with microbial analysis. Sources of appropriate methods can be found in Appendix A. The number of samples to be analyzed is described in section 9.1 above.

Changes in pH can be an indicator of microbial metabolism when microbial populations are not enumerated or if growth is not significant. The pH of foods that are homogeneous and likely have consistent pH throughout the matrix can be measured on a representative sample. In contrast, complex foods consisting of multiple discrete components or ingredients may require multiple pH measurements. For example, a sandwich may require measuring the surface or interface pH of the components in addition to a homogenized sample.

For obvious safety reasons, no sensory assessment other than changes in appearance (phase separation, turbidity, texture, and gas formation) should be performed on challenge test samples. In some instances, the investigator should make a judgment if the product would be considered ''edible'' based on visual and olfactory observations. Note that because pathogens or toxins may be present, olfactory observations may constitute unacceptable risk to the laboratory worker.

10.0. Duration of study and sampling intervals

Challenge studies should be conducted for at least the intended shelf life of the product *(21, 53, 122)*. For some shelf-stable products this may mean holding products for a year or longer. Ideally, products should be held for some period beyond the end of the intended shelf life to account for users who might consume the product past the end of the declared shelf life and to add an additional margin of safety *(53)*. Depending on the shelf life of the product, this time may be 25% (e.g., for products with shelf life of 3 to 6 months) to 50% (e.g., for products with shelf life of 7 to 10 days) longer than the intended shelf life of the food *(53, 91)*. This additional time may be important for recovery of cells injured by heat or by antimicrobials in the product. For some products that still have acceptable sensory properties at the end of the intended shelf life, it may be important to continue studies until overt spoilage occurs, as consumers may consume the product as long as it does not appear spoiled. Samples held under abuse conditions are unlikely to last the full shelf life and are usually sampled for shorter time periods *(53)*. Samples, including controls, should be analyzed initially after inoculation (in some cases, after a short equilibration period) and then five to seven times over the duration of the study *(53)*. For long-shelf-life products, it may be necessary to have more than seven sampling points.

The sampling interval should be determined based on prior experience with similar products and in consideration of the likely duration of survival or rate of growth or inactivation. Depending on the product characteristics and expected outcomes for products with a long shelf life, it may be appropriate to test on a more frequent basis early in the study (e.g., daily) and at longer intervals later in the study *(53)*.

A growth inhibition study may be ended when there is greater than a 1- to 2-log increase in pathogen growth or when toxin is detected in samples for two consecutive sampling intervals (indicating growth of the pathogen of concern) or there is gross spoilage such that the product is no longer fit for consumption. Care should be taken in making this determination, because spoilage and apparent edibility are subjective.

When measuring pathogen inactivation, the study is typically concluded when the pathogen is no longer recovered from the product. However, in some cases (e.g., thermal death time studies) it may be important to take into account the possibility of injured cells and to continue incubation of samples until the end of product shelf life to verify that injured cells do not recover and grow *(91)* or produce toxin in the product over time. Alternatively, attempts to recover the pathogen in noninhibitory enrichment media after a period of incubation in the product may be used to verify the absence of survivors.

11.0. Interpreting test results

Interpreting the results of microbiological growth and inactivation studies requires evaluation by expert microbiologists who will consider all relevant factors *(53, 80, 91)*. In determining whether a product supports growth of a pathogen, it is rarely as simple as comparing final and initial counts. Numbers from different sampling points may vary due to inherent variation in sampling and enumeration procedures, particularly when foods contain antimicrobial compounds that limit growth. It may be difficult to

determine if changes in numbers are real or due to analytical variability. In addition, there may be an initial die-off in some foods following inoculation, and if this die-off is followed by growth that does not exceed the target inoculum level, this growth may not be recognized. This issue may be addressed by allowing a brief time (e.g., 2 h) for the inoculum in the product to equilibrate prior to conducting the initial count *(53)*. Normal sample variation may result in a spike at a sampling interval that may not be significant *(122)*; this variation can often be addressed through testing of multiple samples. Graphical representation of the data to examine trends may be useful in assessing whether actual growth has occurred *(53)*. This is particularly important in cases where the data set contains one or more outlying data points. The interpretation of inconsistent or highly variable results is an important and complicated issue and should be done by an expert microbiologist (see Table 1).

An increase in 1 log cycle over two or more time intervals is generally considered significant by food microbiologists *(122)*. Smaller increases may be significant depending upon the enumeration methods, number of samples and replicates used, and the variability among data points. Thus, in determining that a product does not support growth of a pathogen, in general less than a 1-log increase above the initial inoculum level throughout the intended shelf life of the product and across replicate trials would be an appropriate acceptance criterion *(53, 91)*. This level reflects the inherent variation that exists with enumeration of microorganisms *(53, 103)*.

Statistical methods can also be used to determine whether differences in counts at specific sampling points indicate true growth or are simply due to sampling and measurement errors. Where the repeatability and reproducibility of the enumeration method have been determined through validation studies and the standard deviation of reproducibility can be calculated, a more precise determination of a significant difference may be made. For example, Agence Française de Sécurité Santitaire des Aliments (AFSSA) *(13)* recommends a 0.5-log CFU/g increase between initial and final concentrations as indicating that growth of *L. monocytogenes* has occurred. This value is based on an estimation of measurement uncertainty *(55, 57),* which is determined by doubling the reproducibility standard deviation. It should be noted however that the reproducibility standard deviation can vary. Scotter et al. *(92)* conducted tests to validate the International Organization for Standardization (ISO) method for enumeration of *L. monocytogenes* in foods and found that the reproducibility standard deviation ranged from 0.17 to 0.45 log CFU/g, depending on food product and level of contamination. Thus, depending on the food, inoculum level, and method of enumeration, a difference greater than 0.5 log CFU/g may (or may not) be an appropriate criterion. It should also be noted that statistically significant differences may not always be biologically relevant. An expert microbiologist, using available data and past experience, can best determine if the data represent a trend of increasing numbers or are simply a product of the variation seen in enumeration studies *(91)*.

Where studies have been conducted with *C. botulinum,* detection of toxins is measured rather than growth, as toxin can be produced without an increase in number *(47)*. No toxin should be detected in the product over the duration of the challenge study *(53)*. In lieu of testing for *Staphylococcus* enterotoxins, limiting growth of *S. aureus* to less than 3 log CFU/g may be used *(53)*. This limiting growth level was based on the assumption that the initial population does not exceed 3 log CFU/g and that a minimum of 6 log CFU/g is needed to produce staphylococcal enterotoxins.

Where multiple formulations have been challenged, growth or toxin production in one formulation but not in another may provide useful data on the inhibitory properties of the product with respect to pathogen growth. In this case, the effect of formulation differences will help to identify critical factors necessary to control pathogen growth or toxin production. Similarly, if a product is produced by a manufacturing process that encompasses the point of ''failure,'' this is an indication that the manufacturing variability may be too great to assure the safety of a product formulated in this manner.

For lethality experiments, log reductions should be determined in replicate trials. The log reduction should meet any existing regulatory performance standards that apply to the food product. Where no performance standard exists, the lowest log reduction achieved should exceed the expected contamination level by an amount that incorporates a margin of safety (a 2-log margin is often used) consistent with the variability expected in the product and the process *(91)*.

The discussion above indicates that universally acceptable rules for interpreting test results are not available and points out the need for further consideration to produce clear guidance on this subject.

12.0. Elements to include in the report

In order for others to assess the adequacy of a challenge study, it is imperative that the study report provide appropriate information, including an interpretation of the results. The report should begin with an introduction that includes the purpose of the study and reviews the data supporting the experimental design. The report should include information characterizing the product and process. The materials and methods should be described as they would be in a scientific publication. The results should include both raw and summarized data and should be clearly presented. Any statistical design and analysis of results should be thoroughly described. If statistical analysis was not used, that fact should be clearly stated and justified. A discussion should provide an interpretation of the results and any limitations on the applicability of the data. The conclusions should contain any recommendations and should indicate the types of changes in product formulation or processing that could warrant a new challenge study.

2. What are the appropriate uses of mathematical growth and inactivation models? Under what conditions can these models be used as a substitute for inoculated pack/challenge studies? Of the models

TABLE 3. *Examples of mathematical growth and inactivation models and their applicability to different foods*

Model name	Reference	Applicability
American Meat Institute Foundation Process Lethality Determination Spreadsheet	*4*	The model provides meat processors with a science-based validation tool that can be used to demonstrate the effectiveness of a specific heat process to destroy microorganisms of concern.
ComBase Predictor	*50*	ComBase Predictor models are based on observations made in culture media and comprise a set of 20 growth models, 7 thermal death models, and 2 nonthermal survival models. Temperature, pH, and a_w (usually as a function of NaCl) are the core factors, but for some organisms, the effect of a fourth factor, such as CO_2, nitrite, etc., is also featured.
Isothermal-Based Prediction Tool (IBPT)	*104*	The software can be used to predict whether *Salmonella, E. coli* O157:H7, or *S. aureus* will grow to a ''level of concern'' in raw beef and pork products.
Microbial Responses Viewer (MRV) for ComBase (version beta 1)	*78*	The MRV is a new database consisting of microbial growth–no growth data derived from ComBase. The software allows the user to rapidly view growth–no growth contour plots superimposed with actual ComBase data. Contours of any two of three variables (temperature, pH, and a_w) can be visualized while the third is held constant.
Opti.Form *Listeria* Control Model 2007	*85*	The model predicts *Listeria* growth for both uncured and cured cooked meat products. The model will help to calculate the level of lactate and diacetate needed to control *Listeria* in cured and uncured cooked meat and poultry products for their required shelf life.
Pathogen Modeling Program (PMP)	*106*	This predictive microbiology application was designed as a research and instructional tool for estimating the effects of multiple variables on the growth, inactivation, or survival of foodborne pathogens. Most of the models are based on experimental data of microbial behavior in liquid microbiological media.
Perfringens Predictor	*51*	Perfringens Predictor provides a prediction of growth of *C. perfringens* during the cooling of meats. This model is part of ComBase Predictor and may give more accurate predictions than the *C. perfringens* model included in PMP *(89, 97).*
Seafood Spoilage and Safety Predictor (SSSP, version 3.0)	*77*	Software includes models for relative rates of spoilage, models for growth of spoilage bacteria in specific seafood, models to predict histamine formation by *Morganella* spp., a model to predict the simultaneous growth of *L. monocytogenes* and lactic acid bacteria in lightly preserved seafood, and a model to predict the growth boundary of *L. monocytogenes* in lightly preserved seafood.

currently available, which ones are most suitable for use, and what are the limitations of these models?

Predictive food microbiology is a subdiscipline of food microbiology that uses models (i.e., mathematical equations) to describe the growth, survival, or inactivation of microbes in food systems. Mathematical growth and inactivation models can always be used to help guide the design of product assessments or challenge studies. In these cases, the challenge studies will either substantiate (i.e., agree with or be conservative with respect to) the model predictions or show those predictions to be invalid for the specific product. An example of a conservative model would be one that predicts a 2-log increase when the challenge study shows a 1-log increase. Two ideal uses of predictive models are for narrowing the choices for treatments to be validated for safety and for choosing the appropriate challenge microorganisms.

Intrinsic and extrinsic factors (pH, a_w, temperature, etc.) used as inputs for the model should be chosen with care. The least restrictive parameters determined for the range of processing conditions should be used. If the conditions modeled suggest that growth could occur or that there is limited lethality for the product or process, then additional studies, product reformulation, or modification of target shelf life would be warranted. If there is less confidence in the model, then limited challenge studies may be warranted to verify the prediction from the model *(1).*

Caution should be exercised when models alone are used to make a decision. Use of models requires experience and judgment, both in modeling and food microbiology. When models alone are used to make a decision, those models must be shown to be valid for the food in question and should take into consideration lot-to-lot variation. Validation may be based on published or unpublished data for very similar or identical foods. The data should be generated by a laboratory having personnel with the appropriate knowledge, skills, and abilities in conducting challenge studies (see Table 1) or be obtained from other relevant published studies.

The two best-known multipathogen multifactor models available today are the USDA Agricultural Research Service Eastern Regional Research Center Pathogen Modeling Program (PMP) *(106)* and the ComBase Predictor *(50),* formerly known as the FoodMicroModel (Table 3). Both of these modeling programs make predictions for a wide array of foodborne pathogens and growth factors (temperature, pH, etc.). Both programs are also based on data collected primarily in laboratory media rather than foods and do not always cover the full range of each growth parameter (Tables 4 and 5). Elements of both models have been

TABLE 4. *Pathogen growth ranges used in ComBase and Pathogen Modeling Programs*[a]

	ComBase					PMP				
	Temp (°C)		pH			Temp (°C)		pH		
Pathogen	Minimum	Maximum	Minimum	Maximum	a_w (minimum)	Minimum	Maximum	Minimum	Maximum	a_w (minimum)
B. cereus										
With CO_2	5	34	4.9	7.5	0.974					
Aerobic						5	42	4.7	7.5	0.97
Anaerobic						10	42	5.0	9.0	0.97
C. botulinum (growth only)										
Proteolytic	14	40	4.7	7.2	0.954	15	34	5.0	7.2	0.977
Nonproteolytic	4	30	5.1	7.5	0.974	5	28	5.0	7.0	0.977
C. perfringens	15	52	5	8	0.971	19	37	6.0	6.5	0.983
E. coli O157:H7										
With CO_2	10	30	4.5	7	0.961					
Aerobic						5	42	4.5	8.5	0.97
Anaerobic						5	42	4.5	8.5	0.97
L. monocytogenes										
With CO_2	1	35	4.4	7.5	0.934					
Aerobic						4	37	4.5	7.5	0.928
Anaerobic						4	37	4.5	8.0	0.97
S. aureus (growth only)										
Not specified	7.5	30	4.4	7.1	0.907					
Aerobic						10	42	4.5	9.0	0.911
Anaerobic						12	42	5.3	9.0	0.872
Salmonella										
With CO_2	7	30	3.9	7.4	0.973					
Aerobic						10	30	5.6	6.8	0.974

[a] Limits tested in ComBase *(48)* and the Pathogen Modeling Program (PMP) *(106)* do not necessarily represent limits for growth. See Table 5 for growth limits.

validated (by both published and unpublished studies) to a limited degree in different food systems.

There is also a wide array of computer models developed in laboratory media and food systems that are not part of PMP and ComBase. Examples of several models are shown in Table 3. Some models published in the scientific literature are not available in a user-friendly, downloadable form. These models require some modest modeling or spreadsheet manipulation skills on the part of the user to produce a useful prediction.

Any discussion of modeling and validation of models would be remiss if it did not also mention another tool that is part of the ComBase Modeling Toolbox: the ComBase browser *(48)*. The ComBase browser provides access to the ComBase database of microbial responses to food environments. At the present time the database includes more than 35,000 observations, of which more than 13,000 are from food and the balance (~22,000) are from culture media. Researchers publishing microbial growth or survival data are requested and encouraged to submit the data to ComBase *(49)*. The data contained in ComBase may represent a useful source of published and unpublished data for validating models.

3. What are the limitations for applying the results of an inoculated pack/challenge study conducted on one food to another similar food?

Challenge studies on one product may sometimes be applicable to other products. However, if there are significant differences between the intrinsic properties of the product and those of the food on which the challenge study was conducted, the results of the challenge study may not be applicable. If the challenge study is conducted using parameters or conditions more conducive to growth or survival than those in the food product under consideration, then additional challenge studies may not be needed *(76)*. For example, the results of a challenge study for a specific pathogen in a product formulation with a pH of 5.8 could be applied to a similar formulation where the primary difference is a pH of 5.4. Nevertheless, an expert microbiologist should make the determination of applicability of one challenge study to additional products. The composition of the two foods, e.g., protein content, carbohydrate source, type of organic acid, fat, and moisture, should be considered in determining the applicability of one study to another product. Generally, the more similar the composition the more likely the study will apply.

TABLE 5. *Limits for growth when other conditions are near optimum based on references (54) and (116)*

Pathogen	Source[a]	Temp (°C)		pH		a_w (minimum)	Maximum water phase NaCl (%)
		Minimum	Maximum	Minimum	Maximum		
B. cereus	FDA	4	55	4.3	9.3	0.92	10
	ICMSF	4	55	5.0	8.8	0.93	
C. botulinum (growth only, proteolytic)	FDA	10	48	4.6	9	0.93	10
	ICMSF	10–12		4.6		0.93	10
C. botulinum (growth only, nonproteolytic)	FDA	3.3	45	5	9	0.97	5
	ICMSF	3.3		5.0		0.97	5
C. perfringens	FDA	10	52	5	9	0.93	7
	ICMSF	12	50	5.5–5.8	8.0–9.0	0.97	
Pathogenic *E. coli*	FDA	6.5	49.4	4	9	0.95	6.5
	ICMSF	7–8	44–46	4.4	9.0	0.95	
E. coli O157:H7	ICMSF	8	44–45	4.5			Slow growth at 6.5, no growth at 8.5
L. monocytogenes	FDA	−0.4	45	4.4	9.4	0.92	10
	ICMSF	−0.4	45	4.39	9.4	0.92	
S. aureus (growth only)	FDA	7	50	4	10	0.83	20
Aerobic conditions	ICMSF	7	48	4	10	0.83	
Anaerobic conditions	ICMSF			5.0		0.90	
Salmonella	FDA	5.2	46.2	3.7	9.5	0.94	8
	ICMSF	5.2[b]	46.2	3.8	9.5	0.94	

[a] FDA, U.S. Food and Drug Administration *(116)*; ICMSF, International Commission on Microbiological Specifications for Foods *(54)*.
[b] Most serovars will not grow below 7°C (44.6°F).

4. Of the existing inoculated pack/challenge study protocols, e.g., those published by the American Bakers Association, NSF International, and others, which are most suitable for application to a wide variety of foods, and what are the limitations of these protocols? Are there existing protocols that are appropriate for specific food-pathogen pairs?

The committee agrees with an earlier assessment in the IFT report *(53)* indicating that both the American Bakers Association (ABA) and the National Sanitation Foundation International (NSF) testing protocols suffer from significant weaknesses. These are briefly highlighted below; for more details, see Table 2 in the IFT report *(53)* comparing the NSF, ABA, and expert panel's protocols.

The NSF protocol provides test methods for determining that a product does not require refrigeration for safety. The NSF protocol lacks flexibility and is highly prescriptive in specifying microbial strains and methods. It applies to a limited number of products (breads and pastries with vegetables or soft cheeses added prior to baking; bakery products filled or topped with cream, crème, custard, or cheese after baking; products filled prior to baking, such as pumpkin, sweet potato, custard, or meringue pies; and toppings, glazes, icings, or fillings stored without temperature control) and excludes a number of products of potential concern (e.g., modified-atmosphere-packaged products, all products with a pH $<$ 4.6, and products stored without temperature control for less than 24 h or more than 31 days). The a_w and pH are the only criteria for selection of challenge test organisms, with no consideration of the process given the product in selecting appropriate organisms. In addition, there is no consideration given to challenge tests with *C. botulinum,* only with *C. perfringens*. The recommendations would result in unnecessary and sometimes inappropriate challenge tests. There is no consideration for the need to adapt the inoculum, and the inoculum size is fixed for all products. The protocol does take into consideration the need to inoculate different components and interfaces of multicomponent products and requires testing of duplicate samples per time point with multiple lots of products. Overall, the protocol has significant limitations, even for application to the intended products.

The ABA protocol (Industry Protocol for Establishing the Shelf Stability of Pumpkin Pie) is even more limited in scope (i.e., applies only to pumpkin pie intended for distribution and display without refrigeration). The objective of this protocol is to define the process that a manufacturer can use to demonstrate the shelf stability of a pumpkin pie product in accordance with the then current edition of the FDA Food Code. This protocol is not an inoculated challenge study but rather a method for validating a cooking procedure (product reaches at least 82.2°C [180°F] at the coolest point) with respect to the destruction of naturally occurring microorganisms, both pathogenic and nonpathogenic. However, the absence of a pathogen in such a study cannot be relied on to assess whether or not a pathogen would grow if present in the product, because such a pathogen may or may not have been present initially. Additionally, monitoring the oxidation-reduction potential in the product to ascertain whether *C. botulinum* would grow and produce toxin is inadequate to make such a determination. Thus, the protocol has significant limitations, even for application to the intended product.

The IFT expert panel report *(53)* is written to encompass a wide variety of foods. The guidelines provide a framework for determining whether foods need TCS. The document also describes guidelines for challenge tests for determining the ability of a food to support the growth of one or more pathogens, but it does not address inactivation challenge tests. The guidelines provide flexibility but result in a potential for different interpretations as to what is appropriate for specific food types. This makes it more difficult for those reviewing and evaluating the data to determine if the study itself was adequate, and thus the reviewer may need to have technical expertise for the assessment. This is a weakness inherent to any document that is designed to apply to a broad range of food types.

Notermans et al. *(80)* developed a user's guide to microbial challenge testing for food safety and stability. The document addresses selecting the appropriate microorganism, preparing the inoculum, inoculum size, inoculation procedure, duration of the study, and sampling times. The recommendations are generally consistent with those in this NACMCF document, although less detailed. As with the IFT expert panel report *(53),* technical expertise may be required to interpret the adequacy of studies following these guidelines.

Scott et al. *(91)* published guidelines for conducting *L. monocytogenes* challenge tests for foods. Their article covers guidelines for studies to evaluate both the ability of a food to support the growth of *L. monocytogenes* and the inactivation of the organism in a food. The article in large part applies the recommendations in the IFT report *(53)* to challenge studies involving *L. monocytogenes* and is thus specific to a single organism. The protocols are also limited to those food products in which growth or inactivation of *L. monocytogenes* is a concern. The protocols in general are consistent with those in the present document and are appropriate for *L. monocytogenes* in refrigerated ready-to-eat foods.

AFSSA, a European Union (EU) community reference laboratory for *L. monocytogenes,* has recently published a technical guidance document for conducting shelf-life studies to determine compliance with microbiological criteria for *L. monocytogenes* in ready-to-eat foods set out in EC regulation No. 2073/2005 *(13)*. Similar to Scott et al. *(91),* the scope is limited to *L. monocytogenes,* including information on how to conduct experiments of the shelf life in naturally contaminated and artificially contaminated ready-to-eat products. The document includes determination of shelf life in naturally contaminated foods, called durability studies, which are not addressed in this NACMCF document. The AFSSA document also provides information on how to interpret the results obtained against EU *L. monocytogenes* regulatory criteria in ready-to-eat foods (no more than 100 CFU/g at end of shelf life). The document does not address inactivation of *L. monocytogenes* but does address many of the same key points as this NACMCF document, such as taking into account the product characteristics, batch variability, use of multiple strains, adapting the challenge organisms, and simulating natural conditions when inoculating product. The protocol indicates that to assess growth potential samples need be taken only initially and at the end of the shelf life and that for homogeneous products enumeration of only one sample is needed (three samples for heterogeneous products) at each of these time points. (More sampling times are recommended for studies intended to assess maximum growth rate or lag time.) The methods described in the AFSSA document are appropriate for *L. monocytogenes* in refrigerated ready-to-eat foods; however, the acceptance criteria differ from those proposed here.

In several documents, NACMCF has provided guidance for conducting microbial challenge tests. In 1990, NACMCF *(74)* made recommendations for extended-shelf-life refrigerated cooked meat and poultry products that included appendices on guidelines for thermal inactivation studies using *L. monocytogenes* and for *C. botulinum* inoculation studies. Those recommendations are generally consistent with this NACMCF document. Although the approaches used in the 1990 document are not specific to refrigerated meat and poultry, they are specific for the individual organism for which the guidance was developed. The protocols are appropriate for their intended use.

In 2005, NACMCF *(75)* published an article on considerations for establishing safety-based consume-by date labels for refrigerated ready-to-eat foods; the appendix to that document contained guidance for conducting microbial challenge studies to validate the safety-based use-by date label. This guidance was specific for *L. monocytogenes* and is consistent with the guidance in this NACMCF document. The protocol is appropriate for its intended application (validation of a use-by date).

There are a number of good challenge test protocols useful for specific purposes. This document and the IFT report *(53)* are the most comprehensive, broad-based documents that can be applied to assess the adequacy of microbial challenge studies. Because these documents are not specific to a food category, technical expertise may be needed to assess the adequacy of the challenge study with respect to appropriateness of the challenge organism, storage temperatures, etc. However, a well-written report should provide the rationale for many of the choices, thus assisting in the review to determine study adequacy.

5. Develop a decision tree to aid in the design of an appropriate inoculated pack/challenge study. Test or "desk check" the decision tree using the following five foods: meat-filled puff pastry, (baked) cheese pizza, chopped lettuce, cheese (blocks or slices), and lemon meringue pie.

Due to the complexity of decisions needed, the committee concluded that a decision tree could not be developed. Instead, the committee developed a template containing a series of questions to facilitate the design of an appropriate challenge study. The template was validated using five food products (Appendices E through J).

The examples in Appendices E through J were developed to illustrate the thought processes that expert microbiologists use in approaching the design of microbial

challenge tests. These examples should not be considered complete or accurate with respect to all parameters. Moreover, other approaches to conducting the challenge studies may be applied. The pass-fail criteria used in the examples represent expert opinion and may need to be verified with the appropriate regulatory agency.

6. Identify the basic knowledge, skills, education, training, experience, and abilities necessary for a multidisciplinary work group or individual to be qualified to design, conduct, and evaluate an inoculated pack/challenge study and the pursuant results.

Refer to Table 1, Question 1, Section 1.0 and Appendix B for this information.

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APPENDIX A. SOURCES OF ACCEPTED LABORATORY METHODS*

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* Dates of references current as of publication. Use most current version available.

APPENDIX B. CONSIDERATIONS FOR SELECTING A LABORATORY

Note: The following questions may be useful in comparing the capabilities of different laboratories. The questions are not listed in order of importance. A negative response to one or more of these questions does not necessarily disqualify a laboratory from consideration. The most important considerations are associated with qualifications of personnel for designing, conducting, and evaluating challenge studies.

- Does the microbiologist in charge have experience performing challenge studies including the food types you want to study? If so, ask the laboratory to provide examples of the types of challenge studies performed recently.
- What is the academic education and training of the microbiologist supervising the laboratory operations?
- What is the academic education and training of technicians performing the laboratory experiments?
- Is the laboratory audited periodically or accredited by an independent third party? If so, ask the laboratory to provide a copy of certificates documenting the audit. If not, ask how the laboratory ensures the quality of the processes and results, e.g., appropriate positive and negative controls, and a written, implemented quality control system for the laboratory operations, including a corrective action plan. ISO17025 certification is an example of a third party audit that would verify many of the good laboratory practices that should be implemented. Accreditations and certifications do not necessarily qualify a laboratory to design and conduct microbiological challenge studies. It is important to confirm that the laboratory has the experience and expertise necessary to perform the challenge studies
- Does the laboratory use approved, validated, or widely accepted published methods for the requested analyses? If so, what are the references for the methods used?
- Does the laboratory use certified reference materials (e.g., traceable positive controls) and standards (e.g., NIST calibrated equipment), where applicable, to perform the requested tests?
- Does the laboratory use subcontractors to perform the analyses in question? If so, how does the primary laboratory ensure the subcontract laboratory produces valid results?
- If the protocol involves inoculation with a foodborne pathogen, does the laboratory have appropriate biological safety containment and practices?
- Does the laboratory possess microbial strains that are appropriate for the food to be challenged? How are the stocks maintained and verified for purity and identity prior to the start of the study?
- If the protocol involves testing for a select agent (e.g., *Clostridium botulinum* or botulinum toxin), is the laboratory approved to work with that particular agent? In the United States, laboratories must be approved to work with each select agent on which they perform tests or research.

APPENDIX C. PATHOGENS OF CONCERN AND CONTROL METHODS FOR VARIOUS PRODUCT CATEGORIES THAT MAY NEED A CHALLENGE STUDY (GROWTH INHIBITION, INACTIVATION, OR COMBINATION)[a]

Product category[b] (examples)	Pathogens of concern (alphabetical order)	Examples of process control[c] (alone and in combination, alphabetical order)
Meat and poultry: cooked (e.g., roast beef, deli-style turkey, ham)	*Clostridium botulinum* and *C. perfringens,* enterohemorrhagic *E. coli, L. monocytogenes, Salmonella, Staphylococcus aureus*	Cooling rate, heat treatment,[d] high pressure processing, preservatives, storage time-temperature
Meat and poultry: dried and/or fermented (e.g., fermented sausage, jerky, dry cured ham)	*C. botulinum, C. perfringens,* enterohemorrhagic *E. coli, L. monocytogenes, Salmonella, S. aureus*	a_w, drying, fermentation, heat treatment, humidity, nitrites and other preservatives, pH salting, storage time-temperature, water-phase salt
Fish and seafood (e.g., smoked fish, fresh oysters, pickled herring, pasteurized crab meat)	*B. cereus, C. botulinum*, *L. monocytogenes, Salmonella*, *Shigella* spp., *S. aureus, Vibrio cholerae, V. vulnificus, V. parahaemolyticus*	a_w, drying, harvest site control, heat treatment, high-pressure processing, nitrites, pH, preservatives, salting, storage time-temperature, water-phase salt
Cultured dairy products, pH $\leq$ 4.7 (e.g., yogurt, sour cream, buttermilk)	Enterohemorrhagic *E. coli*, *Salmonella*, *L. monocytogenes*, *S. aureus*	Heat treatment, pH, preservatives, rate of acid production, starter culture activity, storage time-temperature
Cultured dairy products, pH $>$ 4.7 to $\leq$5.4 (e.g., cottage cheese)	*B. cereus*, *C. botulinum*, enterohemorrhagic *E. coli*, *L. monocytogenes*, *Salmonella*, *S. aureus*	Heat treatment, hot fill, preservatives, storage time-temperature
Cheese and cheese products (e.g., natural Swiss cheese, processed cheese slices, processed cheese spread)	*C. botulinum,* enterohemorrhagic *E. coli, L. monocytogenes*, *Salmonella*, *Shigella* spp., *S. aureus*	a_w, emulsifiers, heat treatment, hot fill, moisture content, pH, preservatives, storage time-temperature
Butter and margarine (e.g., light salted butter, whipped butter)	*L. monocytogenes, S. aureus, Yersinia enterocolitica*	a_w, heat treatment, moisture droplet size in the water-in-oil emulsion, water-phase salt
Eggs and egg products (e.g., meringue, pooled pasteurized egg yolks, sliced boiled eggs)	*B. cereus, L. monocytogenes*, *Salmonella*	Heat treatment, preservatives, storage time-temperature
Fruits and vegetables (e.g., peeled carrots, chopped lettuce)	*B. cereus, C. botulinum*, enterohemorrhagic *E. coli, L. monocytogenes, Salmonella*, *Shigella* spp., *Y. enterocolitica*	Heat treatment, storage time-temperature, wash water sanitizers
Fats, oils, and condiments (e.g., garlic in oil)[e]	*B. cereus*, *C. botulinum, S. aureus*, *Salmonella*	a_w, heat treatment, pH, preservatives, salt, storage time-temperature
Acidified sauces, salad dressings, and salsas	Enterohemorrhagic *E. coli, Salmonella*, *S. aureus*	Heat treatment, pH, storage time-temperature, titratable acidity
High a_w syrups (e.g., light maple syrup)	*C. botulinum*[f]	Acidification (light syrups), a_w, heat treatment, preservatives
Confectionery products (e.g., chocolate products)	*Salmonella*	a_w, heat treatment
Cereal grains and related products (e.g., fresh pasta, cooked rice)	*B. cereus, C. botulinum, Salmonella*, *S. aureus*	a_w, heat treatment, pH, preservatives, storage time-temperature

[a] Adapted from IFT report *(53)* Tables 4-1 and 6-1.
[b] Combinations of products, storage in modified atmosphere, and use of novel preservatives or processes require special consideration.
[c] Good agricultural practices where appropriate and good manufacturing practices and HACCP principles would help in reducing the hazards.
[d] Heat treatment includes processes such as cooking, pasteurization, and other thermal processes intended to inactivate pathogens.
[e] Only a concern in anoxic environments.
[f] Only a concern in light syrups and can be controlled by acidification.

APPENDIX D. FDA 2005 MODEL FOOD CODE DEFINITIONS MOST RELEVANT TO CHALLENGE STUDIES

The following definitions were extracted from the 2005 FDA Food Code *(117)*. Note: all paragraph and section references within definitions refer to paragraphs and sections in the 2005 FDA Food Code.

"a_w" means water activity, which is a measure of the free moisture in the food that is available for microbial growth. It is the quotient of the water vapor pressure of the substance divided by the vapor pressure of pure water at the same temperature, and is indicated by the symbol a_w.

"Consumer" means a person who is a member of the public, takes possession of food, is not functioning in the capacity of an operator of a food establishment or food processing plant, and does not offer the food for resale.

"Critical control point" means a point or procedure in a specific food system where loss of control may result in an unacceptable health risk.

Food establishment

(1) ''Food establishment'' means an operation that:
(a) stores, prepares, packages, serves, vends directly to the consumer, or otherwise provides food for human consumption, such as a restaurant, satellite, or catered feeding location; catering operation if the operation provides food directly to a consumer or to a conveyance used to transport people; market; vending location; conveyance used to transport people; institution; or food bank; and

(b) relinquishes possession of food to a consumer directly or indirectly through a delivery service such as home delivery of grocery orders or restaurant takeout orders or delivery service that is provided by common carriers.

(2) ''Food establishment'' includes:
   (a) an element of the operation such as a transportation vehicle or a central preparation facility that supplies a vending location or satellite feeding location unless the vending or feeding location is permitted by the regulatory authority; and
   (b) an operation that is conducted in a mobile, stationary, temporary, or permanent facility or location; where consumption is on or off the premises; and regardless of whether there is a charge for the food.

(3) ''Food establishment'' does not include:
   (a) an establishment that offers only prepackaged foods that are not potentially hazardous (time/temperature control for safety) foods;
   (b) a produce stand that offers only whole, uncut fresh fruits and vegetables;
   (c) a food processing plant including those that are located on the premises of a food establishment;
   (d) a kitchen in a private home if only food that is not potentially hazardous (time-temperature control for safety food) is prepared for sale or service at a function such as a religious or charitable organization's bake sale if allowed by law and if the consumer is informed by a clearly visible placard at the sales or service location that the food is prepared in a kitchen that is not subject to regulation and inspection by the regulatory authority;
   (e) an area where food that is prepared as specified in Subparagraph (3)(d) of this definition (above) is sold or offered for human consumption;
   (f) a kitchen in a private home, such as a small family daycare provider; or a bed-and-breakfast operation that prepares and offers food to guests if the home is owner occupied, the number of available guest bedrooms does not exceed 6, breakfast is the only meal offered, the number of guests served does not exceed 18, and the consumer is informed by statements contained in published advertisements, mailed brochures, and placards posted at the registration area that the food is prepared in a kitchen that is not regulated and inspected by the regulatory authority; or
   (g) a private home that receives catered or home-delivered food.

Food processing plant

(1) ''Food processing plant'' means a commercial operation that manufactures, packages, labels, or stores food for human consumption and provides food for sale or distribution to other business entities such as food processing plants or food establishments.

(2) ''Food processing plant'' does not include a food establishment.

"HACCP plan" means a written document that delineates the formal procedures for following the hazard analysis and critical control point principles developed by The National Advisory Committee on Microbiological Criteria for Foods.

"Hazard" means a biological, chemical, or physical property that may cause an unacceptable consumer health risk.

Packaged

(1) ''Packaged'' means bottled, canned, cartoned, securely bagged, or securely wrapped, whether packaged in a food establishment or a food processing plant.

(2) ''Packaged'' does not include a wrapper, carry-out box, or other nondurable container used to containerize food with the purpose of facilitating food protection during service and receipt of the food by the consumer.

Potentially hazardous food (time/temperature control for safety food)

(1) ''Potentially hazardous food'' (time/temperature control for safety food) means a food that requires time/temperature control for safety (TCS) to limit pathogenic microorganism growth or toxin formation.

(2) ''Potentially hazardous food'' (TCS food) includes:
   (a) an animal food that is raw or heat treated; a plant food that is heat treated or consists of raw seed sprouts, cut melons, cut tomatoes, or mixtures of cut tomatoes that are not modified in a way so that they are unable to support pathogenic microorganism growth or toxin formation or garlic-in-oil mixtures that are not modified in a way that results in mixtures that do not support pathogenic microorganism growth or toxin formation; and
   (b) except as specified in Subparagraph (3)(d) of this definition (below), a food that because of the interaction of its a_w and pH values is designated as Product Assessment Required (PA) in the Food Code Table A or Table B of this definition:

(3) Potentially hazardous food (TCS food) does not include:
   (a) an air-cooled hard-boiled egg with shell intact or an egg with shell intact that is not hard boiled but has been pasteurized to destroy all viable salmonellae;
   (b) a food in an unopened hermetically sealed container that is commercially processed to achieve and maintain commercial sterility under conditions of nonrefrigerated storage and distribution;
   (c) a food that because of its pH or a_w value or the interaction of a_w and pH values is designated as a non-PHF and non-TCS food in Table A or Table B of this definition;
   (d) a food that is designated as Product Assessment Required (PA) in Table A or Table B of this definition and has undergone a Product Assessment showing that the growth or toxin formation of pathogenic microorganisms that are reasonably likely to occur in that food is precluded due to
      (i) intrinsic factors including added or natural characteristics of the food such as preservatives, antimicrobials, humectants, acidulants, or nutrients;
      (ii) extrinsic factors including environmental or operational factors that affect the food such as packaging, modified atmosphere such as reduced oxygen packaging, shelf life and use, or temperature range of storage and use; or
      (iii) a combination of intrinsic and extrinsic factors; or
   (e) a food that does not support the growth or toxin formation of pathogenic microorganisms in accordance with one of the Subparagraphs (3)(a)–(3)(d) of this definition (above) even though the food may contain a pathogenic microorganism or chemical or physical contaminant at a level sufficient to cause illness or injury.

TABLE A. *Interaction of pH and a_w for control of spores in food that has been heat treated to destroy vegetative cells and then packaged*[a]

a_w values	pH values:		
	≤4.6	>4.6–5.6	>5.6
≤0.92	Non-PHF, non-TCS food	Non-PHF, non-TCS food	Non-PHF, non-TCS food
>0.92–0.95	Non-PHF, non-TCS food	Non-PHF, non-TCS food	PA
>0.95	Non-PHF, non-TCS food	PA	PA

[a] PHF, potentially hazardous food; TCS food, time/temperature control for safety food; PA, product assessment required.

TABLE B. *Interaction of pH and a_w for control of vegetative cells and spores in food that has not been heat treated or has been heat treated but not packaged*[a]

a_w values	pH values:			
	<4.2	4.2–4.6	>4.6–5.0	>5.0
<0.88	Non-PHF, non-TCS food	Non-PHF, non-TCS food	Non-PHF, non-TCS food	Non-PHF, non-TCS food
0.88–0.90	Non-PHF, non-TCS food	Non-PHF, non-TCS food	Non-PHF, non-TCS food	PA***
>0.90–0.92	Non-PHF, non-TCS food	Non-PHF, non-TCS food	PA	PA
>0.92	Non-PHF, non-TCS food	PA	PA	PA

[a] PHF, potentially hazardous food; TCS food, time/temperature control for safety food; PA, product assessment required.

Ready-to-eat food

(1) ''Ready-to-eat food'' means food that
   (a) is in a form that is edible without additional preparation to achieve food safety, as specified under one of the following parts of the Food Code: Paragraph 3-401.11(A) or (B), Section 3-401.12, or Section 3-402.11, or as specified in Paragraph 3-401.11(C); or
   (b) is a raw or partially cooked animal food and the consumer is advised as specified in the Food Code, Subparagraphs 3-401.11(D)(1) and (2); or
   (c) is prepared in accordance with a variance that is granted as specified in the Food Code, Subparagraphs 3-401.11(D) and (3); and
   (d) may receive additional preparation for palatability or aesthetic, epicurean, gastronomic, or culinary purposes.

(2) ''Ready-to-eat food'' includes:
   (a) raw animal food that is cooked as specified in the Food Code under Section 3-401.11 or 3-401.12, or frozen as specified under Section 3-402.11;
   (b) raw fruits and vegetables that are washed as specified in the Food Code under Section 3-302.15;
   (c) fruits and vegetables that are cooked for hot holding as specified in the Food Code under Section 3-401.13;
   (d) all potentially hazardous food (TCS food) that is cooked to the temperature and time required for the specific food in the Food Code under Subpart 3-401 and cooled as specified under Section 3-501.14;
   (e) plant food for which further washing, cooking, or other processing is not required for food safety and from which rinds, peels, husks, or shells, if naturally present, are removed;
   (f) substances derived from plants such as spices, seasonings, and sugar;
   (g) a bakery item such as bread, cakes, pies, fillings, or icing for which further cooking is not required for food safety;
   (h) the following products that are produced in accordance with USDA guidelines and that have received a lethality treatment for pathogens: dry fermented sausages such as dry salami or pepperoni; salt-cured meat and poultry products such as prosciutto ham, country cured ham, and Parma ham; and dried meat and poultry products such as jerky or beef sticks; and
   (i) foods manufactured as specified in 21 CFR Part 113 ''Thermally Processed Low-Acid Foods Packaged in Hermetically Sealed Containers.''

Reduced oxygen packaging

(1) ''Reduced oxygen packaging'' means:
   (a) the reduction of the amount of oxygen in a package by removing oxygen, displacing oxygen and replacing it with another gas or combination of gases, or otherwise controlling the oxygen content to a level below that normally found in the atmosphere (approximately 21% at sea level); and
   (b) a process as specified in Subparagraph (1)(a) of this definition (above) that involves a food for which the hazards *Clostridium botulinum* or *Listeria monocytogenes* require control in the final packaged form.

(2) ''Reduced oxygen packaging'' includes:
   (a) vacuum packaging, in which air is removed from a package of food and the package is hermetically sealed so that a vacuum remains inside the package;
   (b) modified atmosphere packaging, in which the atmosphere of a package of food is modified so that its composition is different from air but the atmosphere may change over time due to the permeability of the packaging material or the respiration of the food (modified atmosphere packaging includes reduction in the proportion of oxygen, total replacement of oxygen, or an increase in the proportion of other gases such as carbon dioxide or nitrogen);
   (c) controlled atmosphere packaging, in which the atmosphere of a package of food is modified so that until the package is opened, its composition is different from air, and continuous control of that atmosphere is maintained, such as by using oxygen scavengers, or a combination of total replacement of oxygen, nonrespiring food, and impermeable packaging material;

(d) cook chill packaging, in which cooked food is hot filled into impermeable bags that have the air expelled and are then sealed or crimped closed, and the bagged food is rapidly chilled and refrigerated at temperatures that inhibit the growth of psychrotrophic pathogens; or

(e) sous vide packaging, in which raw or partially cooked food is placed in a hermetically sealed, impermeable bag, cooked in the bag, rapidly chilled, and refrigerated at temperatures that inhibit the growth of psychrotrophic pathogens.

"Regulatory authority" means the local, state, or federal enforcement body or authorized representative having jurisdiction over the food establishment.

"Risk" means the likelihood that an adverse health effect will occur within a population as a result of a hazard in a food.

"Variance" means a written document issued by the regulatory authority that authorizes a modification or waiver of one or more requirements of this code if, in the opinion of the regulatory authority, a health hazard or nuisance will not result from the modification or waiver.

APPENDIX E. FOOD PRODUCT CHECKLIST: MOZZARELLA SLICES

Evaluation of Mozzarella slices packaged under modified atmosphere packaging and stored at ambient temperatures for up to 2 wk to enhance sales

	Considerations	*Response*	*Additional comments*
1	**Determine the purpose of the study**		
1.a	***Exempt from time/temperature control for safety (no refrigeration required)***	NA[a]	
1.b	***Variance from any regulatory requirements (e.g., holding for >4 h without temperature control)***	Extended out-of-refrigeration storage of modified-atmosphere or vacuum-packaged Mozzarella slices for 2 wk; Food Code variance.	
1.c	***Validate lethality***	NA (used pasteurized milk in production of cheese).	
1.d	***Verify that formulation will inhibit microbial growth in refrigerated foods or under mild temperature abuse***	NA	
2	**Collect information regarding the product**		
2.a	***What are the ingredients?***	Cheese (pasteurized milk, salt, rennet, starter cultures)	
2.a.1	*How consistent are the ingredients from various sources, lot to lot?*	Ingredients same or similar lot to lot; pH, moisture, salt can vary slightly but in accordance with Standard of Identity (SOI) as defined in 21 CFR 133.155–158 *(119)*.	
2.a.2	*What are the pH, a_w, and proximate analysis (moisture, salt, fat, protein, residual nitrite, etc.) for product and/or individual components?*	pH 5.3–5.4; a_w 0.96; proximate analysis at end of production, 46–52% moisture, 1.0% NaCl, 30% fat. Homogeneous throughout.	Note: a_w is not measured or controlled in typical production but is a function of moisture and salt content; moisture is limited by SOI. Starter culture activity (acid development, measured by pH) is a critical control point.
2.a.3	*Do any of these values change from preparation to consumption?*	Once the cheese is sliced and packaged, the pH may increase from 5.4 to 5.9 during refrigerated storage over a 3-mo period if lactic acid bacteria starter cultures are killed by heat used in molding.	The pH will not increase during the 2-wk holding period at 23°C (73°F) at retail.
2.a.4	*If applicable, what are the dimensions of cuts, pieces, etc?*	NA	
2.a.5	*What is the normal microbial load (species, etc.) at the beginning and end of production?*	Microbial load: lactic acid bacteria starter culture 7 log CFU/ml of milk; residual cultures 2 log CFU/g. Reduction due to heating at 70°C (158°F) during molding step.	
2.a.6	*Is there likelihood that contamination may be internalized in or distributed throughout individual components?*	Unlikely if produced under Good Manufacturing Practices (GMPs), HACCP using pasteurized milk; contamination by nonsporeformers would be on the surface.	
2.b	***What are the preparation steps?***		
2.b.1	*Is the product an assembled (multicomponent) product?*	Product is not an assembled nor a multicomponent product.	
2.b.2	*Is there a microbial reduction step that is validated? What are the parameters associated with the microbial reduction step? Are there different microbial reduction steps for different components?*	Pasteurization is a validated heat inactivation step for milk used to make the cheese; no kill step for surface contamination of the cheese.	

APPENDIX E. *Continued*

Evaluation of Mozzarella slices packaged under modified atmosphere packaging and stored at ambient temperatures for up to 2 wk to enhance sales			
	Considerations	***Response***	***Additional comments***
2.b.3	*Is there a potential for recontamination?*	Yes. Potential for recontamination during slicing and packaging.	
2.b.4	*What is the variability in parameters that affect lethality or growth?*	Little variability for lethality if prepared under GMPs HACCP; growth potential can vary depending on moisture and pH at the end of production for high-moisture versus low-moisture product.	
2.b.5	*How is the product packaged?*	After slicing or cutting, slices or blocks will be vacuum packaged or modified-atmosphere packaged with nitrogen–carbon dioxide mix.	
2.b.6	*Is the product cultured or fermented? Does it contain starter culture intentionally added?*	Product made with starter culture but populations reduced by heating at 70°C (158°F) for molding step.	
2.b.7	*Does the product contain antimicrobials (preservatives) or other ingredients that might be inhibitory, such as spices?*	NaCl is present but not at inhibitory levels. No antimicrobials are added to cheese, but natamycin may be added to the surface of cut or shredded cheese to inhibit mold.	
2.c	***What are the storage conditions?***		
2.c.1	*How will the product be displayed for sale? Any changes to packaging for display?*	Slices or blocks will be packaged under vacuum or modified atmosphere (nitrogen–carbon dioxide mixture) for storage; product may be displayed unrefrigerated for increased sales but will otherwise be held refrigerated to extend shelf life.	
2.c.2	*What temperatures (and times) are expected during production, preparation, and storage or display?*	During cheese production, milk will be cultured and curd cooked at ≤40°C (104°F); curd will be heated to 70°C (158°F) for molding step; cheese cured at 3°C (37°F) for up to 2 wk and distributed to retailers typically at <7°C (45°F); maximum storage at 23°C (73°F) at retail for 2 wk.	Product quality will deteriorate rapidly if temperature exceeds 23°C (73°F). However, temperatures as high as 27°C (81°F), e.g., during transportation, will have limited effect on quality if the time does not exceed 4 h.
2.c.3	*What potential is there for storage or display at temperatures greater than those listed above in 2.c.2?*	Product is unlikely to be stored at temperatures greater than described; temperatures exceeding 30°C (86°F) will result in a significant decrease in product quality (melting, fat separation).	
2.c.4	*Are there other hazards that may be created by preparation, storage, or display?*	No, but molds may grow on the surface if oxygen is present and when natamycin is not used.	
2.c.5	*What is the estimated maximum time from production to consumption?*	9 mo if stored at refrigeration temperatures; 2 wk for unrefrigerated storage.	
2.c.6	*What is the time to spoilage or unacceptable quality?*	2 wk unrefrigerated storage if held at 20–23°C (68–73°F), shorter if temperatures exceed 23°C; 9 mo refrigerated storage.	
3	**Determine if product assessment for growth or inactivation is needed**		
3.a	***Is a product assessment for growth necessary based on pH and a_w? (See Appendix D, Tables A and B.) If yes, also answer 4.e and 5.a.***	Yes, product assessment required. Food Code Table B is applicable because of potential recontamination and survival of spores. pH > 5.4 and a_w 0.96.	
3.b	***Is an inactivation study needed? If yes, also answer 4.f and 5.b.***	No, the purpose of this study is to determine if pathogens likely to be present will grow in the product if stored out of refrigeration; milk has been previously pasteurized.	

APPENDIX E. *Continued*

Evaluation of Mozzarella slices packaged under modified atmosphere packaging and stored at ambient temperatures for up to 2 wk to enhance sales			
	Considerations	***Response***	***Additional comments***
3.c	***Are there any regulations applicable for lethality (inactivation) or TCS (growth)?***	Latest edition Food Code for TCS.	
4	**Determine pathogens of concern to include in the challenge study**		
4.a	***According to Table 2 and Appendix C, which pathogens are of concern? If food is not seafood,* Vibrio *spp. may be excluded from consideration.***	Given a product pH of 5.4 and an a_w of 0.96, the pathogens of concern are *B. cereus*, *C. botulinum,* pathogenic *E. coli, L. monocytogenes, Salmonella, S. aureus, Vibrio parahaemolyticus,* and *V. vulnificus*.	
4.b	***Considering the ecology, product, and epidemiological history, what pathogens are reasonably likely to occur? (Also see Appendix C.)***	*B. cereus* spores survive pasteurization; pathogenic *E. coli, L. monocytogenes, Salmonella,* and *S. aureus* from postprocessing handling. *Salmonella* has been associated with Mozzarella due to contamination during production, not postprocess contamination; illness associated with survival, not growth; no outbreak has been reported with *B. cereus, L. monocytogenes,* or *S. aureus (22).*	The most likely vegetative pathogens to recontaminate the product are *L. monocytogenes* and *S. aureus. L. monocytogenes* is a more likely pathogen to recontaminate the product due to its ubiquity in the environment. *S. aureus* is a likely contaminant from workers' hands. *Vibrio* spp. were excluded from consideration since seafood is not a component. *C. botulinum* was excluded from consideration because the spores are rare in the ecology of dairy products.
4.c	***What pathogens are likely to recontaminate the product after the inactivation step?***	Recontamination can occur as indicated above.	
4.d	***Are there any baseline surveys that indicate prevalence of pathogens for the target product or a related product?***	No.	
4.e	***For growth inhibition (TCS) studies:***		
4.e.1	*Which pathogen(s) will grow the fastest? Consider gram positive versus gram negative; vegetative microorganisms versus spore formers. If food is not seafood,* Vibrio *may be excluded from consideration. Use a predictive model or cite applicable literature. Consider growth potential through 1.5 times the shelf life, if appropriate.*	Please see 4.e.2, 4.e.3, and 4.e.4.	

APPENDIX E. *Continued*

Evaluation of Mozzarella slices packaged under modified atmosphere packaging and stored at ambient temperatures for up to 2 wk to enhance sales			
	Considerations	***Response***	***Additional comments***
4.e.2	*Predictive model*	At pH 5.4, a_w 0.96, 27°C (80.6°F): PMP 7.0 version 1.1 predicts a 3-log *S. aureus* increase within 29 h (22 h without lag) under aerobic conditions; ComBase Predictor predicts a 3-log *S. aureus* increase within 18 h for the same conditions. For *L. monocytogenes*, PMP predicts a 1-log increase within 42 h for the same conditions (7 h without lag); ComBase Predictor with 5,000 ppm of lactic acid predicts a 1-log *L. monocytogenes* increase within 33 h for the same conditions. PMP does not include *B. cereus* predictions at a_w 0.96 but ComBase Predictor with 40% CO_2 predicts a 3-log *B. cereus* increase within 101 h.	Modeling was conservatively done at the highest expected exposure temperature. Of the likely contaminants, *L. monocytogenes* and *S. aureus* will grow fastest at this a_w and pH; *S. aureus* is generally not a good competitor in cheese made with starter cultures, but starter cultures are reduced by heating and molding step. If *B. cereus* growth occurred, it would be at a slower rate than that of *L. monocytogenes* or *S. aureus*.
4.e.3	*Compare choice with literature*	Stecchini et al. *(101)* indicated a 5-log increase of *L. monocytogenes* during storage at 5°C (41°F) for 21 days (pH and moisture not reported).	
4.e.4	*Any further information on growth or survival?*	Data presented at the 2003 International Association for Food Protection Annual Meeting 2003 *(29)* on cheese shreds for *L. monocytogenes* and *Salmonella* demonstrated no growth on low-moisture Mozzarella stored at 15°C (59°F) for 2 mo (pH 5.0–5.5; 47% moisture; a_w 0.965).	
4.e.5	*Based on the above analysis, what challenge organisms are chosen for growth inhibition studies?*	*L. monocytogenes* and *S. aureus*.	
4.f	***If inactivation studies:***	NA	
4.f.1	*What is the lethal treatment? (HPP, heat, acid, etc.)*		
4.f.2	*Which microorganisms are most resistant to the lethal treatment? (HPP, heat, acid, etc.)*		
4.f.3	*Will the lethality be delivered to all areas of the product that may contain the pathogen? Account for all surface and internalized contamination.*		
4.f.4	*What is in the formulation that may affect inactivation? (Intrinsic factors may contribute to lethality or resistance: a_w, moisture, salt, pH, fat, etc.)*		
4.f.5	*Are there any data on pathogen levels in the product?*		
4.f.6	*Is there a regulatory requirement or policy for log reduction for this product? Cite requirement.*		
4.f.7	*If there is no regulatory requirement for log reduction, use scientific basis for determining acceptable reduction (21, 76).*		

APPENDIX E. *Continued*

Evaluation of Mozzarella slices packaged under modified atmosphere packaging and stored at ambient temperatures for up to 2 wk to enhance sales			
	Considerations	***Response***	***Additional comments***
4.f.8	*Based on the above analysis, what challenge organisms are chosen for inactivation studies?*		
5	**Determine appropriate time and sampling intervals for challenge study**		
5.a	***For growth inhibition (TCS) studies, use 1.25–1.5 times the shelf life as testing time***	14 days × 1.5 = 21 days.	
5.a.1	*Maximum time from production to consumption*	Maximum 9 mo if refrigerated; 21 days if not refrigerated.	
5.a.2	*Actual time to spoilage or unacceptable quality*	Point of unacceptable quality: 21 days.	
5.a.3	*For growth inhibition studies, determine appropriate sampling intervals for microbial analysis; use five to seven (preferred) sampling intervals; fewer sampling intervals should be justified, e.g., using results from similar products.*	Sample at 0, 1, 2, 3, 4, 7, 14, 21 days.	Based on predictive models, growth could occur within 24–48 h at 27°C (81°F); more than seven sampling intervals are appropriate to ensure the ability to identify minimum time to growth.
5.b	***For inactivation studies determine appropriate sampling points considering the process and formulation.*** *Identify populations at 0 time and end of processing; whenever possible, include intermediate sampling intervals to determine death curve.*	NA	
5.b.1	*When inactivation treatments may result in sublethal injury, repair and growth of microorganisms during product shelf life should be considered (21).*		
6	**Determine inoculation, storage, and testing procedures**		
6.a	***Determine strains for use in study (multiple strains for each species are recommended; consider use of appropriate food or clinical isolates)***	*L. monocytogenes* and *S. aureus* will be tested individually using three-strain mixtures. Each mixture will include isolates from cheese or other dairy isolates or clinical isolates.	
6.b	***Determine if adaptation is required for inoculum preparation***	No adaptation necessary; product is low acid, high a_w at ambient temperatures.	
6.c	***Determine method of inoculation (surface, mixing, dipping, liquid, dry, etc.)***	Surface inoculation of individual 25-g slices; two slices per package with inoculum on inner surface between the two slices.	Using two slices per package with inoculum in between will retain moisture and provide a worst-case scenario for growth.
6.d	***Determine size of inoculum (populations, e.g., log CFU/g, CFU per package, percentage of inoculum vol/wt or vol/vol)***	3 log CFU/g; 0.05 ml (50 μl) per package. Each organism will be inoculated independently (separate samples) to avoid possible antagonistic effect between different organisms.	Inoculum level is high considering likelihood of contamination but will allow enumeration by direct plating and detection of growth and low levels of inactivation by formulation during storage; inoculum volume 1% of sample size; preliminary data suggest inoculum does not change pH and a_w appreciably.

APPENDIX E. *Continued*

Evaluation of Mozzarella slices packaged under modified atmosphere packaging and stored at ambient temperatures for up to 2 wk to enhance sales			
	Considerations	***Response***	***Additional comments***
6.e	***Determine packaging to be used***	Two inoculated slices will be used per package unit; slices will be packaged with 60% nitrogen and 40% carbon dioxide mixture and sealed; packaging material will be gas-moisture impermeable.	Packaging is the same as commercial product.
6.f	***Determine the incubation temperature for growth inhibition studies or temperature(s) for thermal inactivation studies***	23°C (73°F).	23°C (73°F) is the maximum temperature to which the product will be exposed without adverse changes in product quality that would deter purchase and consumption.
6.g	***Determine sampling method and sample size***	Entire sample (two slices) will be mixed in the bag, and 25-g portions will be removed for microbial analysis; sample will be homogenized with equal volume of 0.1% peptone buffer, and serial dilutions will be plated on selective agar as appropriate per FDA BAM methods *(115)*.	
6.h	***How many replicates are needed to ensure confidence in data?*** *Does variability in proximate analysis or production warrant more than two or three replicate trials? Will multiple variations of similar formulations be tested? Has a statistical design for choosing formulations been used (block design, central composite, etc.)?*	Two replicate (unique production) lots using highest moisture and pH combination; triplicate samples per testing interval.	
7	**Determine other controls**		
7.a	***Is use of surrogates appropriate or necessary? If so, justify.***	Surrogates are not appropriate or necessary.	
7.b	***Are uninoculated controls needed to assess spoilage or competitive microflora or for other purposes?***	Uninoculated controls will be used to monitor growth of molds or yeasts and other spoilage microorganisms that can change pH during testing interval and for proximate analysis at the beginning of the study.	
7.c	***What other controls are necessary (including negative or positive growth controls)?***	Not required for this study; anticipate growth if samples were held for sufficient time.	
8	**Determine pass-fail criteria**		
8.a	***What are the pass-fail criteria?***	No more than 1-log increase for *L. monocytogenes*; no more than 3-log increase for *S. aureus*.	A 1-log increase in *L. monocytogenes* is considered significant growth, but any detectable presence of *L. monocytogenes* in a ready-to-eat food renders the product adulterated.
8.b	***What are the limits for use of the results?***	Data apply only to Mozzarella with the maximum moisture-pH-temperature-time limits tested in this study.	

[a] NA, not applicable.

APPENDIX F. FOOD PRODUCT CHECKLIST: CHOPPED LETTUCE

Evaluation to determine the absence of measurable growth (<1 log) of pathogens of concern in chopped lettuce held out of refrigeration for up to 8 h

	Considerations	*Response*	*Additional comments*
1	**Determine the purpose of the study**		
1.a	***Exempt from time/temperature control for safety (no refrigeration required)***	NA[a]	
1.b	***Variance from any regulatory requirements (e.g., holding for >4 h without temperature control)***	Yes. The purpose of the study is to allow chopped lettuce to be held out of temperature control (at room temperature) for a period of up to 8 h. This is a salad bar product consumed on premises. Once the lettuce has been removed from temperature control it will be used or discarded within 8 h. Product will not be re-refrigerated and offered for service at a later time.	
1.c	***Validate lethality***	NA	
1.d	***Verify that formulation will inhibit microbial growth in refrigerated foods or under mild temperature abuse***	NA	
2	**Collect information regarding the product**		
2.a	***What are the ingredients?***	The single ingredient is heads of whole Romaine lettuce that are chopped and washed in water containing a wash water sanitizer at concentrations specified on the label.	
2.a.1	*How consistent are the ingredients from various sources, lot to lot?*	Total plate count on the product varies widely from batch to batch.	
2.a.2	*What are the pH, a_w, and proximate analysis (moisture, salt, fat, protein, residual nitrite, etc.) for product and/or individual components?*	The pH is estimated to be 5.8–6.2 *(118)*. Water activity in iceberg lettuce is 0.995–0.998, and this is assumed to hold true for Romaine *(33)*. The product is very high in water, with minimal amounts of salt, fat, and protein.	
2.a.3	*Do any of these values change from preparation to consumption?*	The pH is not likely to change. The a_w may decrease slightly as the product dries out, but we have elected to ignore the impact this drying would have on pathogen growth.	
2.a.4	*If applicable, what are the dimensions of cuts, pieces, etc.?*	Heads arrive whole and are chopped into pieces about 5 by 5 cm.	
2.a.5	*What is the normal microbial load (species, etc.) at the beginning and end of production?*	No published data are available on incoming heads of lettuce. Internal company data on the lettuce after chopping shows the following trends, based on several years of sample collection, where sample size was 25 g. Total aerobic plate counts are normally distributed with a mean of 5.5 log CFU/g and a standard deviation of 1.5 log CFU/g. *S. aureus* has been found in 1 of 50 samples, *Salmonella* in 1 of 200 samples, *B. cereus* in 1 of 10 samples. *E. coli* is generally absent, but 1 of 20 samples had >2 log MPN/g. *L. monocytogenes* was not detected in tests of more than 200 samples.	Data presented here were collected after the lettuce had been washed and cut.

APPENDIX F. *Continued*

Evaluation to determine the absence of measurable growth (<1 log) of pathogens of concern in chopped lettuce held out of refrigeration for up to 8 h			
	Considerations	***Response***	***Additional comments***
2.a.6	*Is there likelihood that contamination may be internalized in or distributed throughout individual components?*	Published laboratory data show that internalization in fresh cut lettuce is possible *(93)*. The extent to which this happens under real world conditions is not clear.	
2.b	***What are the preparation steps?***	Receive lettuce from vendor, store in cooler until use, remove from cooler, remove and discard outer leaves, cut off bottom end, separate remaining leaves and wash them in water containing wash water sanitizer at label concentrations, spin to remove excess water, chop into pieces approximately 5 by 5 cm.	
2.b.1	*Is the product an assembled (multicomponent) product?*	The product is not assembled and is not multicomponent.	
2.b.2	*Is there a microbial reduction step that is validated? What are the parameters associated with the microbial reduction step? Are there different microbial reduction steps for different components?*	The wash step has been shown to result in a 1- to 2-log reduction in aerobic plate count.	The microbial reduction reported here is not considered in the design of the challenge study for this product.
2.b.3	*Is there a potential for recontamination?*	There is a slight potential for recontamination. Lettuce is hand chopped in a food service kitchen environment. Data on actual product (see above) indicate that *S. aureus* may contaminate the product but that *L. monocytogenes* does not represent a significant risk. Employees receive annual food safety training, and managers are certified by accredited Food Managers Certification testing. Standard procedures are in place to prevent cross-contamination of this product during preparation.	
2.b.4	*What is the variability in parameters that affect lethality or growth?*	The product does vary due to normal biological variation. The pH and a_w values are very permissive to growth, so variability is unlikely to influence pathogen growth.	
2.b.5	*How is the product packaged?*	The product is not packaged but may be placed in plastic bins and covered with plastic wrap for refrigeration prior to display.	
2.b.6	*Is the product cultured or fermented? Does it contain starter culture intentionally added?*	No.	
2.b.7	*Does the product contain antimicrobials (preservatives) or other ingredients that might be inhibitory, such as spices?*	There are no antimicrobials, preservatives, or other inhibitory ingredients.	
2.c	***What are the storage conditions?***		
2.c.1	*How will the product be displayed for sale? Any changes to packaging for display?*	Display in open containers on salad bar.	

APPENDIX F. *Continued*

Evaluation to determine the absence of measurable growth (<1 log) of pathogens of concern in chopped lettuce held out of refrigeration for up to 8 h			
	Considerations	***Response***	***Additional comments***
2.c.2	*What temperatures (and times) are expected during production, preparation, and storage or display?*	Product is stored below 5°C (41°F) prior to preparation. Preparation takes approximately 2 h per batch and takes place at room temperature (21.1°C, 70°F). Product may either be covered with plastic wrap and refrigerated after preparation or placed at room temperature for sale and consumption.	The 8 h starts from the time of preparation unless the product will be rapidly cooled to 5°C (41°F) within 4 h after preparation, in which case the 8 h starts when the chopped product is removed from refrigeration.
2.c.3	*What potential is there for storage or display at temperatures greater than those listed above in 2.c.2?*	The restaurant is climate controlled. Our data show that the room temperature is usually 21.1°C (70°F) but can in some cases increase to 23.9°C (75°F) for short periods of time.	
2.c.4	*Are there other hazards that may be created by preparation, storage, or display?*	Recontamination by the consumer during serving is possible, but sneeze guards and tongs are used, as per normal Food Code practice.	
2.c.5	*What is the estimated maximum time from production to consumption?*	The maximum amount of time the product will be out of temperature control is 8 h.	
2.c.6	*What is the time to spoilage or unacceptable quality?*	The product is overtly spoiled after 24 h at room temperature.	
3	**Determine if product assessment for growth or inactivation is needed**		
3.a	***Is a product assessment for growth necessary based on pH and a_w? (See Appendix D, Tables A and B.) If yes, also answer 4.e and 5.a.***	Yes, product assessment is required according to Food Code Table B.	
3.b	***Is an inactivation study needed? If yes, also answer 4.f and 5.b.***	No.	
3.c	***Are there any regulations applicable for lethality (inactivation) or TCS (growth)?***	The Food Code defines this product as requiring temperature control for safety. There are no requirements for lethality on this product.	
4	**Determine pathogens of concern to include in the challenge study**		
4.a	***According to Table 2 and Appendix C, which pathogens are of concern? If food is not seafood,* Vibrio *spp. may be excluded from consideration.***	Based on pH and a_w, *B. cereus, C. botulinum, C. perfringens, L. monocytogenes,* pathogenic *E. coli, Salmonella, S. aureus, Shigella* spp., and *Y. enterocolitica* should be considered.	
4.b	***Considering the ecology, product, and epidemiological history, what pathogens are reasonably likely to occur? (Also see Appendix C.)***	Product testing shows that *B. cereus* and *S. aureus* are present. Epidemiological data would suggest *E. coli* O157:H7 as the primary concern, followed by *Salmonella* and *Shigella*. *C. botulinum* and *C. perfringens* were excluded based on the nature of the finished product (loosely packed chopped leaves). Although *L. monocytogenes* will grow on chopped lettuce *(62), L. monocytogenes* was excluded based on lack of epidemiological evidence *(41),* as was *Y. enterocolitica* and *B. cereus*.	
4.c	***What pathogens are likely to recontaminate the product after the inactivation step?***	See response to 2.b.3.	
4.d	***Are there any baseline surveys that indicate prevalence of pathogens for the target product or a related product?***	See response to 2.a.5.	

APPENDIX F. *Continued*

Evaluation to determine the absence of measurable growth (<1 log) of pathogens of concern in chopped lettuce held out of refrigeration for up to 8 h			
	Considerations	***Response***	***Additional comments***
4.e	***For growth inhibition (TCS) studies:***		
4.e.1	*Which pathogen(s) will grow the fastest? Consider gram positive versus gram negative; vegetative microorganisms versus spore formers. If food is not seafood,* Vibrio *spp. may be excluded from consideration. Use a predictive model or cite applicable literature. Consider growth potential through 1.5 times the shelf life, if appropriate.*	See response to 4.e.2 and 4.e.3.	
4.e.2	*Predictive model*	A temperature of 21°C (69.8°F), pH 6.2, and a_w 0.995 were assumed for the following predictions: When typical lag time values are assumed, ComBase Predictor shows a 1-log increase after 6.5 h (*E. coli* O157:H7), 8.2 h (*Salmonella*), 9.4 h (*S. aureus*), 12 h (*L. monocytogenes*), and 18.5 h (*Shigella*). PMP 7.0 predicted a 1-log increase (including lag) in 9.9 h (*E. coli* O157:H7), 8.3 h (*Salmonella*), 9.1 h (*S. aureus*), 9.5 h (*L. monocytogenes*), and 15.9 h (*Shigella*). When lag time is assumed to be zero, ComBase Predictor shows a 1-log increase after 3.4 h (*E. coli* O157:H7), 3.6 h (*Salmonella*), 4.1 h (*S. aureus*), 4.6 h (*L. monocytogenes*), and 10 h (*Shigella*). PMP shows a 1-log increase (excluding lag) after 3.6 h (*E. coli* O157:H7), 3.0 h (*Salmonella*), 5.6 h (*S. aureus*), 3.2 h (*L. monocytogenes*), and 6.7 h (*Shigella*).	
4.e.3	*Compare choice with literature*	Literature data (four growth rates) for *E. coli* O157:H7 growth in cut iceberg lettuce were extracted from published studies *(1, 23, 67)*. The four data points were fit to a simple literature-based model, and growth rate at 21°C (69.8°F) was estimated. The literature-based model predicted about 0.86-log increase in *E. coli* O157:H7 after 8 h at 21°C (69.8°F). Note that this prediction considers only growth rate and neglects lag time.	
4.e.4	*Any further information on growth or survival?*	No.	
4.e.5	*Based on the above analysis, what challenge organisms are chosen for growth inhibition studies?*	Results from the modeling and epidemiology show *E. coli* O157:H7 and *Salmonella* to represent the greatest risk. Also, modeling results presented above demonstrate that the growth of the two organisms is similar. Challenge studies will be done with *E. coli* O157:H7 due to the greatest epidemiological link to illness.	The ComBase modeling analysis above shows that the product could be of questionable safety when held at room temperature for 8 h. Literature-based model suggests that the 8-h holding might be acceptable based on a <1-log growth.

APPENDIX F. *Continued*

Evaluation to determine the absence of measurable growth (<1 log) of pathogens of concern in chopped lettuce held out of refrigeration for up to 8 h			
	Considerations	***Response***	***Additional comments***
			A challenge study was justified in order to provide a more conclusive answer. The study will be designed to identify the period of time the growth remains below 1 log CFU/g.
4.f	***If inactivation studies:***	NA	
4.f.1	*What is the lethal treatment? (HPP, heat, acid, etc.)*		
4.f.2	*Which microorganisms are most resistant to the lethal treatment? (HPP, heat, acid, etc.)*		
4.f.3	*Will the lethality be delivered to all areas of the product that may contain the pathogen? Account for all surface and internalized contamination.*		
4.f.4	*What is in the formulation that may affect inactivation? (Intrinsic factors may contribute to lethality or resistance: a_w, moisture, salt, pH, fat, etc.)*		
4.f.5	*Are there any data on pathogen levels in the product?*		
4.f.6	*Is there a regulatory requirement or policy for log reduction for this product? Cite requirement.*		
4.f.7	*If there is no regulatory requirement for log reduction, use scientific basis for determining acceptable reduction (21, 76).*		
4.f.8	*Based on the above analysis, what challenge organisms are chosen for inactivation studies?*		
5	**Determine appropriate time and sampling intervals for challenge study**		
5.a	***For growth inhibition (TCS) studies, use 1.25–1.5 times the shelf life as testing time***	Assuming the product is to be held for 8 h, the product should be tested for 8 × 1.5 = 12 h.	
5.a.1	*Maximum time from production to consumption*	8 h.	See comment to 2.c.2.
5.a.2	*Actual time to spoilage or unacceptable quality*	Prior data indicate 24 h at room temperature results in an unacceptable product.	
5.a.3	*For growth inhibition studies, determine appropriate sampling intervals for microbial analysis; use five to seven (preferred) sampling intervals; fewer sampling intervals should be justified, e.g., using results from similar products.*	Test at 0, 2, 4, 6, 8, 10, 12 h.	If cost is an issue, a fewer number of time points could be evaluated (e.g., 0, 3, 6, 9, and 12 h).

APPENDIX F. *Continued*

Evaluation to determine the absence of measurable growth (<1 log) of pathogens of concern in chopped lettuce held out of refrigeration for up to 8 h			
	Considerations	***Response***	***Additional comments***
5.b	***For inactivation studies determine appropriate sampling points considering the process and formulation.*** *Identify populations at 0 time and end of processing; whenever possible, include intermediate sampling intervals to determine death curve.*	NA	
5.b.1	*When inactivation treatments may result in sublethal injury, repair and growth of microorganisms during product shelf life should be considered (21).*		
6	**Determine inoculation, storage, and testing procedures**		
6.a	***Determine strains for use in study (multiple strains for each species are recommended; consider use of appropriate food or clinical isolates)***	A cocktail of marked strains will be used. *E. coli* O157:H7 strains will be a combination of human isolates and food isolates associated with sporadic illnesses or outbreaks where leafy greens were implicated.	In order to easily enumerate the *E. coli* O157:H7 amid a high natural background population, the selected strain will be modified to express an appropriate marker (e.g., antibiotic resistance, green fluorescent protein).
6.b	***Determine if adaptation is required for inoculum preparation***	Adaptation of inoculum not needed.	*(42)*
6.c	***Determine method of inoculation (surface, mixing, dipping, liquid, dry, etc.)***	Chopped leaves will be spot inoculated on both uncut surface and cut edges, briefly air dried in a biosafety cabinet, and then stored at refrigeration temperature until the following day.	Dip inoculation would add excess moisture that is difficult to remove without a salad spinner. Salad spinners used to remove moisture from inoculated lettuce generate potentially dangerous aerosols in a laboratory, and it is difficult to decontaminate the spinner. The lettuce is refrigerated at 5°C (41°F) after inoculation to duplicate the temperature profile of the restaurant lettuce.
6.d	***Determine size of inoculum (population, e.g., log CFU/g, CFU per package, percentage of inoculum vol/wt or vol/vol)***	Spot inoculum (approximately 10 μl for a 10-g sample) will be applied in multiple (four or more) spots. The target final concentration will be 3 log CFU/10-g sample.	
6.e	***Determine packaging to be used***	Samples will be stored in loosely sealed plastic containers.	
6.f	***Determine the incubation temperature for growth inhibition studies or temperature(s) for thermal inactivation studies***	Although the product is typically held at 21°C (70°F), the product will be incubated at 25°C (77°F) to represent the worst-case condition.	
6.g	***Determine sampling method and sample size***	Each 10-g sample will be combined with 90 ml of 0.1% peptone and homogenized for 1 min at high speed prior to dilution and plating onto appropriate selective media.	

APPENDIX F. *Continued*

Evaluation to determine the absence of measurable growth (<1 log) of pathogens of concern in chopped lettuce held out of refrigeration for up to 8 h			
	Considerations	***Response***	***Additional comments***
6.h	***How many replicates are needed to ensure confidence in data?*** *Does variability in proximate analysis or production warrant more than two or three replicate trials? Will multiple variations of similar formulations be tested? Has a statistical design for choosing formulations been used (block design, central composite, etc.)?*	Two replicate trials will be conducted, and three samples will be analyzed at each time point and plated in duplicate. Each trial will use fresh lettuce from a different batch and fresh inoculum and will be conducted on a different day.	
7	**Determine other controls**		
7.a	***Is use of surrogates appropriate or necessary? If so, justify.***	The use of surrogates is not appropriate or necessary.	
7.b	***Are uninoculated controls needed to assess spoilage or competitive microflora or for other purposes?***	Uninoculated controls (one) will be sampled at each time point. They will be plated on tryptic soy agar and on the selective agar used for the study. The visual appearance of the control lettuce will be described at each time point.	
7.c	***What other controls are necessary (including negative or positive growth controls)?***	The concentration of *E. coli* O157:H7 will be determined in the freshly prepared inoculum as well as the freshly inoculated lettuce at time 0.	
8	**Determine pass-fail criteria**		
8.a	***What are the pass-fail criteria?***	Less than a 1-log increase for *E. coli* O157:H7 at the end of study (12 h).	
8.b	***What are the limits for use of the results?***	Results are applicable to similarly prepared Romaine and iceberg lettuce. These data do not apply to finely chopped or shredded Romaine and iceberg lettuce, which are likely to support more rapid growth.	Given the results of this study, it may not be necessary to conduct full studies on other leafy greens, but some study is needed before data can be more widely applied.

[a] NA, not applicable.

APPENDIX G. FOOD PRODUCT CHECKLIST: MEAT-FILLED PASTRY

Evaluation of display of fully cooked meat-filled pastry for up to 12 h at room temperature			
	Considerations	***Response***	***Additional comments***
1	**Determine the purpose of the study**		
1.a	***Exempt from time/temperature control for safety (no refrigeration required)***	NA[a]	Not a shelf-stable product.
1.b	***Variance from any regulatory requirements (e.g., holding for >4 h without temperature control)***	Want to hold a fully cooked meat product up to 12 h at room temperature (assuming consumption within 2 h after purchase).	Discarded if not served within 12 h.
1.c	***Validate lethality***	NA	Processed in state or federally inspected food processing establishment meeting regulatory cook and cool requirements.
1.d	***Verify that formulation will inhibit microbial growth in refrigerated foods or under mild temperature abuse***	NA	
2	**Collect information regarding the product**		
2.a	***What are the ingredients?***	Ready-to-eat product that contains cooked ground beef, spices, salt, pastry dough.	
2.a.1	*How consistent are the ingredients from various sources, lot to lot?*	Highly consistent lot to lot.	Product specifications in place, produced at a food processing establishment under GMPs.
2.a.2	*What are the pH, a_w, and proximate analysis (moisture, salt, fat, protein, residual nitrite, etc.) for product and/or individual components?*	Beef filling: pH 6.2, a_w 0.97. Pastry dough: pH 7.0, a_w at interface 0.97; a_w at exterior surface 0.75.	If this were an inactivation study, percent fat content may be important; not relevant for this growth study.
2.a.3	*Do any of these values change from preparation to consumption?*	No change of pH. The exterior of the pastry may increase above a_w 0.75 the longer the product is held.	Potential for a_w to increase on external surface if condensate forms between the package and pastry surface.
2.a.4	*If applicable, what are the dimensions of cuts, pieces, etc?*	NA	Component dimensions consistent with product specifications.
2.a.5	*What is the normal microbial load (species, etc.) at the beginning and end of production?*	Vegetative pathogens are inactivated during cooking. There is a potential for spore-forming pathogens to survive cooking. There is a potential for low levels of microorganisms (aerobic plate count of up to 2 log CFU/g).	Fully cooked at processing establishment.
2.a.6	*Is there likelihood that contamination may be internalized in or distributed throughout individual components?*	Yes, spores surviving the cooking process could be distributed throughout the product.	Internal and external vegetative pathogens are destroyed by cooking process. However, vegetative pathogens could be introduced on external surfaces during handling or packaging.
2.b	***What are the preparation steps?***		
2.b.1	*Is the product an assembled (multicomponent) product?*	Yes.	See product ingredients and description above.
2.b.2	*Is there a microbial reduction step that is validated? What are the parameters associated with the microbial reduction step? Are there different microbial reduction steps for different components?*	Yes. Adequate lethality and cooling to result in a ready-to-eat product (meets all regulatory requirements for cooking and cooling). One cook and cool process for the multicomponent product.	Achieving minimum internal temperature of 73.9°C (165°F), resulting in at least a 6.5-log reduction of *Salmonella* for lethality, see *(108)*; for proper cooling, see *(109)*.

APPENDIX G. *Continued*

Evaluation of display of fully cooked meat-filled pastry for up to 12 h at room temperature			
	Considerations	***Response***	***Additional comments***
2.b.3	*Is there a potential for recontamination?*	Yes, *L. monocytogenes* is a potential recontaminant.	Although product is individually wrapped, vegetative pathogens could be introduced on external surfaces during handling or packaging. Control of this potential *L. monocytogenes* postlethality contamination is managed per 9 CFR 430 *(113)*.
2.b.4	*What is the variability in parameters that affect lethality or growth?*	Limited variability in production of cooked product due to controls in a regulated food processing establishment. Limited variability during refrigerated distribution and storage up to the time of display for sale.	
2.b.5	*How is the product packaged?*	Individually hand wrapped in the inspected establishment in a clear plastic wrap. Wrapped pastries are placed in labeled boxes.	Provides protection from moisture and air.
2.b.6	*Is the product cultured or fermented? Does it contain starter culture intentionally added?*	No.	
2.b.7	*Does the product contain antimicrobials (preservatives) or other ingredients that might be inhibitory, such as spices?*	No.	Low level of spices and salt would not likely be inhibitory to pathogen growth.
2.c	***What are the storage conditions?***		
2.c.1	*How will the product be displayed for sale? Any changes to packaging for display?*	Product will remain individually wrapped.	
2.c.2	*What temperatures (and times) are expected during production, preparation, and storage or display?*	Delivered refrigerated at or below 5°C (41°F) to the retail establishment and kept refrigerated until moved out for display. Held at room temperature for display to customers. Displayed for up to 12 h at room temperature: 24°C (75°F). The product is expected to be consumed or refrigerated within 2 h of purchase.	
2.c.3	*What potential is there for storage or display at temperatures greater than those listed above in 2.c.2?*	Higher temperatures are possible if product is heated and displayed under a heat lamp.	A separate study may be required for product stored under a heat lamp.
2.c.4	*Are there other hazards that may be created by preparation, storage, or display?*	No.	
2.c.5	*What is the estimated maximum time from production to consumption?*	7 days (refrigerated).	Labeled use-by date is 7 days after production.
2.c.6	*What is the time to spoilage or unacceptable quality?*	10 days (refrigerated) or 2 days at ambient temperatures.	Product is to be discarded after 12 h of ambient display but may continue to have an acceptable appearance and odor at the end of the display period. Storage under a heat lamp may lead to unacceptable organoleptic quality.
3	**Determine if product assessment for growth or inactivation is needed**		
3.a	***Is a product assessment for growth necessary based on pH and a_w? (See Appendix D, Tables A and B.) If yes, also answer 4.e and 5.a.***	Yes, for beef filling, pH 6.2, a_w 0.97.	The outer pastry component with an a_w of 0.75 does not require product assessment for growth.

APPENDIX G. *Continued*

Evaluation of display of fully cooked meat-filled pastry for up to 12 h at room temperature			
	Considerations	***Response***	***Additional comments***
3.b	***Is an inactivation study needed? If yes, also answer 4.f and 5.b.***	No.	
3.c	***Are there any regulations applicable for lethality (inactivation) or TCS (growth)?***	Yes, maximum 4-h holding time limit when there are no temperature controls for safety (Food Code).	
4	**Determine pathogens of concern to include in the challenge study**		
4.a	***According to Table 2 and Appendix C, which pathogens are of concern? If food is not seafood,* Vibrio *spp. may be excluded from consideration.***	*B. cereus, C. botulinum, C. perfringens, L. monocytogenes,* pathogenic *E. coli, Salmonella, S. aureus, V. parahaemolyticus,* and *V. vulnificus.*	
4.b	***Considering the ecology, product, and epidemiological history, what pathogens are reasonably likely to occur? (Also see Appendix C.)***	*C. perfringens, C. botulinum,* and *B. cereus.*	Vegetative cells are not a concern due to USDA FSIS validated cooking process. Postprocess contamination would be limited to the outside of the pastry shell, which has very low a_w and would not support growth. Standard GMPs will also reduce likelihood of pathogen recontamination.
4.c	***What pathogens are likely to recontaminate the product after the inactivation step?***	Recontamination of meat filling is not likely because it is encased within a pastry shell.	
4.d	***Are there any baseline surveys that indicate prevalence of pathogens for the target product or a related product?***	No, not for meat-filled pastry products.	
4.e	***For growth inhibition (TCS) studies:***		
4.e.1	*Which pathogen(s) will grow the fastest? Consider gram positive versus gram negative; vegetative microorganisms versus spore formers. If food is not seafood,* Vibrio *may be excluded from consideration. Use a predictive model or cite applicable literature. Consider growth potential through 1.5 times the shelf life, if appropriate.*	*B. cereus* based on predictive models (see 4.e.2).	
4.e.2	*Predictive model*	Predictive models were used to gauge comparative growth of *C. perfringens*, *C. botulinum,* and *B. cereus* in the meat filling. The PMP predicts a 1-log increase of *C. perfringens* in approximately 32 h based on pH 6.2, a_w 0.983 (lowest a_w in program), and 37°C (highest temperature in program). ComBase Predictor predicts a 1-log increase in approximately 13 h, assuming pH 6.2, a_w 0.971, and 37°C (98.6°F).	All modeling included the lag phase. This was considered appropriate given that spore-forming organisms require both germination and outgrowth. Predictive models estimate that *B. cereus* will grow faster than *C. perfringens* and that both organisms would grow faster than *C. botulinum.*

APPENDIX G. *Continued*

Evaluation of display of fully cooked meat-filled pastry for up to 12 h at room temperature			
	Considerations	***Response***	***Additional comments***
		The PMP predicts growth of *C. botulinum* in >10 days at 26.7°C (estimated room temperature of 80°F) based on pH 6.2, a_w 0.977 (lowest a_w in program). ComBase predicts a lag time for proteolytic *C. botulinum* of about 2 days, assuming pH 6.2, a_w 0.97, and 37°C (98.6°F), and a slightly shorter lag time for nonproteolytic *C. botulinum* at 30°C (86°F) and a_w 0.974 (the least permissive conditions allowed by the model). The PMP predicts a 1-log increase in *B. cereus* in 5 h at pH 6.2, a_w 0.97, and 37°C (98.6°F) under aerobic conditions and approximately 12 h under anaerobic conditions. ComBase predicts a 1-log increase in approximately 14 h at pH 6.2, a_w 0.97, and 37°C (98.6°F) with 0% CO_2.	
4.e.3	*Compare choice with literature*	Spices are an ingredient in the meat filling. *B. cereus* is a known contaminant of spices *(88).*	
4.e.4	*Any further information on growth or survival?*	No.	
4.e.5	*Based on the above analysis, what challenge organisms are chosen for growth inhibition studies?*	*B. cereus.*	
4.f	***If inactivation studies:***	NA	
4.f.1	*What is the lethal treatment? (HPP, heat, acid, etc.)*		
4.f.2	*Which microorganisms are most resistant to the lethal treatment? (HPP, heat, acid, etc.)*		
4.f.3	*Will the lethality be delivered to all areas of the product that may contain the pathogen? Account for all surface and internalized contamination.*		
4.f.4	*What is in the formulation that may affect inactivation? (Intrinsic factors may contribute to lethality or resistance: a_w, moisture, salt, pH, fat, etc.)*		
4.f.5	*Are there any data on pathogen levels in the product?*		
4.f.6	*Is there a regulatory requirement or policy for log reduction for this product? Cite requirement.*		
4.f.7	*If there is no regulatory requirement for log reduction, use scientific basis for determining acceptable reduction (21, 76).*		
4.f.8	*Based on the above analysis, what challenge organisms are chosen for inactivation studies?*		

APPENDIX G. *Continued*

Evaluation of display of fully cooked meat-filled pastry for up to 12 h at room temperature			
	Considerations	***Response***	***Additional comments***
5	**Determine appropriate time and sampling intervals for challenge study**		
5.a	***For growth inhibition (TCS) studies, use 1.25–1.5 times the shelf life as testing time***	1.5 × 14 h (target shelf life plus up to 2 h in the hands of the consumer) = 21 h.	
5.a.1	*Maximum time from production to consumption*	Refrigerated up to 7 days, 12 h at room temperature and 2 h to consumption after leaving the store. May or may not be heated prior to consumption.	
5.a.2	*Actual time to spoilage or unacceptable quality*	Expected to have 7-day shelf life in refrigerator. At room temperature product may appear to be spoiled after 2 days.	
5.a.3	*For growth inhibition studies, determine appropriate sampling intervals for microbial analysis; use five to seven (preferred) sampling intervals; fewer sampling intervals should be justified, e.g., using results from similar products.*	Times 0, 14, and 21 h.	Due to the very short shelf life of this product and the fact that predictive models estimated limited growth in the time frames of the study (e.g., approximately 1 log unit of growth), sampling times were set at 0, 14 (the end of the target shelf life), and 21 h (1.5 times the target shelf life).
5.b	***For inactivation studies determine appropriate sampling points considering the process and formulation.*** *Identify populations at 0 time and end of processing; whenever possible, include intermediate sampling intervals to determine death curve.*	NA	
5.b.1	*When inactivation treatments may result in sublethal injury, repair and growth of microorganisms during product shelf life should be considered (21).*	NA	
6	**Determine inoculation, storage, and testing procedures**		
6.a	***Determine strains for use in study (multiple strains for each species are recommended; consider use of appropriate food or clinical isolates)***	Use at least three strains of *B. cereus*.	This should include a composite of clinical strains from foodborne illness outbreaks as well as isolates from food.
6.b	***Determine if adaptation is required for inoculum preparation***	Adaptation not required.	
6.c	***Determine method of inoculation (surface, mixing, dipping, liquid, dry, etc.)***	Injection of inoculum into the meat filling through the pastry.	Inoculum spores mixed with meat filling supplied by manufacturer.
6.d	***Determine size of inoculum (populations, e.g., log CFU/g, CFU per package, percentage of inoculum vol/wt or vol/vol)***	2–3 log CFU/g not to exceed 0.1% of filling volume.	Inoculum size is verified with time 0 sample of filling.
6.e	***Determine packaging to be used***	Plastic cellophane wrap.	
6.f	***Determine the incubation temperature for growth inhibition studies or temperature(s) for thermal inactivation studies***	Incubate at 30°C (86°F).	This represents a reasonable maximum ambient temperature.

APPENDIX G. *Continued*

Evaluation of display of fully cooked meat-filled pastry for up to 12 h at room temperature			
	Considerations	***Response***	***Additional comments***
6.g	***Determine sampling method and sample size***	Duplicate filled pastries will be sampled from each of the three replicate lots at each time point. Each sample in its entirety will be blended or stomached in a 1:10 dilution of buffer. Duplicate plate counts will be run for each sample.	
6.h	***How many replicates are needed to ensure confidence in data?*** *Does variability in proximate analysis or production warrant more than two or three replicate trials? Will multiple variations of similar formulations be tested? Has a statistical design for choosing formulations been used (block design, central composite, etc.)?*	Three replicate production lots are to be tested, preferably lots made with separate batches of ingredients or on separate days.	If different formulations, three replicates per formulation.
7	**Determine other controls**		
7.a	***Is use of surrogates appropriate or necessary? If so, justify.***	Surrogates are not appropriate or necessary.	
7.b	***Are uninoculated controls needed to assess spoilage or competitive microflora or for other purposes?***	An uninoculated control is needed for each replicate lot to monitor for natural contamination.	
7.c	***What other controls are necessary (including negative or positive growth controls)?***	Not required for this study; anticipate growth if samples were held for sufficient time.	
8	**Determine pass-fail criteria**		
8.a	***What are the pass-fail criteria?***	No more than a 3-log increase of *B. cereus*.	The 3-log increase level selected for *B. cereus* is based on the increase suggested in the IFT report *(53)*. Some regulatory agencies may consider a lower log increase to be appropriate.
8.b	***What are the limits for use of the results?***	These results cannot be applied to pastries held at higher than ambient temperatures, e.g., holding under a heat lamp.	

[a] NA, not applicable.

APPENDIX H. FOOD PRODUCT CHECKLIST: LEMON MERINGUE PIE

Validate that the formulation of lemon meringue pie will inhibit pathogen growth under nonrefrigerated conditions			
	Considerations	***Response***	***Additional comments***
1	**Determine the purpose of the study**		
1.a	***Exempt from time/temperature control for safety (no refrigeration required)***	Exempt from time/temperature control after opening.	Labeled shelf life of 3 days.
1.b	***Variance from any regulatory requirements (e.g., holding for >4 h without temperature control)***	NA[a]	
1.c	***Validate lethality***	NA	
1.d	***Verify that formulation will inhibit microbial growth in refrigerated foods or under mild temperature abuse***	NA	
2	**Collect information regarding the product**		
2.a	***What are the ingredients?***	Pie crust: flour, shortening, water, salt. Filling: water, sugar, modified food starch, corn syrup solids, margarine, lemon juice solids, high fructose corn syrup, sodium citrate, agar agar, potassium sorbate, natural flavor, locust bean gum, artificial color (FD&C yellow no. 5). Meringue: unpasteurized egg whites, sugar, cream of tartar.	
2.a.1	*How consistent are the ingredients from various sources, lot to lot?*	Very consistent, same or similar lot to lot.	
2.a.2	*What are the pH, a_w, and proximate analysis (moisture, salt, fat, protein, residual nitrite, etc.) for product and/or individual components?*	Baked crust: pH 6.2, a_w 0.45. Cooked filling: pH 4.2, a_w 0.88. Meringue: pH 4.6, a_w 0.93.	Values are after baking.
2.a.3	*Do any of these values change from preparation to consumption?*	No.	
2.a.4	*If applicable, what are the dimensions of cuts, pieces, etc?*	NA	
2.a.5	*What is the normal microbial load (species, etc.) at the beginning and end of production?*	After baking, aerobic plate count of <10 CFU/g.	
2.a.6	*Is there likelihood that contamination may be internalized in or distributed throughout individual components?*	Yes, during slicing the contamination may occur along the sliced edge of all three components (crust, filling, and meringue).	
2.b	***What are the preparation steps?***	Mix dough, sheet, form, bake. Cook the filling to set the starch, fill the baked crust, cool to ambient temperature, spread meringue evenly over filling and bake. Cool to ambient temperature, package.	Product is prepared in a commercial manufacturing facility, cooled to room temperature, packaged, and shipped at ambient temperature.
2.b.1	*Is the product an assembled (multicomponent) product?*	Yes.	
2.b.2	*Is there a microbial reduction step that is validated? What are the parameters associated with the microbial reduction step? Are there different microbial reduction steps for different components?*	All three components have heat inactivation steps.	

APPENDIX H. *Continued*

Validate that the formulation of lemon meringue pie will inhibit pathogen growth under nonrefrigerated conditions			
	Considerations	***Response***	***Additional comments***
2.b.3	*Is there a potential for recontamination?*	Yes, contamination may occur after opening and slicing.	
2.b.4	*What is the variability in parameters that affect lethality or growth?*	Low variability.	
2.b.5	*How is the product packaged?*	Paperboard box or plastic dome over an aluminum pie plate.	
2.b.6	*Is the product cultured or fermented? Does it contain starter culture intentionally added?*	No.	
2.b.7	*Does the product contain antimicrobials (preservatives) or other ingredients that might be inhibitory, such as spices?*	Sodium citrate and potassium sorbate in the filling.	
2.c	***What are the storage conditions?***		
2.c.1	*How will the product be displayed for sale? Any changes to packaging for display?*	Refrigerated or ambient, no change to packaging.	
2.c.2	*What temperatures (and times) are expected during production, preparation, and storage or display?*	Cooled to ambient temperature after baking, shipped and displayed at ambient temperatures 20–35°C (68–95°F) until the end of labeled shelf life of 3 days.	Unacceptable quality at 5 days.
2.c.3	*What potential is there for storage or display at temperatures greater than those listed above in 2.c.2?*	Unlikely.	
2.c.4	*Are there other hazards that may be created by preparation, storage, or display?*	No.	However, hazards may be introduced during slicing and serving.
2.c.5	*What is the estimated maximum time from production to consumption?*	3 days.	
2.c.6	*What is the time to spoilage or unacceptable quality?*	5 days.	
3	**Determine if product assessment for growth or inactivation is needed**		
3.a	***Is a product assessment for growth necessary based on pH and a_w? (See Appendix D, Tables A and B.) If yes, also answer 4.e and 5.a.***	Yes, according to Table B, a product assessment is required for the meringue component but not the crust or the filling.	
3.b	***Is an inactivation study needed? If yes, also answer 4.f and 5.b.***	Yes, a separate inactivation study is being conducted on the meringue.	
3.c	***Are there any regulations applicable for lethality (inactivation) or TCS (growth)?***	Yes, purpose of study is to get a variance from need for time/temperature control for safety.	

APPENDIX H. *Continued*

Validate that the formulation of lemon meringue pie will inhibit pathogen growth under nonrefrigerated conditions			
	Considerations	***Response***	***Additional comments***
4	**Determine pathogens of concern to include in the challenge study**		
4.a	***According to Table 2 and Appendix C, which pathogens are of concern? If food is not seafood,* Vibrio *spp. may be excluded from consideration.***	From Appendix C, pathogens of concern in egg products are *Salmonella* and *Listeria*. From Table 2, for pH 4.6 and a_w 0.94, *L. monocytogenes* and *Salmonella* would be the organisms of concern.	
4.b	***Considering the ecology, product, and epidemiological history, what pathogens are reasonably likely to occur? (Also see Appendix C.)***	*Salmonella* and *L. monocytogenes* are known to be in retail and food service environments.	
4.c	***What pathogens are likely to recontaminate the product after the inactivation step?***	*Salmonella* and *L. monocytogenes*.	
4.d	***Are there any baseline surveys that indicate prevalence of pathogens for the target product or a related product?***	There are studies documenting the presence of *Listeria* in the retail deli environment *(68)*.	
4.e	***For growth inhibition (TCS) studies:***		
4.e.1	*Which pathogen(s) will grow the fastest? Consider gram positive versus gram negative; vegetative microorganisms versus spore formers. If food is not seafood,* Vibrio *may be excluded from consideration. Use a predictive model or cite applicable literature. Consider growth potential through 1.5 times the shelf life, if appropriate.*	See 4.e.2.	
4.e.2	*Predictive model*	The PMP indicates that *L. monocytogenes* will not grow, but the model does not go below pH 5.6 for *Salmonella,* so growth rate under pH and a_w conditions in meringue is unknown.	
4.e.3	*Compare choice with literature*	Literature shows *Salmonella* and *L. monocytogenes* growth can occur at pH 4.6; most of these studies were in laboratory media with high a_w.	
4.e.4	*Any further information on growth or survival?*	No.	
4.e.5	*Based on the above analysis, what challenge organisms are chosen for growth inhibition studies?*	*Salmonella*.	Since we are unable to determine the likelihood of growth of *Salmonella* from predictive models or from the literature, this organism was chosen for a challenge study.
4.f	***If inactivation studies:***	NA	
4.f.1	*What is the lethal treatment? (HPP, heat, acid, etc.)*		
4.f.2	*Which microorganisms are most resistant to the lethal treatment? (HPP, heat, acid, etc.)*		
4.f.3	*Will the lethality be delivered to all areas of the product that may contain the pathogen? Account for all surface and internalized contamination.*		

APPENDIX H. *Continued*

Validate that the formulation of lemon meringue pie will inhibit pathogen growth under nonrefrigerated conditions			
	Considerations	***Response***	***Additional comments***
4.f.4	*What is in the formulation that may affect inactivation? (Intrinsic factors may contribute to lethality or resistance: a_w, moisture, salt, pH, fat, etc.)*		
4.f.5	*Are there any data on pathogen levels in the product?*		
4.f.6	*Is there a regulatory requirement or policy for log reduction for this product? Cite requirement.*		
4.f.7	*If there is no regulatory requirement for log reduction, use scientific basis for determining acceptable reduction (21, 76).*		
4.f.8	*Based on the above analysis, what challenge organisms are chosen for inactivation studies?*		
5	**Determine appropriate time and sampling intervals for challenge study**		
5.a	***For growth inhibition (TCS) studies, use 1.25–1.5 times the shelf life as testing time***	3 days × 1.5 = 4.5 days (round to 5 days).	
5.a.1	*Maximum time from production to consumption*	3 days.	
5.a.2	*Actual time to spoilage or unacceptable quality*	5 days.	
5.a.3	*For growth inhibition studies, determine appropriate sampling intervals for microbial analysis; use five to seven (preferred) sampling intervals; fewer sampling intervals should be justified, e.g., using results from similar products.*	Sample at time 0, then at days 1, 2, 3, 4, and 5.	
5.b	***For inactivation studies determine appropriate sampling points considering the process and formulation.*** *Identify populations at 0 time and end of processing; whenever possible, include intermediate sampling intervals to determine death curve.*	NA	
5.b.1	*When inactivation treatments may result in sublethal injury, repair and growth of microorganisms during product shelf life should be considered (21).*	NA	
6	**Determine inoculation, storage, and testing procedures**		
6.a	***Determine strains for use in study (multiple strains for each species are recommended; consider use of appropriate food or clinical isolates)***	A mixture of at least five strains of *Salmonella* isolated from eggs or egg products and including at least one *Salmonella* Enteritidis strain isolated from clinical or egg samples associated with outbreaks.	
6.b	***Determine if adaptation is required for inoculum preparation***	Not necessary for this study.	

APPENDIX H. *Continued*

Validate that the formulation of lemon meringue pie will inhibit pathogen growth under nonrefrigerated conditions			
	Considerations	***Response***	***Additional comments***
6.c	***Determine method of inoculation (surface, mixing, dipping, liquid, dry, etc.)***	Inoculate the cut face of the meringue for single slices of pie by distributing 25 μl of liquid inoculum from the filling-meringue interface to the surface of the meringue; a noninhibitory dye will be added to inoculum to facilitate identification of the sampling area.	A preliminary study should be conducted to ensure that the dye is not inhibitory to *Salmonella* unless previously documented in the scientific literature.
6.d	***Determine size of inoculum (populations, e.g., log CFU/g, CFU per package, percentage of inoculum vol/wt or vol/vol)***	Target 2–3 log CFU per site for each slice.	
6.e	***Determine packaging to be used***	Packed in a ventilated plastic container that prevents contamination of the slice but allows exchange of air.	
6.f	***Determine the incubation temperature for growth inhibition studies or temperature(s) for thermal inactivation studies***	35°C (95°F).	
6.g	***Determine sampling method and sample size***	For each sample, the entire slice (approximately 100 g) will be placed in a sterile plastic sampling bag. The sample will be homogenized with an equal volume of 0.1% peptone buffer, and serial dilutions will be plated on appropriate *Salmonella* selective agar using the FDA BAM method *(7)*.	
6.h	***How many replicates are needed to ensure confidence in data?*** *Does variability in proximate analysis or production warrant more than two or three replicate trials? Will multiple variations of similar formulations be tested? Has a statistical design for choosing formulations been used (block design, central composite, etc.)?*	Three replicate trials, pies made from different batches of ingredients for each trial, triplicate slices per trial. Separate slices will be assayed for each sampling interval ($n = 9$ for each sampling interval).	Three replicate trials with three samples at each interval were chosen because of the inherent variability of inoculating individual slices for each sampling time interval.
7	**Determine other controls**		
7.a	***Is use of surrogates appropriate or necessary? If so, justify.***	No surrogates are necessary.	
7.b	***Are uninoculated controls needed to assess spoilage or competitive microflora or for other purposes?***	Yes.	An uninoculated pie for aerobic plate counts and counts of yeasts and molds.
7.c	***What other controls are necessary (including negative or positive growth controls)?***	NA	
8	**Determine pass-fail criteria**		
8.a	***What are the pass-fail criteria?***	Must show <1-log growth of *Salmonella* throughout the 5-day testing period.	
8.b	***What are the limits for use of the results?***	Would be applicable only to meringue pies with very similar pH and a_w in both the filling and the meringue.	

[a] NA, not applicable.

APPENDIX I. FOOD PRODUCT CHECKLIST: MERINGUE TOPPING

Evaluation of the adequacy of thermal inactivation of pathogens of concern in meringue topping for lemon meringue pie			
	Considerations	***Response***	***Additional comments***
1	**Determine the purpose of the study**		
1.a	***Exempt from time/temperature control for safety (no refrigeration required)***	NA[a]	
1.b	***Variance from any regulatory requirements (e.g., holding for >4 h without temperature control)***	NA	
1.c	***Validate lethality***	Validate lethality of meringue topping heat treatment (baking).	
1.d	***Verify that formulation will inhibit microbial growth in refrigerated foods or under mild temperature abuse***	NA	
2	**Collect information regarding the product**		
2.a	***What are the ingredients?***	Pie crust: flour, shortening, water, salt. Filling: water, sugar, modified food starch, corn syrup solids, margarine, lemon juice solids, high fructose corn syrup, sodium citrate, agar agar, potassium sorbate, natural flavor, locust bean gum, artificial color (FD&C yellow no. 5). Meringue: unpasteurized egg whites, sugar, cream of tartar.	2007 supplement to the 2005 Food Code specifies that pasteurized egg white be used for meringue. This is an example illustrating an inactivation challenge study and could potentially be used to obtain a variance for the use of unpasteurized egg whites.
2.a.1	*How consistent are the ingredients from various sources, lot to lot?*	Very consistent, same or similar lot to lot.	
2.a.2	*What are the pH, a_w, and proximate analysis (moisture, salt, fat, protein, residual nitrite, etc.) for product and/or individual components?*	Baked crust: pH 6.2, a_w 0.45. Cooked filling: pH 4.2, a_w 0.88. Raw meringue: pH 4.6, a_w 0.93.	
2.a.3	*Do any of these values change from preparation to consumption?*	a_w may decrease for the meringue at the exposed surface.	
2.a.4	*If applicable, what are the dimensions of cuts, pieces, etc?*	NA	
2.a.5	*What is the normal microbial load (species, etc.) at the beginning and end of production?*	Before cooking: aerobic plate count <1,000 CFU/g. After baking: aerobic plate count <10 CFU/g.	
2.a.6	*Is there likelihood that contamination may be internalized in or distributed throughout individual components?*	*Salmonella* may be present in unpasteurized egg whites used for meringue topping.	

APPENDIX I. *Continued*

Evaluation of the adequacy of thermal inactivation of pathogens of concern in meringue topping for lemon meringue pie			
	Considerations	***Response***	***Additional comments***
2.b	***What are the preparation steps?***	Mix dough, sheet, form, bake. Cook the filling to set the starch, fill the baked crust, cool to ambient temperature, spread meringue evenly over filling and bake. Cool to ambient temperature, package.	Product is prepared in a commercial manufacturing facility, cooled to room temperature, packaged and shipped at ambient temperature.
2.b.1	*Is the product an assembled (multicomponent) product?*	Yes.	
2.b.2	*Is there a microbial reduction step that is validated? What are the parameters associated with the microbial reduction step? Are there different microbial reduction steps for different components?*	Purpose of this study. All three components (crust, filling, meringue) have heat inactivation steps, but the crust gets heat treated twice, the filling gets heat treated twice and there is an added inactivation due to the pH, and the meringue gets heat treated once.	
2.b.3	*Is there a potential for recontamination?*	Very unlikely, controlled through GMPs at the commercial manufacturing facility.	
2.b.4	*What is the variability in parameters that affect lethality or growth?*	Low variability.	
2.b.5	*How is the product packaged?*	Paperboard box or plastic dome over an aluminum pie plate.	
2.b.6	*Is the product cultured or fermented? Does it contain starter culture intentionally added?*	No.	
2.b.7	*Does the product contain antimicrobials (preservatives) or other ingredients that might be inhibitory, such as spices?*	Sodium citrate and potassium sorbate in the filling.	
2.c	***What are the storage conditions?***		
2.c.1	*How will the product be displayed for sale? Any changes to packaging for display?*	Refrigerated or ambient, no change to packaging.	
2.c.2	*What temperatures (and times) are expected during production, preparation, and storage or display?*	Crust cook: 85°C (185°F) final temperature, 15 min total cooking time in 176.7°C (350°F) nonhumidified oven. Filling set: 90.6°C (195°F) for 10 min. Meringue set: 15 min total cooking time in a preheated 176.7°C (350°F) oven.	The cook time for the meringue is based on the time required to achieve the characteristic browning.
2.c.3	*What potential is there for storage or display at temperatures greater than those listed above in 2.c.2?*	NA; purpose of this study is to validate microbial reduction.	
2.c.4	*Are there other hazards that may be created by preparation, storage, or display?*	No.	
2.c.5	*What is the estimated maximum time from production to consumption?*	NA	
2.c.6	*What is the time to spoilage or unacceptable quality?*	NA	

APPENDIX I. *Continued*

Evaluation of the adequacy of thermal inactivation of pathogens of concern in meringue topping for lemon meringue pie			
	Considerations	***Response***	***Additional comments***
3	**Determine if product assessment for growth or inactivation is needed**		
3.a	***Is a product assessment for growth necessary based on pH and a_w? (See Appendix D, Tables A and B.) If yes, also answer 4.e and 5.a.***	NA	
3.b	***Is an inactivation study needed? If yes, also answer 4.f and 5.b.***	Yes.	
3.c	***Are there any regulations applicable for lethality (inactivation) or TCS (growth)?***	2007 supplement to the 2005 Food Code specifies that pasteurized egg white be used for meringue.	
4	**Determine pathogens of concern to include in the challenge study**		
4.a	***According to Table 2 and Appendix C, which pathogens are of concern? If food is not seafood,* Vibrio *spp. may be excluded from consideration.***	From Appendix C, pathogens of concern in egg products are *B. cereus, Salmonella,* and *L. monocytogenes*.	
4.b	***Considering the ecology, product, and epidemiological history, what pathogens are reasonably likely to occur? (Also see Appendix C.)***	*B. cereus* spores would be expected to survive the heat treatment but would not grow out due to the a_w of meringue. *Salmonella* is more prevalent and present in higher numbers than *L. monocytogenes* in unpasteurized liquid egg products. *Salmonella* has been associated with numerous products containing undercooked egg ingredients, including meringue pie *(72)*.	For this study, we are concerned with pathogen survival, not growth; therefore, a pathogen with a low infectious dose was chosen as the challenge organism.
4.c	***What pathogens are likely to recontaminate the product after the inactivation step?***	NA	The objective of the study is to evaluate inactivation and not recontamination.
4.d	***Are there any baseline surveys that indicate prevalence of pathogens for the target product or a related product?***	In FSIS *(111)* risk assessments regarding eggs and egg products, *Salmonella* estimates in unpasteurized liquid whole egg product were 0 and 100 cells per ml, on average, in pooled product from multiple eggs (see Fig. 3-45 from the FSIS risk assessment). In addition, estimates in unpasteurized liquid egg white product were less than 10 cells per ml, on average, in pooled product from multiple eggs (see Fig. 3-44 from the FSIS risk assessment). On some occasions MPNs of *Salmonella* can exceed 1,000 CFU/ml for both unpasteurized liquid whole egg and liquid egg white products, but these events appear to be rare. The FSIS identified *Salmonella* Enteritidis in some but not all samples collected and analyzed. Thus, the *Salmonella* Enteritidis levels used in the inactivation study described below would represent a worst case.	For an inactivation study, quantitative levels are more important than qualitative prevalence, as levels help estimate the amount of kill necessary to protect public health.
4.e	***For growth inhibition (TCS) studies:***	NA	

APPENDIX I. *Continued*

Evaluation of the adequacy of thermal inactivation of pathogens of concern in meringue topping for lemon meringue pie			
	Considerations	***Response***	***Additional comments***
4.e.1	*Which pathogen(s) will grow the fastest? Consider gram positive versus gram negative; vegetative microorganisms versus spore formers. If food is not seafood,* Vibrio *may be excluded from consideration. Use a predictive model or cite applicable literature. Consider growth potential through 1.5 times the shelf life, if appropriate.*		
4.e.2	*Predictive model*		
4.e.3	*Compare choice with literature*		
4.e.4	*Any further information on growth or survival?*		
4.e.5	*Based on the above analysis, what challenge organisms are chosen for growth inhibition studies?*		
4.f	***If inactivation studies:***		
4.f.1	*What is the lethal treatment? (HPP, heat, acid, etc.)*	Heat.	
4.f.2	*Which microorganisms are most resistant to the lethal treatment? (HPP, heat, acid, etc.)*	Sporeformers would be most heat resistant but have been eliminated as a risk due to inability to grow in the product. *Salmonella* Enteritidis is the most appropriate challenge organism.	
4.f.3	*Will the lethality be delivered to all areas of the product that may contain the pathogen? Account for all surface and internalized contamination.*	Yes.	
4.f.4	*What is in the formulation that may affect inactivation? (Intrinsic factors may contribute to lethality or resistance: a_w, moisture, salt, pH, fat, etc.)*	Relatively high sugar content of the meringue will reduce the a_w, potentially leading to increased heat resistance.	
4.f.5	*Are there any data on pathogen levels in the product?*	See 4.d.	
4.f.6	*Is there a regulatory requirement or policy for log reduction for this product? Cite requirement.*	No.	
4.f.7	*If there is no regulatory requirement for log reduction, use scientific basis for determining acceptable reduction (21, 76).*	Using 2-log reduction as worst case and building in a 2-log margin of safety, the target reduction is 4-log units.	
4.f.8	*Based on the above analysis, what challenge organisms are chosen for inactivation studies?*	*Salmonella* Enteritidis.	
5	**Determine appropriate time and sampling intervals for challenge study**		
5.a	***For growth inhibition (TCS) studies, use 1.25–1.5 times the shelf life as testing time***	NA	
5.a.1	*Maximum time from production to consumption*		

APPENDIX I. *Continued*

Evaluation of the adequacy of thermal inactivation of pathogens of concern in meringue topping for lemon meringue pie			
	Considerations	***Response***	***Additional comments***
5.a.2	*Actual time to spoilage or unacceptable quality*		
5.a.3	*For growth inhibition studies, determine appropriate sampling intervals for microbial analysis; use five to seven (preferred) sampling intervals; fewer sampling intervals should be justified, e.g., using results from similar products.*		
5.b	***For inactivation studies determine appropriate sampling points considering the process and formulation.*** *Identify populations at 0 time and end of processing; whenever possible, include intermediate sampling intervals to determine death curve.*	Times 0 and 15 min.	Sampling at more than three time points would allow a *D*-value to be calculated that may be of use in further defining the cook process but is not in the current study design.
5.b.1	*When inactivation treatments may result in sublethal injury, repair and growth of microorganisms during product shelf life should be considered (21).*	NA	
6	**Determine inoculation, storage, and testing procedures**		
6.a	***Determine strains for use in study (multiple strains for each species are recommended; consider use of appropriate food or clinical isolates)***	A mixture of at least five strains of *Salmonella* isolated from eggs or egg products and including at least one *Salmonella* Enteritidis isolated from clinical or egg samples associated with outbreaks.	
6.b	***Determine if adaptation is required for inoculum preparation***	Not necessary for this study.	
6.c	***Determine method of inoculation (surface, mixing, dipping, liquid, dry, etc.)***	Will mix concentrated, washed inoculum into the egg whites before beating to a meringue. Finished meringue will be weighed and spread evenly over the surface of a cooked lemon filling that has been cooled to room temperature. The concentration of *Salmonella* in the uncooked finished meringue will be determined as described below.	Because of the potential for aerosols, beating of egg whites should be done in a biological safety cabinet. The assumption is also made that the filling will not be chilled below ambient temperature prior to application of the meringue.
6.d	***Determine size of inoculum (populations, e.g., log CFU/g, CFU per package, percentage of inoculum vol/wt or vol/vol)***	A final target level of at least 4 log CFU per pie.	
6.e	***Determine packaging to be used***	NA	
6.f	***Determine the incubation temperature for growth inhibition studies or temperature(s) for thermal inactivation studies***		

APPENDIX I. *Continued*

Evaluation of the adequacy of thermal inactivation of pathogens of concern in meringue topping for lemon meringue pie			
	Considerations	***Response***	***Additional comments***
6.g	***Determine sampling method and sample size***	The sample size will be the whole meringue from a single pie. The whole meringue will be enriched for *Salmonella* using the BAM method *(7)*. In addition, at T_0 one pie will be used to determine the initial number of *Salmonella* recovered in the meringue prior to baking by removing the meringue from the pie, mixing thoroughly, and taking three 10-g samples of the meringue for enumeration.	
6.h	***How many replicates are needed to ensure confidence in data?*** *Does variability in proximate analysis or production warrant more than two or three replicate trials? Will multiple variations of similar formulations be tested? Has a statistical design for choosing formulations been used (block design, central composite, etc.)?*	There will be three replicate trials. Each trial will consist of three inoculated baked pies plus one T_0 unbaked pie; thus, a total of 12 pies will be needed for the study.	
7	**Determine other controls**		
7.a	***Is use of surrogates appropriate or necessary? If so, justify.***	No surrogates are appropriate.	
7.b	***Are uninoculated controls needed to assess spoilage or competitive microflora or for other purposes?***	NA	
7.c	***What other controls are necessary (including negative or positive growth controls)?***	Temperature will be verified in several places in the oven during baking.	
8	**Determine pass-fail criteria**		
8.a	***What are the pass-fail criteria?***	Must achieve >4-log reduction within 15 min in a 176.7°C (350°F) oven.	Based on nondetection of *Salmonella* upon enrichment of the meringue.
8.b	***What are the limits for use of the results?***	Limitations of this study include the volume and depth of the meringue on the pie. The temperature of the filling may impact the results. The data will apply for longer but not shorter cook times at the oven temperature indicated. These data could apply to other types of filling.	

[a] NA, not applicable.

APPENDIX J. FOOD PRODUCT CHECKLIST: BAKED CHEESE PIZZA

Evaluation of baked cheese pizza held out of refrigeration for up to 8 h			
	Considerations	***Response***	***Additional comments***
1	**Determine the purpose of the study.**		
1.a	***Exempt from time/temperature control for safety (no refrigeration required)***	NA[a]	
1.b	***Variance from any regulatory requirements (e.g., holding for >4 h without temperature control)***	Holding of baked cheese (sliced) pizza without refrigeration for up to 8 h.	
1.c	***Validate lethality***	NA	Assembled at another facility (central commissary) and held refrigerated until baked at retail store.
1.d	***Verify that formulation will inhibit microbial growth in refrigerated foods or under mild temperature abuse***	NA	
2	**Collect information regarding the product**		
2.a	***What are the ingredients?***	Pizza crust: flour, salt, shortening. Cheese: pasteurized milk, salt, rennet, starter cultures. Tomato sauce: canned tomato paste, water, oregano, basil, garlic.	
2.a.1	*How consistent are the ingredients from various sources, lot to lot?*	Ingredients same; relatively consistent composition of sauce and cheese but preparation of pizza may vary considerably with respect to amounts of ingredients.	
2.a.2	*What are the pH, a_w, and proximate analysis (moisture, salt, fat, protein, residual nitrite, etc.) for product and/or individual components?*	Proximate analysis: 10% protein, 8% fat, 1% salt, 46–49% moisture pH: crust, 6.8; sauce, 4.5; cheese, 5.4 a_w: crust, 0.70; sauce, 0.98; cheese, 0.95–0.96.	
2.a.3	*Do any of these values change from preparation to consumption?*	No.	
2.a.4	*If applicable, what are the dimensions of cuts, pieces, etc?*	NA	
2.a.5	*What is the normal microbial load (species, etc.) at the beginning and end of production?*	Microbial load: <100 CFU/g after baking; primarily spore-forming microorganisms potentially including *B. cereus, C. perfringens,* and *C. botulinum.*	
2.a.6	*Is there likelihood that contamination may be internalized in or distributed throughout individual components?*	Yes, each of the components is likely to contain pathogenic bacterial spores.	
2.b	***What are the preparation steps?***		
2.b.1	*Is the product an assembled (multicomponent) product?*	Yes. Product consists of a thin crust covered with sauce and topped with a layer of cheese.	

APPENDIX J. *Continued*

Evaluation of baked cheese pizza held out of refrigeration for up to 8 h			
	Considerations	***Response***	***Additional comments***
2.b.2	*Is there a microbial reduction step that is validated? What are the parameters associated with the microbial reduction step? Are there different microbial reduction steps for different components?*	No further kill step after pizza is baked. Baking has been validated to eliminate all vegetative bacterial pathogens.	
2.b.3	*Is there a potential for recontamination?*	Yes, there is potential for recontamination once the pizza cools from the baking process and is handled by food service workers.	Due to the large surface area, the pizza is expected to cool to room temperature rapidly after baking; therefore, growth of *C. perfringens* is not a concern.
2.b.4	*What is the variability in parameters that affect lethality or growth?*	Little variability in parameters that affect lethality if baked to an endpoint of visual doneness.	
2.b.5	*How is the product packaged?*	Not packaged. Trays containing pizzas are shipped from commissary to food service establishment.	
2.b.6	*Is the product cultured or fermented? Does it contain starter culture intentionally added?*	No.	
2.b.7	*Does the product contain antimicrobials (preservatives) or other ingredients that might be inhibitory, such as spices?*	NaCl is present but not at inhibitory levels. No antimicrobials are added.	
2.c	***What are the storage conditions?***		
2.c.1	*How will the product be displayed for sale? Any changes to packaging for display?*	Held in an enclosed display cabinet where the maximum temperature is 30°C (86°F).	
2.c.2	*What temperatures (and times) are expected during production, preparation, and storage or display?*	Only the baked product holding temperature is relevant; in this instance, 30°C (86°F) for up to 8 h at retail.	
2.c.3	*What potential is there for storage or display at temperatures greater than those listed above in 2.c.2?*	There is the possibility that product will be held at temperatures as great as 40°C (104°F), but quality deterioration would occur in less than 8 h.	
2.c.4	*Are there other hazards that may be created by preparation, storage, or display?*	*L. monocytogenes* contamination from the environment may occur; handling can result in contamination with *S. aureus*.	
2.c.5	*What is the estimated maximum time from production to consumption?*	Maximum 8 h store display; 2 h from sale to consumption (total of 10 h).	
2.c.6	*What is the time to spoilage or unacceptable quality?*	Product is of acceptable quality for the duration of the study, even though it may appear to be dried out. Little is known about unacceptable quality parameters for pizza and what consumers may determine to be of unacceptable quality. In accordance with general food safety practices, food should be consumed or refrigerated within 2 h of purchase.	

APPENDIX J. *Continued*

Evaluation of baked cheese pizza held out of refrigeration for up to 8 h			
	Considerations	***Response***	***Additional comments***
3	**Determine if product assessment for growth or inactivation is needed**		
3.a	***Is a product assessment for growth necessary based on pH and a_w? (See Appendix D, Tables A and B.) If yes, also answer 4.e and 5.a.***	Yes, product assessment required; Food Code Table B is applicable because of potential recontamination and survival of spores; pH $>$ 5.0 and $a_w > 0.92$ in parts of the product and product not protected from recontamination.	Multicomponent product. Crust has low a_w, but a_w will be increased by moisture from sauce. Sauce also lowers the pH of the crust. Moisture loss of product occurs over time.
3.b	***Is an inactivation study needed? If yes, also answer 4.f and 5.b.***	No, the purpose of this study is to determine if pathogens likely to be present will grow in the product if stored out of refrigeration.	
3.c	***Are there any regulations applicable for lethality (inactivation) or TCS (growth)?***	Latest edition of the Food Code for TCS.	
4	**Determine pathogens of concern to include in the challenge study**		
4.a	***According to Table 2 and Appendix C, which pathogens are of concern? If food is not seafood,* Vibrio *spp. may be excluded from consideration.***	Based on a measured pH of 5.4 and a maximum a_w of 0.96 for cheese, the organisms of concern are *B. cereus, C. botulinum,* pathogenic *E. coli, L. monocytogenes, Salmonella,* and *S. aureus.* Based on a measured pH of 5.3 and a maximum a_w of 0.98 at the cheese-sauce interface, same organisms as above. Based on a measured pH of 5.0 and a maximum a_w of 0.97 at the sauce-crust interface, same organisms as above.	*Vibrio* spp. were excluded from consideration since seafood is not involved.
4.b	***Considering the ecology, product, and epidemiological history, what pathogens are reasonably likely to occur? (Also see Appendix C.)***	*B. cereus* and *C. botulinum* spores survive baking; *L. monocytogenes* and *S. aureus* may be present from postprocessing handling. No known illnesses have occurred from consumption of cheese pizza. However, illnesses due to *E. coli* O157:H7 were associated with frozen pepperoni pizza, although the cause of the outbreak was undetermined *(70).*	Pathogenic *E. coli* and *Salmonella* are inactivated during adequate baking. They are also not likely to be present in the environment and therefore recontamination of the cheese pizza with these organisms is unlikely. *C. botulinum* was excluded from consideration because of the aerobic conditions and the reduced pH levels and because spores of *B. cereus* are more common and likely to grow faster.
4.c	***What pathogens are likely to recontaminate the product after the inactivation step?***	Study is designed to determine safety if recontamination should occur.	
4.d	***Are there any baseline surveys that indicate prevalence of pathogens for the target product or a related product?***	No.	
4.e	***For growth inhibition (TCS) studies:***		
4.e.1	*Which pathogen(s) will grow the fastest? Consider gram positive versus gram negative; vegetative microorganisms versus spore formers. If food is not seafood,* Vibrio *may be excluded from consideration. Use a predictive model or cite applicable literature. Consider growth potential through 1.5 times the shelf life, if appropriate.*	No one organism was determined to grow faster. See 4.e.2.	

APPENDIX J. *Continued*

Evaluation of baked cheese pizza held out of refrigeration for up to 8 h			
	Considerations	***Response***	***Additional comments***
4.e.2	*Predictive model*	Cheese surface at pH 5.4, a_w 0.96, 27°C (80.6°F): PMP 7.0 version 1.1 predicts a 3-log increase in *S. aureus* within 29 h (22 h without lag) under aerobic conditions; ComBase Predictor predicts a 3-log increase in *S. aureus* within 18 h for the same conditions. For *L. monocytogenes*, PMP predicts a 1-log increase within 42 h for the same conditions (7 h without lag); ComBase Predictor with 5,000 ppm of lactic acid predicts a 1-log increase within 33 h for the same conditions. PMP does not include *B. cereus* predictions at a_w 0.96, but ComBase Predictor with 40% CO_2 predicts a 3-log increase in *B. cereus* within 101 h. For the sauce-crust interface (pH 5.0, a_w 0.97), PMP predicts a 3-log increase in *S. aureus* within 34 h (approximately 23 h without lag) under aerobic conditions; ComBase Predictor predicts a 3-log increase in *S. aureus* within 23 h (approximately 16 h without lag) for the same conditions. For *L. monocytogenes,* PMP predicts a 1-log increase within 52 h for the same conditions (approximately 9 h without lag); ComBase Predictor with 5,000 ppm of lactic acid predicts a 1-log increase in 34 h (within 13 h without lag) for the same conditions. PMP predicts a 3-log increase in *B. cereus* in approximately 21 h (8.5 h without lag); ComBase Predictor with 40% CO_2 predicts a 3-log increase in *B. cereus* in 85 h (in 41 h without lag) for the same conditions. For the cheese-sauce interface (pH 5.3, a_w 0.98), PMP predicts a 3-log increase in *S. aureus* within 22 h (approximately 16 h without lag) under aerobic conditions; ComBase Predictor predicts a 3-log increase in *S. aureus* within 15 h (10 h without lag) for the same conditions. For *L. monocytogenes,* PMP predicts a 1-log increase within 22 h for the same conditions (approximately 5 h without lag); ComBase Predictor with 5,000 ppm of lactic acid predicts a 1-log increase in 19 h (within 8 h without lag) for the same conditions. PMP predicts a 3-log increase in *B. cereus* in approximately 15 h (10 h without lag); ComBase Predictor with 40% CO_2 predicts a 3-log increase in *B. cereus* within 42 h (21 h without lag).	
4.e.3	*Compare choice with literature*		
4.e.4	*Any further information on growth or survival?*		

APPENDIX J. *Continued*

Evaluation of baked cheese pizza held out of refrigeration for up to 8 h			
	Considerations	***Response***	***Additional comments***
4.e.5	*Based on the above analysis, what challenge organisms are chosen for growth inhibition studies?*	*L. monocytogenes, S. aureus,* and *B. cereus.*	Modeling results suggest that *L. monocytogenes, S. aureus,* and *B. cereus* are all likely candidates for a challenge study, and none could be completely excluded from consideration based on modeling alone.
4.f	***If inactivation studies:***	NA	
4.f.1	*What is the lethal treatment? (HPP, heat, acid, etc.)*		
4.f.2	*Which microorganisms are most resistant to the lethal treatment? (HPP, heat, acid, etc.)*		
4.f.3	*Will the lethality be delivered to all areas of the product that may contain the pathogen? Account for all surface and internalized contamination.*		
4.f.4	*What is in the formulation that may affect inactivation? (Intrinsic factors may contribute to lethality or resistance: a_w, moisture, salt, pH, fat, etc.)*		
4.f.5	*Are there any data on pathogen levels in the product?*		
4.f.6	*Is there a regulatory requirement or policy for log reduction for this product? Cite requirement.*		
4.f.7	*If there is no regulatory requirement for log reduction, use scientific basis for determining acceptable reduction (21, 76).*		
4.f.8	*Based on the above analysis, what challenge organisms are chosen for inactivation studies?*		
5	**Determine appropriate time and sampling intervals for challenge study**		
5.a	***For growth inhibition (TCS) studies, use 1.25–1.5 times the shelf life as testing time***	10 h × 1.5 = 15 h.	
5.a.1	*Maximum time from production to consumption*	Maximum 10 h.	
5.a.2	*Actual time to spoilage or unacceptable quality*	NA	
5.a.3	*For growth inhibition studies, determine appropriate sampling intervals for microbial analysis; use five to seven (preferred) sampling intervals; fewer sampling intervals should be justified, e.g., using results from similar products.*	Sample at 0, 4, 8, 10, 15 h.	

APPENDIX J. *Continued*

Evaluation of baked cheese pizza held out of refrigeration for up to 8 h			
	Considerations	***Response***	***Additional comments***
5.b	***For inactivation studies determine appropriate sampling points considering the process and formulation.*** *Identify populations at 0 time and end of processing; whenever possible, include intermediate sampling intervals to determine death curve.*	NA	
5.b.1	*When inactivation treatments may result in sublethal injury, repair and growth of microorganisms during product shelf life should be considered (21).*		
6	**Determine inoculation, storage, and testing procedures**		
6.a	***Determine strains for use in study (multiple strains for each species are recommended; consider use of appropriate food or clinical isolates)***	Multistrain mixtures for *L. monocytogenes, S. aureus,* and *B. cereus* will be used. Each pathogen composite will be tested individually (i.e., inoculate one set of samples with *L. monocytogenes* composite, inoculate a different set of samples with *S. aureus* composite, etc.).	
6.b	***Determine if adaptation is required for inoculum preparation***	No.	Although sauce pH is low, *L. monocytogenes* comes from the environment and would not be adapted to acid. Adaptation is not a concern for *S. aureus* or *B. cereus.*
6.c	***Determine method of inoculation (surface, mixing, dipping, liquid, dry, etc.)***	Slice an entire pizza into 16 individual slices (approximately 75 g each). (Assume that the pizza is approximately 1,200 g.) Individual slices of pizza will be inoculated on the surface and the sliced edge with *S. aureus, L. monocytogenes,* or *B. cereus.*	Each replicate will require 10 inoculated slices (2 for each sampling time interval) and 5 control slices for each organism tested.
6.d	***Determine size of inoculum (populations, e.g., log CFU/g, CFU per package, percentage of inoculum vol/wt or vol/vol)***	Not less than 2 log CFU/g for *L. monocytogenes, S. aureus,* or *B. cereus,* surface inoculated, including the cut surface, delivered by spot inoculation (several 50-μl spots). As noted above, each organism will be inoculated independently to avoid possible antagonist effect between different organisms.	Inoculum level is high considering the likelihood of contamination but will allow enumeration by direct plating and detection of growth and low levels of inactivation by formulation during storage; inoculum volume no more than 1% of sample size; preliminary data suggest inoculum does not change pH and a_w appreciably.
6.e	***Determine packaging to be used***	Product is not packaged during typical display but should be protected from the environment during the study by placing in a cardboard or plastic pizza container with a loose fitting lid.	
6.f	***Determine the incubation temperature for growth inhibition studies or temperature(s) for thermal inactivation studies***	Incubated at 30°C (86°F).	Maximum temperature product will be exposed without adverse changes in product quality that would deter purchase and consumption.
6.g	***Determine sampling method and sample size***	Analyze an entire slice of pizza (approximately 75 g).	Slices will be tested for *S. aureus, B. cereus,* and *L. monocytogenes* according to methods provided in Appendix A.

APPENDIX J. *Continued*

Evaluation of baked cheese pizza held out of refrigeration for up to 8 h			
	Considerations	***Response***	***Additional comments***
6.h	***How many replicates are needed to ensure confidence in data?*** *Does variability in proximate analysis or production warrant more than two or three replicate trials? Will multiple variations of similar formulations be tested? Has a statistical design for choosing formulations been used (block design, central composite, etc.)?*	Three replicate (unique production) lots (i.e., three whole pizzas) per organism tested; duplicate samples (slices) per testing interval.	Greatest variability likely occurs in the production of different lots of pizza.
7	**Determine other controls**		
7.a	***Is use of surrogates appropriate or necessary? If so, justify.***	No surrogates used.	
7.b	***Are uninoculated controls needed to assess spoilage or competitive microflora or for other purposes?***	Uninoculated controls will be used to monitor other spoilage microorganisms that can change pH during testing interval.	
7.c	***What other controls are necessary (including negative or positive growth controls)?***		
8	**Determine pass-fail criteria**		
8.a	***What are the pass-fail criteria?***	No more than a 1-log increase for *L. monocytogenes*; no more than a 3-log increase for *S. aureus* or *B. cereus*.	A 1-log increase in *L. monocytogenes* is considered significant growth, but note that *L. monocytogenes* detectable in 25 g of a ready-to-eat food would render the product adulterated. Maximum 3-log increase selected for *S. aureus* and *B. cereus* are based on increases suggested in the IFT report *(53)*. Some regulatory agencies may consider a lower log increase to be actionable.
8.b	***What are the limits for use of the results?***	These results are applicable only to cheese pizza with tomato sauce and not to pizza containing meat or vegetable toppings.	Minor variations in the amount of cheese or tomato sauce are not likely to have a significant impact on growth of the test organisms.

[a] NA, not applicable.

doi:10.4315/0362-028X.74.4.522

ERRATUM

In the article ''Parameters for Determining Inoculated Pack/Challenge Study Protocols,'' by the National Advisory Committee on Microbiological Criteria for Foods, which appeared in the *Journal of Food Protection,* 73(1):140–202, there are additions that need to be made to Table 2 on p. 144.

For an a_w value of >0.96 and a pH value of 4.2–4.6, *L. monocytogenes* should be added to the list of potential pathogens so that it includes Pathogenic *E. coli, Salmonella, S. aureus,* and *L. monocytogenes*.[d]

The footnote designator *d* should be added to all three *L. monocytogenes* entries in the pH 4.2–4.6 column and the following footnote added: [d] *L. monocytogenes* should not be used if the pH of the product is <4.4.