National Advisory Committee on Microbiological Criteria for Foods

Parameters for Determining Inoculated Pack/Challenge Study Protocols

Final Draft Document

March 19, 2009

Abstract

The National Advisory Committee on Microbiological Criteria for Foods (NACMCF or Committee) 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.

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Scope of Document

This document was prepared at the request of the sponsoring agencies of the National Advisory Committee on Microbiological Criteria for 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. 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 food (TCS food), the National Advisory Committee on Microbiological Criteria for Foods (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 one(s) 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 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, 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 U.S., and their suppliers routinely use inoculation/challenge testing to determine whether a specific food requires time-temperature control for safety (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 Food and Drug Administration (FDA) Food Code.

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.

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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 product handling is under appropriate control using a 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 un-refrigerated; sliced pasteurized processed cheese held at ambient temperature for more than 4 hours; certain cheeses held unrefrigerated; *etc*. 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 potentially hazardous food (PHF) or time/temperature control for safety food (TCS food) was amended in the 2005 FDA Food Code to include pH and a_w interaction tables, allowing the hurdle concept to be used in the determination of whether TCS is necessary (Chapter 1 Definitions, 2005 FDA Food Code, available at http://www.cfsan.fda.gov/~dms/fc05-toc.html). The two interaction tables, as well as a decision making framework were developed by the Institute of Food Technologists (IFT) and provided to FDA in the report, "Definition and Evaluation of Potentially Hazardous Food," December 31, 2001, IFT/FDA Contract No. 223-98-2333, Task Order No. 4 (available at http://www.cfsan.fda.gov/~comm/ift4-toc.html). 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 with its recommendations to FDA left a number of unanswered questions regarding the understanding and implementation of a product assessment when pH and a_w are unable to determine if TCS is required. This was confirmed in a 2005 survey of stakeholders conducted by the Conference for Food Protection (CFP).

THE COMMITTEE'S RESPONSE

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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 time/temperature control for safety (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 that can affect the validity and interpretability of results. However, due to resource constraints, this is generally addressed through the use of worst case scenarios, which should provide conservative results. Although this document encompasses a variety of sources, those 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.

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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 vary 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 the hazard analysis of the product.

Food safety-related challenge studies include the following:

Pathogen growth inhibition study – a study that evaluates 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).

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Pathogen inactivation study – a study that evaluates 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 study - These studies may be combined 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

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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 (4). 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 (4,10). In 2000, FDA requested 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 if time/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 (10). This concept was subsequently adopted as the basis for defining when foods need refrigeration or some other form of time/temperature control in FDA's 2005 Model Food Code (16). 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 to control growth of various pathogens can be found in the literature, e.g., International Commission on Microbiological Specifications for Foods (ICMSF) *Microorganisms in Foods 5*, *Characteristics of Microbial Pathogens* (15). 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 (1). For example, there would be no need to assess whether a product with a pH of 3.5 supports growth of *Salmonella*, since 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* (75). Determination of the need for a challenge study is referred to as "product assessment" in the IFT and Food Code tables (10, 16).

When growth inhibition occurs due to factors other than, or in addition to, pH and a_w, such as the addition of preservatives, e.g., lactate and diacetate, the literature may provide information relevant to the pathogen and food product. However, it is necessary to ensure 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 (33, 38) and sorbate (45, 85). Conversely, a low pH may potentiate the activity of antimicrobials such as sorbate and benzoate (35). 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 "product assessment" is needed have a long history of safe use. However, safe history of a food product is only relevant if 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 (21, 43, 44).

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 (20). Parameters that should be considered when designing a microbial challenge study are outlined below (1, 4, 6, 10, 20).

1.0 Obtaining expert advice and identifying a laboratory

2.0 Type of study

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. If 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 as well as have it

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reviewed by the regulatory body or intended recipient of the study. Suggested modifications can then be incorporated before the study is executed.

Choosing a laboratory requires careful consideration as 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 personnel are experienced and qualified (Table 1) to conduct the types of analyses needed for the challenge studies and 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 references listed in Appendix A. In situations where approved methods are not available or applicable, labs 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.

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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. Food and Drug Administration (FDA), US Department of Agriculture/Food Safety and Inspection Service (USDA/FSIS), state laws based on the Pasteurized Milk Ordinance (PMO), etc.].

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2.1 Growth inhibition studies

The objective of a growth study may be to request exemption (variance) from TCS or from other requirements defined by the Food Code, PMO, 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 if formulation or processing changes are required if 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 if 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 *E. coli* O157:H7 in fermented, dry sausage) (86). Inactivation studies may also be used to determine if non-thermal technologies or if 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 processed meat manufacturer wishing to have a product line classified as Alternative 1 for control of *Listeria monocytogenes* by FSIS regulation (9 CFR 430) may undertake a study to demonstrate a 2-log post-lethality 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 (68ºF) for 3 days, as well as no recovery or growth of the pathogen during ambient-temperature storage for 1 year.

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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 on product variability below).

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Multi-component 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., the measurements that 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.

Wherever 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/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 to measure and control by the producer than a_w 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, since pathogens may have increased heat resistance under these conditions (11, 12, 28, 34, 87). Similarly, increased solute content has been shown to protect *L. monocytogenes* against high hydrostatic pressure (64, 65). 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 it 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 (49, 50, 52, 62, 63). As a result, if the challenge study was conducted on a product formulated with acetic acid (vinegar) it may not be valid for a reformulated product containing citric acid (lemon juice) even if the final pH is 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, within the first 10% of its shelf-life should be used; for example, if 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 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 (66).

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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/FDA report on Evaluation and Definition of Potentially Hazardous Foods (10); specifically, see Table 1, Table A, Table B, Table 4-1, and Table 6-1. 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, since 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*, due to epidemiological attribution, while 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 *S. aureus* for a food with a pH of 4.3 and a_w of 0.98 because it is generally considered to be more resistant to acid.

Ideally, in 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 in 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 it may be considered a better choice for a challenge study among the organisms of concern for that product.

While Table 2 is similar to Table B in the Food Code (Appendix D) and the IFT report (10), 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 and the Food Code are specifically focused on foods that require temperature control for safety, while the focus of this document is broader. Finally, this report 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*. While *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 in identifying appropriate pathogens of concern for particular pH and a_w combinations. However, it should not typically be used for the selection of organisms for use in process inactivation (e.g., thermal) 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, non-proteolytic 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 non-proteolytic *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-plant, where the introduction of the pathogen would pose an unacceptable risk. Surrogates may also be useful to select the study parameters before conducting the full study with the pathogen. Care should be taken when using surrogates for in-plant challenge studies, as they 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 (53).

Clostridium sporogenes 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 to reduce the number of formulations to be verified using *C. botulinum* because they 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 product for inhibition of botulinum toxin production (41). Other examples of surrogate/pathogen pairs include *Listeria innocua/L. monocytogenes* (27) and non-pathogenic *Escherichia coli/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 (24), so while *C. sporogenes* may be the preferred surrogate for *C. botulinum* for canning

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processes, another organism, such as *Bacillus amyloliquefaciens* may be appropriate as a surrogate for *C. botulinum* for high hydrostatic pressure studies (24, 25).

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 strains either individually or in combination (1, 3, 10). Where there is considerable variability among strains or if 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. cocktail) of a given pathogen is preferred, as it will help to encompass the variability among organisms and may reduce the number of required tests. Prior to the use in the study, the strains selected should be screened for antagonism that can be caused by production of bacteriocins or other antimicrobial factors (10). 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 conduct the challenge studies using that single strain (1, 20). 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 (72). 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. The determination of 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 to confirm 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 which may be encountered during the challenge study.

Isolates should be appropriate for the food product being challenged (1, 4, 10). This includes using isolates from the food type, the food processing environment and from 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 (11, 12, 13, 24). 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, atmosphere, etc.).

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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 (1).

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, since this allows enumeration by direct plating (1, 10). Lower concentrations may be used if documentation of low levels of natural contamination exists, as this will more accurately represent the product's ability to support growth (1). 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 (e.g., from three to six samples) analyzed, 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 or concentration 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 size, leading to the incorrect conclusion that the formulation does not inhibit growth (4, 10). 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 (56, 57). In contrast, a high inoculum level of vegetative cells (e.g. 5-7 log CFU/g) in a growth study may also mimic the population nearing stationary phase. This may result in an apparent no-growth or low-growth observation.

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5.2 Inactivation studies

When conducting inactivation studies, high numbers of organisms are typically used, e.g., 6 to 7 log CFU/g (1, 10), 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); 4-log reduction for treatment of almonds (7 CFR 981) to inactivate *Salmonella*; 7-log reduction for *Salmonella* in poultry products (9 CFR 381.150). 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 in pathogen inactivation during storage of a food product. In the former case, relatively large 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, as 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 (17, 54, 55) 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, and 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 non-selective growth medium. Working cultures, e.g., refrigerated slants, may be prepared and used for a period of time (e.g., 7-30 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 (1). AOAC International Guidelines for Laboratories indicate there should be not more than five passages from the primary reference material (14). In some instances even fewer transfers may be appropriate, as organisms may readily lose extrachromosomal elements such as plasmids and phage.

For challenge studies using vegetative cells, stationary phase (18-24 h) cells grown on non-selective media under conditions suitable for optimal growth of the specific challenge culture should generally be used (10). 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 (22, 37, 42, 100), which can often be accomplished by growing the culture in tryptic soy broth with 1% glucose (18, 19). Cold adaptation at 7-8°C (44.6 – 46.4ºF) for 7 days may reduce the lag phase for pathogens (5), 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 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 (55), or acid stressing cells prior to a heat treatment (23).

For inactivation studies, cells that are grown at greater than optimum temperatures may become more resistant to heat than cells grown at optimal temperatures (72, 99). Increased heat resistance can also be observed with brief exposure to sublethal temperatures (heat shock) (69, 70, 71). 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

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under specific growth conditions or by turbidity measurements (e.g., optical density, McFarland Standards).

Spores of pathogens such as *C. botulinum, C. perfringens* and *B. cereus* can be prepared, washed and suspended in sterile water and frozen, preferably at -20°C (-4ºF) 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 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/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). Where 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 bacteriocidal ingredients such as nisin or other commercial fermentation byproducts 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 fermentate or nisin (31, 32).

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 (4, 10), or dried on a product similar to the challenge food (10). When preparing a dehydrated inoculum, the organism may require several days to months to stabilize (e.g., *Salmonella* in skim milk powder, 88). 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/FDA report (10). 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, 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 (10). 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 Storage conditions – packaging). Ideally, product should be first inoculated and then packaged under 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 for 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 are considered.

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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 the 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 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 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 post-process 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 modified atmospheres, 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 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 (10). 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 should be conducted at 7°C (44.6ºF) to account for expected consumer storage temperature in the United States (3). Refrigerated studies may incorporate additional temperatures (e.g., 4-6°C or 10-12°C) (39.2-42.8ºF or 50-53.6ºF) 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 (1, 9).

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 (10). For shelf stable products, typical temperatures range from 24 to 35°C (75.2 to 95ºF) depending on expected storage room temperatures (9). 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 (4).

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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 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 (2). For end-point lethality determination, 5-10 samples per time interval may be appropriate. If supporting data from other studies exist, the need for replication may be reduced (1). 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.

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The sample preparation method should be selected based on the type/properties of the food and the method of inoculation, which depend on the food product and the inoculation procedure (1, 10). 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/sponged, washed/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 (Section 9.2). 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 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.

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Caution should be exercised when considering analysis of composited samples in challenge studies. Compositing multiple samples for pathogen enumeration eliminates detection of variability among discreet samples and may reduce sensitivity of the analysis. Furthermore, composited samples may dilute toxins to less than detectable levels if 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 which will later allow one to identify 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 their metabolic activity to lead to detectable colonies or other measurements indicating activity and leading to 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 cells. Enrichment procedures for the target pathogen should be considered at time points where 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.

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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 it 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; they include volume or weight, surface area, composition, and properties (e.g., pH) (20).

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For growth studies, pathogens should be enumerated on appropriate selective agar (see Appendix A). Inactivation studies may result in injured cells where direct plating 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 non-selective media such as tryptic soy agar (TSA) or Plate Count Agar overlaid with selective agar after 2 to 4 h incubation at optimum temperature (29, 36); by using selective agar overlaid with non-selective agar (47); by using agar underlay techniques (64, 65); or by replica plating from a non-selective agar such as TSA to selective agar (46). 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 their changes during product shelf-life (1, 10). 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

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874 populations. Thus it is recommended that microbiological numbers such as aerobic plate count
875 and spoilage organisms typical for the product (e.g., lactic acid bacteria or yeast and mold) be
876 obtained. Testing for these or other indicator microorganisms cannot substitute for pathogen
877 testing. In addition, the presence or absence of spoilage bacteria cannot be used as an indicator of
878 safety.
879
880 Lactic acid bacteria (LAB) are expected in fermented or cultured food products at relatively high
881 populations (e.g., 6 log CFU/g), but indigenous populations are low in most processed foods.
882 This group of bacteria is known to compete well with low levels of pathogens for nutrients, can
883 grow over a wide range of temperatures, can reduce the pH of the food through acid production,
884 and some strains can produce bacteriocins that may inhibit some pathogens. Relying on the
885 presence of naturally-occurring background levels of LAB in foods is an unreliable method to
886 control pathogens. Conversely, competitive microflora may inhibit growth of specific
887 pathogens, and failure to account for this interaction could lead to erroneous conclusions. Thus,
888 it may be important in some circumstances to monitor LAB growth during the challenge study to
889 determine if competition may contribute to inhibition of pathogens during the trial.
890
891 Although they may be present, molds and yeasts may not be initially visible on the food.
892 Deamination of food proteins by molds can produce ammonia and a localized increase in pH that
893 can increase the potential for pathogen growth in that microenvironment (61). Populations of
894 molds and yeasts can be enumerated by using a variety of selective plating media or by other
895 validated procedures.
896
897 *9.4. Determination of physical parameters*
898 Food properties such as proximate composition (protein, fat, moisture), pH, titratable acidity, a_w,
899 salt content and residual nitrite can influence the behavior of pathogens. It may be important to
900 measure these factors as part of the challenge study. Some parameters that may change during
901 the study, such as pH, may need to be monitored at appropriate points throughout the study in
902 parallel with microbial analysis. Sources of appropriate methods can be found in Appendix A.
903 The number of samples to be analyzed is described in section 9.1 above.
904
905 Changes in pH can be an indicator of microbial metabolism when microbial populations are not
906 enumerated or if growth is not significant. The pH of foods that are homogeneous and likely
907 have consistent pH throughout the matrix can be measured on a representative sample. In
908 contrast, complex foods consisting of multiple discreet components or ingredients may require
909 multiple pH measurements. For example, a sandwich may require measuring the surface or
910 interface pH of the components in addition to a homogenized sample.
911
912 For obvious safety reasons, no sensory assessment other than changes in appearance (phase
913 separation, turbidity, texture, gas formation) should be performed on challenge test samples. In
914 some instances, the investigator should make a judgment if the product would be considered
915 "edible" based on visual and olfactory observations. Note that because pathogens or toxins may
916 be present, olfactory observations may constitute unacceptable risk to the laboratory worker.
917
918 **10.0 Duration of study and sampling intervals**

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Challenge studies should be conducted for at least the intended shelf-life of the product (6, 9. 10). 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 (10). Depending on the shelf-life of the product, this may be 25% (e.g., for products with shelf-life of 3-6 months) to 50% (e.g., for products with shelf-life of 7-10 days) longer than the intended shelf-life of the food (1, 10). 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 (10). 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 (10). 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 (10).

A growth inhibition study may be ended when there is greater than a 1- to 2-log increase in pathogen growth or toxin is detected in samples for two consecutive sampling intervals (indicating growth of the pathogen of concern) or if 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., TDT 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 (1) or produce toxin in the product over time. Alternatively, attempts to recover the pathogen in non-inhibitory 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 (1, 4, 10). 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; if this is followed by growth that does not exceed the target inoculum level, this growth may not be recognized; this may be addressed by allowing a brief equilibration time (e.g., 2 h) for the

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inoculum in the product prior to conducting the initial count (10). Normal sample variation may result in a spike at a sampling interval that may not be significant (6); this 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 (10). 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 one log cycle over two or more time intervals is generally considered significant by food microbiologists (6). 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 (1, 10). This reflects the inherent variation that exists with enumeration of microorganisms (10, 67).

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, 48) 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 (101, 102), which is determined by doubling the "reproducibility standard deviation." It should be noted however that the reproducibility standard deviation can vary. Scotter et al. (89) conducted tests to validate the 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 is simply a product of the variation seen in enumeration studies (1).

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 (8). No toxin should be detected in the product over the duration of the challenge study (10). In lieu of testing for *Staphylococcus* enterotoxins, limiting growth of *S. aureus* to less than 3 log CFU/g may be used (10). 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

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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 (1).

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 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 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 currently available, which one(s) are most suitable for use and what are the limitations of these models?

Predictive food microbiology is a sub-discipline 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 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 to narrow 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/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 other relevant published studies.

The two best known multi-pathogen multi-factor models available today are the USDA Agricultural Research Service (ARS) Eastern Regional Research Center (ERRC) Pathogen Modeling Program, PMP (http://ars.usda.gov/Services/docs.htm?docid=11550), and the ComBase Predictor, CBP (http://www.combase.cc/predictor.html), formerly known as 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 (Table 4). Elements of both models have been validated (by both published and unpublished studies) to a limited degree in different food systems.

There are 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 (http://combase.arserrc.gov/BrowserHome/SearchOptions/Search.aspx). 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) from culture media. Researchers publishing microbial growth or survival data are requested and encouraged to submit the data to ComBase (http://www.combase.cc/faq.html). 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 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 in 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

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to growth or survival than those in the food product under consideration, then additional challenge studies may not be needed (7). 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.

4. If 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 (10) indicating that both the American Bakers Association (ABA) and the NSF International (NSF) testing protocols suffer from significant weaknesses. These are briefly highlighted below; for more details, see Table 2 in the IFT report (10) 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/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 less than 24 h or more than 31 days). Water activity 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 multi-component 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 82.2C (180ºF) at the

coolest point] with respect to the destruction of naturally-occurring microorganisms, both pathogenic and non-pathogenic. 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, since it 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 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/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. (4) 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, technical expertise may be required to interpret the adequacy of studies following these guidelines.

Scott et al. (1) published guidelines for conducting *L. monocytogenes* challenge tests for foods. This paper 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 paper in large part applies the recommendations in the IFT report to challenge studies involving *L. monocytogenes*, and are 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 this document and are appropriate for *L. monocytogenes* in refrigerated ready-to-eat foods.

AFSSA, an 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 (48). Similar to Scott et al. (1), 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 document also provides information on how to interpret the results obtained against *L. monocytogenes* regulatory criteria (EU) 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,

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adapting the challenge organisms, simulating natural conditions when inoculating product, etc. The protocol indicates that to assess growth potential samples need only be taken 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.

NACMCF has provided guidance for conducting microbial challenge tests in several documents. In 1990, NACMCF (2) 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. While 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 published a paper (3) 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 are the most comprehensive, broad-based documents that can be applied to assess the adequacy of microbial challenge studies. Because they 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 the five food products. See Appendix E.

The examples in Appendix E 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

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

	Design	Conduct[2]	Evaluate
Knowledge and skills	Knowledge of food products and pathogens likely to be encountered in different foods. Knowledge in 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.[3]	Knowledge of basic microbiological techniques. Able to work using aseptic technique, ability to perform serial dilutions, able to work at biosafety level 2 (84).	Knowledge of food products and pathogens likely to be encountered in different foods. Knowledge in the fundamental microbial ecology of foods, factors that influence microbial behavior in foods, and quantitative aspects of microbiology. Knowledge of statistical analysis.[3]
Education and training	Ph.D. in Food Science, Microbiology or a related degree/field or an equivalent combination of education and experience.	B.S. in Food Science, Microbiology, a related degree 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 degree/field or an equivalent combination of education and experience.
Experience	Two years of experience in conducting challenge studies independently and experience in design of challenge studies under the guidance of an expert food microbiologist.	Two years of experience in conducting challenge studies is useful, however close supervision by an expert food microbiologist may substitute.	Two years of experience in 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.

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[1]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's food microbiologists through their Regional Retail Food specialist.

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

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

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Table 2. Potential pathogens[1] of concern for growth studies based on interaction of product pH and a_w[2].

a_w values	pH values					
	<3.9	3.9 - <4.2	4.2 – 4.6	>4.6 – 5.0	>5.0 – 5.4	>5.4
< 0.88	NG[3]	NG	NG	NG	NG	NG
0.88 – 0.90	NG	NG	NG	NG	*S. 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*	*B. cereus* *C. 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* *V. 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*

[1]*Campylobacter* spp., *Shigella*, and *Yersinia enterocolitica* do not appear in this table because they are typically controlled when the pathogens in the table are addressed.

[2]Data are based on the PMP, ComBase Predictor, ComBase Database, or peer reviewed publications (51,59, 60).
[3]Where no pathogen growth expected, formulation or process inactivation studies may still be needed.

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

Model name	URL	Applicability
American Meat Institute Process Lethality Determination Spreadsheet	http://www.amif.org/ht/d/sp/i/1870/pid/1870	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	http://www.combase.cc/toolbox.html	ComBase Predictor models are based on observations made in culture media, and comprise a set of 20 growth models, seven thermal death models and two non-thermal 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	http://www.meathaccp.wisc.edu/ibm.htm	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)	http://cbnfri.dc.affrc.go.jp/	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 by actual ComBase data. Contours of any two of three variables (temperature, pH and water activity) can be visualized, while the third is held constant.
OptiForm *Listeria* Control Model 2007	http://www.purac.com/purac_com/a5348511153c582f5bd69fd6bd64bb49.php	The model predicts *Listeria* growth based on 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	http://ars.usda.gov/Services/docs.htm?docid=11550	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.

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Perfringens Predictor	http://www.ifr.ac.uk/safety/growthpredictor/	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 (68, Schaffner, personal communication).
Seafood Safety and Spoilage Predictor, SSSP v 3.0	http://sssp.dtuaqua.dk/	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 *Listeria monocytogenes* and lactic acid bacteria in lightly preserved seafood, and a model to predict the growth boundary of *L. monocytogenes* in lightly preserved seafood

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Table 4. Pathogen growth ranges used in ComBase and Pathogen Modeling Programs[1].

	ComBase[2]					**PMP[3]**				
	Temperature (°C)		pH		a_w	Temperature (°C)		pH		a_w
	Min	Max	Min	Max	Min	Min	Max	Min	Max	Min
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
non-proteolytic	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 spp.										
with CO_2	7	30	3.9	7.4	0.973					
aerobic						10	30	5.6	6.8	0.974

[1]Limits tested in ComBase and PMP do not necessarily represent limits for growth. See Table 5 for growth limits.
[2]ComBase, http://combase.arserrc.gov/
[3]PMP, Pathogen Modeling Program http://ars.usda.gov/Services/docs.htm?docid=11550

Table 5. Limits for growth when other conditions are near optimum (based on references 15 and 58).

Pathogen	Source	Temperature (ºC)		pH		a_w	Water Phase NaCl (%)
		Min	Max	Min	Max	Min	Max
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)	FDA	10	48	4.6	9	0.93	10
(Proteolytic)	ICMSF	10 - 12		4.6		0.93	10
C. botulinum (growth only)	FDA	3.3	45	5	9	0.97	5
(Non-proteolytic)	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[3]	46.2	3.8	9.5	0.94	

[1]US FDA CFSAN. 2001. (58)
[2]ICMSF. 1998. (15)
[3]Most serovars will not grow below 7°C (44.6ºF).

APPENDIX A

Sources of Accepted Laboratory Methods*

- American Public Health Association. 2001. Compendium of Methods for the Microbiological Examination of Foods, 4th ed., Washington, D.C.
- American Public Health Association. 2004. Standard methods for the examination of dairy products. 2004. 17th ed. Washington, D.C.
- Association of Official Analytical Chemists. 2007. Official Methods of Analysis, 18th ed., Revision 2, Arlington, VA.
- Health Canada. 2008. The compendium of analytical methods. http://www.hc-sc.gc.ca/fn-an/res-rech/analy-meth/microbio/index-eng.php
- International Organization for Standardization (ISO) International Standards. 67.050: General methods of tests and analysis for food products. Listing of standards accessed at: http://www.iso.org/iso/catalogue_ics_browse?ICS1=67&ICS2=050&
- United States Department of Agriculture, Food Safety Inspection Service. 1998. Microbiology Laboratory Guidebook Accessed at: http://www.fsis.usda.gov/Science/Microbiological_Lab_Guidebook/index.asp.
- United States Food and Drug Administration. 2001. Bacteriological Analytical Manual. Accessed at: http://www.cfsan.fda.gov/~ebam/bam-toc.html

*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 in 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 their processes and results, e.g., appropriate positive and negative controls; 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?

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Does the laboratory have a corrective action plan to address quality control issues within the laboratory operations?¶
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? Do the backgrounds meet the qualifications of an expert microbiologist and technicians as described in Table 1?¶

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If the protocol involves testing for a select agent (e.g., *C. botulinum* or botulinum toxin), is the laboratory approved to work with that particular agent? In the U.S., 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)[1]

Product Category[2] (examples of possible foods for evaluation)	Pathogens of Concern (in alphabetical order)	Examples of Process Control[3] (alone and in combination, in alphabetical order)
Meat and poultry - cooked (e.g., roast beef, deli-style turkey, ham)	*C. botulinum* and *C. perfringens*, enterohemorrhagic *E. coli*, *L. monocytogenes*, *Salmonella*, *S. aureus*	Cooling rate, heat treatment[4], 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 ≤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 ≤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

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Cheese and cheese products (e.g., natural Swiss cheese, process cheese slices, process 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*, *Y. 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, condiments (e.g., garlic-in-oil)[5]	*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*[6]	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

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[1] Adapted from reference 10, Tables 4-1 and 6-1.
[2]Combinations of products, storage in modified atmosphere and use of novel preservatives or processes require special consideration.

[3] Good Agricultural Practices where appropriate, and Good Manufacturing Practices and Hazard Analysis and Critical Control Point principles would help in reducing the hazards.
[4]Heat treatment means processes such as cooking, pasteurization and other thermal processes intended to inactivate pathogens
[5] Only a concern in anoxic environments.
[6]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 (www.cfsan.fda.gov/~ear/fc05-toc.html). 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 only offers 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 is sold or offered for human consumption;

(f) A kitchen in a private home, such as a small family day-care 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 (time/temperature control for safety 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, a food that because of the interaction of its a_w and pH values is designated as Product Assessment Required (PA) in Food Code Table A or B of this definition:

Table A. Interaction of pH and A_w for control of spores in food heat-treated to destroy vegetative cells and subsequently packaged.

a_w values	pH values		
	4.6 or less	> 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

*PHF means potentially hazardous food
**TCS food means time/temperature control for safety food
***PA means Product Assessment is required

Table B. Interaction of pH and a_w for control of vegetative cells and spores in food not heat-treated or heat-treated but not packaged.

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

*PHF means potentially hazardous food
**TCS food means time/temperature control for safety food
***PA means Product Assessment required

(3) Potentially hazardous food (time/temperature control for safety 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 s*almonellae*;

(b) A food in an unopened hermetically sealed container that is commercially processed to achieve and maintain commercial sterility under conditions of non-refrigerated storage and distribution;

(c) A food that because of its pH or a_w value, or interaction of a_w and pH values, is designated as a non-PHF/non-TCS food in Table A or B of this definition;

(d) A food that is designated as Product Assessment Required (PA) in Table A or 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 even though the food may contain a pathogenic microorganism or chemical or physical contaminant at a level sufficient to cause illness or injury.

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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: 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) in the Food Code; or

(b) Is a raw or partially cooked animal FOOD and the consumer is advised as specified in Subparagraphs 3-401.11(D)(1) and (2) in the Food Code; or

(c) Is prepared in accordance with a variance that is granted as specified in Subparagraphs 3-401.11(D) and (3) in the Food Code; 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 under Section 3-401.11 or 3-401.12, or frozen as specified under Section 3-402.11 in the Food Code;

(b) Raw fruits and vegetables that are washed as specified under Section 3-302.15 in the Food Code;

(c) Fruits and vegetables that are cooked for hot holding, as specified under Section 3-401.13 in the Food Code;

(d) All potentially hazardous food (time/temperature control for safety food) that is cooked to the temperature and time required for the specific food under Subpart 3-401 and cooled as specified under Section 3-501.14 in the Food Code;

(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 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, non-respiring food, and impermeable packaging material;

(d) Cook chill packaging, in which cooked food is hot filled into impermeable bags which have the air expelled and are then sealed or crimped closed. 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 Checklists

Table 5: Evaluation of Mozzarella slices packaged under MAP and stored at ambient temperatures for up to 2 weeks 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)***	N/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 weeks; Food Code variance.	
1.c	***Validate lethality***	N/A (used pasteurized milk in production of cheese).	
1.d	***Verify that formulation will inhibit microbial growth in refrigerated foods or under mild temperature abuse***	N/A	
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/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. (97)	
2.a.2	*What are the pH, a_w, and proximate analysis (moisture,*	pH 5.3-5.4; a_w 0.96; Proximate analysis:	Note: a_w is not measured or controlled in typical production but is a function of moisture and salt

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	salt, fat, protein, residual nitrite, etc) for product and/or individual components?	At end of production, 46-52% moisture, 1.0% NaCl, 30% fat. Homogeneous throughout.	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 3 month period if lactic acid bacteria starter cultures are killed by heat used in molding.	The pH will not increase during the two week holding period at 23°C (73°F) at retail.
2.a.4	*If applicable, what are the dimensions of cuts, pieces, etc?*	N/A	
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 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 non-sporeformers 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.	

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 vs. 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/display?*	During cheese production, milk will be cultured and curd cooked at ≤40°C (104°F); curd will be heated	Product quality will deteriorate rapidly if temperature exceeds 23°C (73°F). However, temperatures as high as 27ºC (81ºF), e.g. during

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		to 70°C (158°F) for molding step; cheese cured at 3°C (37°F) for up to 2 weeks and distributed to retailers typically at <7°C (45°F); maximum storage at 23°C (73°F) at retail for 2 weeks.	transportation, will have limited effect on quality if the time does not exceed 4 h.
2.c.3	*What potential is there for storage/display at temperatures greater than those listed above?*	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?*	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 months if stored at refrigeration temperatures, 2 weeks unrefrigerated storage.	
2.c.6	*What is the time to spoilage or unacceptable quality?*	2 weeks unrefrigerated storage if held between 20-23°C (68-73°F); shorter if temperatures exceed 23°C (73°F); 9 months 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.	
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*, and *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)***	*B. cereus* spores survive pasteurization; pathogenic *E. coli*, *L. monocytogenes*, *Salmonella*, and *S. aureus* from post-processing handling. *Salmonella* has been associated with Mozzarella due to contamination during production not post-process contamination; illness associated with survival not growth; no outbreak has been reported with *B. cereus*, *L. monocytogenes*, or *S. aureus* (93).	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 worker's 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 vs. Gram negative; vegetative microorganisms vs. 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 shelf-life, if appropriate.*	Please see 4.e.2, 4.e.3, and 4.e.4.	
4.e.2	*Predictive Model*	At pH 5.4, a_w 0.96, 27°C (80.6ºF): 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 5000 ppm 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.*	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/molding step. If *B. cereus* growth occurred, it would be at a slower rate than *L. monocytogenes* or *S. aureus.*

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		cereus increase within 101 h.	
4.e.3	*Compare choice with literature*	Stecchini et al. (78) indicated a 5-log increase of *L. monocytogenes* when stored at 5°C (41°F) for 21 days. (pH and moisture not reported)	
4.e.4	*Any further information on growth/survival?*	Data presented at IAFP 2003 (94) on cheese shreds for *L. monocytogenes* and *Salmonella* demonstrated no growth on low moisture Mozzarella stored at 15°C (59°F) for 2 months. (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***	N/A	
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 that may contribute to lethality/resistance (a_w, moisture, salt, pH, fat, etc)*		
4.f.5	*Are there any data on pathogen*		

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	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, see reference(7).*		
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 "shelf-life" as testing time***	14 days x 1.5 = 21 days.	
5.a.1	*Maximum time from production to consumption*	Maximum 9 months 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 5-7 (preferred) sampling intervals; fewer sampling intervals should be justified, e.g., using results from similar products.*	Sample 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 7 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*	N/A	

	0-time and end of processing; whenever possible include intermediate sampling intervals to determine death curve		
5.b.1	*When inactivation treatments may result in sublethal injury, repair and growth of microorganisms during product shelf-life should be considered(7).*		
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 3-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; 2 slices/package with inoculum on inner surface between the two slices.	Using 2 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 or CFU/package, percentage of inoculum v/w or v/v)***	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 suggests inoculum does not change pH and a_w appreciably.
6.e	***Determine packaging to be used***	Two inoculated slices will be used per package unit; slices will be	Packaging is the same as commercial product.

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		packaged with 60% nitrogen-40% carbon dioxide mixture and sealed; packaging material will be gas-moisture impermeable.	
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 (2 slices) will be mixed in the bag and 25 g portions removed for microbial analysis; sample will be homogenized with equal volume of 0.1% peptone buffer and serial dilutions plated on selective agar as appropriate per FDA BAM methods.	
6.h	***How many replicates are needed to ensure confidence in data***? *Does variability in proximate analysis/production warrant > 2-3 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	***Are use of surrogates appropriate or necessary? If so, justify.***	Surrogates are not appropriate or necessary.	
7.b	***Are uninoculated controls needed to assess spoilage, competitive microflora, or for other purposes?***	Uninoculated controls will be used to monitor growth of molds/yeasts and other spoilage microorganisms	

		which 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 applies only to Mozzarella with the maximum moisture-pH-temperature-time limits tested in this study.	

Table 6. 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)***	N/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***	N/A	
1.d	***Verify that formulation will inhibit microbial growth in refrigerated foods or under mild temperature abuse***	N/A	
2	**Collect information regarding the product**		
2.a	***What are the ingredients?***	The single ingredient is heads of whole Romaine lettuce which are chopped, 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 (http://www.cfsan.fda.gov/~comm/lacf-phs.html)(80). Water activity in iceberg lettuce is 0.995 to 0.998 and this is assumed to hold true for Romaine (74). The product is very high in water, with minimal amounts of salt, fat or 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 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 x 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. Log CFU/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 one of 50 samples, *Salmonella* in one of 200 samples, *B.*	Data presented here are collected after the lettuce has been washed and cut.

		cereus in one of 10 samples. Generic *E. coli* is generally absent but one of 20 samples had greater than 2 log MPN/g. *L. monocytogenes* was not detected in tests of more than 200 samples.	
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 (96). 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 in water containing wash water sanitizer at label concentrations, spin to remove excess water, chop into approximately 5 x 5 cm pieces.	
2.b.1	*Is the product an assembled (multicomponent) product?*	The product is not assembled, and is not multi-component.	
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 foodservice kitchen	

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		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.	
2.c.2	*What temperatures (and times)*	Product is stored below 5ºC (41°F)	The 8 h starts from the time of preparation

	are expected during production, preparation, and storage/display?	prior to preparation. Preparation takes approximately 2 h per batch, and takes place at room temperature (21.1ºC; 70°F). Product may either be covered with plastic wrap and refrigerated after preparation, or placed at room temperature for sale/consumption.	unless the product will be rapidly cooled to 5ºC (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/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 21.1ºC (70°F) but can in some cases increase to 23.9ºC (75°F) for short periods of time.	
2.c.4	*Are there other hazards that may be created by preparation/storage/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.***	The product is clearly: "PA 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	

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		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 *Yersinia 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 (91), *L. monocytogenes* was excluded based on lack of epidemiological evidence (92) 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.	
4. e	***For growth inhibition (TCS studies):***		

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4.e.1	*Which pathogen(s) will grow the fastest? Consider Gram positive vs. Gram negative; vegetative microorganisms vs. 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 shelf-life, if appropriate.*	See response to 4.e.2 and 4.e.3.	
4.e.2	*Predictive Model*	A temperature of 21°C (69.8ºF), 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	

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		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 (77, 79, 83). The four data points were fit to a simple literature-based model and growth rate at 21°C (69.8ºF) was estimated. The literature-based model predicted about 0.86 log CFU increase in *E. coli* O157:H7 after 8 h at 21°C (69.8ºF). Note that this prediction considers only growth rate and neglects lag time.	
4.f.4	*Any further information on growth/survival?*	No.	
4.f.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. 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. g	***If inactivation studies***	N/A	

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4.g.1	*What is the lethal treatment? (HPP, heat, acid, etc.)*		
4.g.2	*Which microorganisms are most resistant to the lethal treatment? (HPP, heat, acid, etc.)*		
4.g.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.g.4	*What is in the formulation that may affect inactivation? intrinsic factors that may contribute to lethality/resistance (a_w, moisture, salt, pH, fat, etc)*		
4.g.5	*Are there any data on pathogen levels in the product?*		
4gh.6	*Is there a regulatory requirement or policy for log reduction for this product? Cite requirement*		
4.g.7	*If there is no regulatory requirement for log reduction, use scientific basis for determining acceptable reduction, see NACMCF (9)*		
4gh.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 "shelf-life" as***	Assuming the product is to be held for 8 h, the product should be tested for	

	testing time	8 x 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 5-7 (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).
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.*	N/A	
5.b.1	*When inactivation treatments may result in sublethal injury, repair and growth of microorganisms during product shelf-life should be considered (7)*		
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, from patients where leafy greens were	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.,

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		implicated, or food isolates from leafy green outbreaks.	antibiotic resistance, green fluorescent protein).
6.b	***Determine if adaptation is required for inoculum preparation***	Adaptation of inoculum not needed.	Harris, unpublished data.
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 5ºC (41ºF) after inoculation to duplicate the temperature profile of the restaurant lettuce.
6.d	***Determine size of inoculum (populations e.g., log CFU/g or CFU/package, percentage of inoculum v/w or v/v)***	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.	
6.h	***How many replicates are needed to ensure confidence in data***? *Does variability in proximate*	Two replicate trials will be conducted and three samples will be analyzed at each time point and plated in	

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	analysis/production warrant > 2-3 replicate trials? Will multiple variations of similar formulations be tested? Has a statistical design for choosing formulations been used (block design, central composite, etc)?	duplicate. Each trial will use fresh lettuce from a different batch, fresh inoculum and will be conducted on a different day.	
7	**Determine other controls**		
7.a	***Are 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, 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 zero.	
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.

Table 7: Evaluation of display of fully cooked meat-filled pastry for up to 12 hours 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)***	N/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***	N/A	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***	N/A	
2	**Collect information regarding the product**		
2.a	***What are the ingredients?***	RTE 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,*	Beef filling: pH 6.2, a_w 0.97 Pastry dough: pH 7.0, a_w at	If this were an inactivation study, percent fat content may be important; not relevant for this

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	salt, fat, protein, residual nitrite, etc) for product and/or individual components?	interface is 0.97; a_w at exterior surface is 0.75.	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?*	N/A	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 (up to 2 log CFU/g Aerobic Plate Count).	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/packaging.
2.b	***What are the preparation steps?***		
2.b.1	*Is the product an assembled (multicomponent) product?*	Yes.	See product ingredients/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 RTE product (meets all regulatory requirements for cooking and cooling). One cook and cool process for the multi-component 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 81; for proper cooling, see 82)
2.b.3	*Is there a potential for*	Yes, *L. monocytogenes* is a potential	Although individually wrapped, vegetative

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	recontamination?	recontaminant.	pathogens could be introduced on external surfaces during handling/packaging. Control of this potential post-lethality contamination with *L. monocytogenes* is managed per 9 CFR 430.
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/display?*	Delivered refrigerated at or below 5ºC (41ºF) 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	

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		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/display at temperatures greater than those listed above?*	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?*	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 Tables A and B). If yes, also answer 4.g 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.
3.b	***Is an inactivation study needed? If yes, also answer 4.h 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?***	*B. cereus, C. botulinum, C. perfringens, L. monocytogenes,*	

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	If food is not seafood,* Vibrio *spp. may be excluded from consideration.	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. Post-process contamination would be limited to the outside of the pastry shell which has very low water activity 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 vs. Gram negative; vegetative microorganisms vs. 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 shel- 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	All modeling including the lag phase. This was considered appropriate given that spore-forming organisms require both germination

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		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), at 37°C (highest temp in program). ComBase Predictor predicts a 1-log increase in approximately 13 h, assuming pH 6.2, a_w 0.971 and 37°C (98.6ºF). The PMP predicts growth of *C. botulinum* in >10 days at 26.7°C (estimated room temp 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 (98.6ºF), and a slightly shorter lag time for non-proteolytic *C. botulinum* at 30°C (86ºF) and 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 at 37°C (98.6ºF) under aerobic conditions, and approximately 12 h under anaerobic conditions. ComBase predicts a 1 log increase in	and outgrowth. Predictive models estimate that *B. cereus* will grow faster than *C. perfringens* and that both organisms would grow faster than *C. botulinum*.

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		approximately 14 h at pH 6.2, a_w 0.97 and 37°C (98.6ºF) 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 (98).	
4.g.4	*Any further information on growth/survival?*	No.	
4.g.5	*Based on the above analysis, what challenge organisms are chosen for growth inhibition studies?*	*B. cereus.*	
4. h	***If inactivation studies***	N/A	
4.h.1	*What is the lethal treatment? (HPP, heat, acid, etc.)*	N/A	
4.h.2	*Which microorganisms are most resistant to the lethal treatment? HPP, heat, acid, etc.*	N/A	
4.h.3	*Will the lethality be delivered to all areas of the product that may contain the pathogen? Account for all surface and internalized contamination*	N/A	
4.h.4	*What is in the formulation that may affect inactivation? intrinsic factors that may contribute to lethality/resistance (a_w, moisture, salt, pH, fat, etc)*	N/A	
4.h.5	*Are there any data on pathogen levels in the product?*	N/A	
4.h.6	*Is there a regulatory requirement or policy for log reduction for this*	N/A	

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	product? Cite requirement		
4.h.7	*If there is no regulatory requirement for log reduction, use scientific basis for determining acceptable reduction, see NACMCF (9)*	N/A	
4.h.8	*Based on the above analysis, what challenge organisms are chosen for inactivation studies?*	N/A	
5	**Determine appropriate time and sampling intervals for challenge study**		
5.a	***For growth inhibition (TCS) studies, use 1.25 – 1.5 times "shelf-life" as testing time.***	1.5 x 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 temp 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 5-7 (preferred) sampling intervals; fewer sampling intervals should be justified, e.g., using results from similar products.*	Time 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 of growth), sampling times were set at 0 h, 14 h (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***	N/A	

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	formulation		
5.b.1	*Identify populations at 0-time and end of processing (minimum).*	N/A	
5.b.2	*Whenever possible include intermediate sampling intervals to determine death curve.*	N/A	
5.b.3	*When inactivation treatments may result in sublethal injury, repair and growth of microorganisms during product shelf-life should be considered (9).*	N/A	
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 or CFU/package, percentage of inoculum v/w or v/v)***	2-3 log CFU/g not to exceed 0.1% of filling volume.	Inoculum size is verified with time zero 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.
6.g	***Determine sampling method and***	Duplicate filled pastries will be	

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	sample size	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/production warrant > 2-3 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/formulation.
7	**Determine other controls**		
7.a	***Are use of surrogates appropriate or necessary? If so, justify.***	Surrogates are not appropriate or necessary.	
7.b	***Are uninoculated controls needed to assess spoilage, 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*.	Three log increase level selected for *B. cereus* is based on the increase suggested in the IFT Report (10). Some regulatory agencies may consider a lower

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			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.	

Table 8: Validate that the formulation of lemon meringue pie will inhibit pathogen growth under non-refrigerated 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)***	N/A	
1.c	***Validate lethality***	N/A	
1.d	***Verify that formulation will inhibit microbial growth in refrigerated foods or under mild temperature abuse***	N/A	
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*	Very consistent, same or similar lot-	

	ingredients from various sources, lot-to-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?*	N/A	
2.a.5	*What is the normal microbial load, species, etc. at the beginning and end of production?*	After baking, Aerobic Plate Count (APC) 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*	All 3 components have heat inactivation steps.	

	there different microbial reduction steps for different components?		
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/display?*	Cooled to ambient temperature after baking, shipped and displayed at ambient temperatures 20-35ºC (68-95ºF) until the end of labeled shelf-life of 3 days.	Unacceptable quality at 5 days.
2.c.3	*What potential is there for storage/display at temperatures greater than those listed above?*	Unlikely.	
2.c.4	*Are there other hazards that may be created by preparation/storage?*	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.	
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 a pH of 4.6, a_w of 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.*	

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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 (76).	
4. e	***For growth inhibition (TCS studies):***		
4.e.1	*Which pathogen(s) will grow the fastest? Consider Gram positive vs. Gram negative; vegetative microorganisms vs. 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 shelf-life, if appropriate.*	see 4.e.2.	
4.e.2	*Predictive Model*	The PMP indicates that LM will not grow, and 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 and with high a_w.	
4.e.4	*Any further information on growth/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***		

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4.f.1	*What is the lethal treatment? (HPP, heat, acid, etc.)*	N/A	
4.f.2	*Which microorganisms are most resistant to the lethal treatment? (HPP, heat, acid, etc.)*	N/A	
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.*	N/A	
4.f.4	*What is in the formulation that may affect inactivation? intrinsic factors that may contribute to lethality/resistance (a_w, moisture, salt, pH, fat, etc).*	N/A	
4.f.5	*Are there any data on pathogen levels in the product?*	N/A	
4.f.6	*Is there a regulatory requirement or policy for log reduction for this product? Cite requirement*	N/A	
4.f.7	*If there is no regulatory requirement for log reduction, use scientific basis for determining acceptable reduction, see (9).*	N/A	
4.f.8	*Based on the above analysis, what challenge organisms are chosen for inactivation studies?*	N/A	
5	**Determine appropriate time and sampling intervals for challenge study**		
5.a	***For growth inhibition (TCS) studies, use 1.25 – 1.5 times "shelf-life" as testing time***	3 days X 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 5-7 (preferred) sampling intervals; fewer sampling intervals should be justified, e.g., using results from similar products.*	Sample at time 0, then day 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.*	N/A	
5.b.1	*When inactivation treatments may result in sublethal injury, repair and growth of microorganisms during product shelf-life should be considered (9).*	N/A	
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.	

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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.***	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 non-inhibitory 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 or CFU/package, percentage of inoculum v/w or v/v)***	Target log 2-3 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 which allows exchange of air.	
6.f	***Determine the incubation temperature for growth inhibition studies or temperature(s) for thermal inactivation studies***	35°C (95ºF).	
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 plated on appropriate *Salmonella* selective agar using the FDA BAM method (95).	
6.h	***How many replicates are needed to***	3 replicate trials, pies made from	Three replicate trials with three samples at

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	ensure confidence in data? *Does variability in proximate analysis/production warrant > 2-3 replicate trials? Will multiple variations of similar formulations be tested? Has a statistical design for choosing formulations been used (block design, central composite, etc)?*	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).	each interval were chosen because of the inherent variability of inoculating individual slices for each sampling time interval.
7	**Determine other controls**		
7.a	***Are use of surrogates appropriate or necessary? If so, justify.***	No surrogates are necessary.	
7.b	***Are uninoculated controls needed to assess spoilage, competitive microflora, or for other purposes?***	Yes.	An uninoculated pie for APC and yeast and mold counts.
7.c	***What other controls are necessary? (including negative or positive growth controls)***	N/A	
8	**Determine pass-fail criteria**		
8.a	***What are the pass-fail criteria?***	Must show < 1 log growth of *Salmonella* throughout the 5 d 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.	

Table 9: 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.)***	N/A	
1.b	***Variance from any regulatory requirements (e.g., holding for >4 h without temperature control).***	N/A	
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.***	N/A	
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?*	N/A	
2.a.5	*What is the normal microbial load, species, etc. at the beginning and end of production?*	Before cooking: Aerobic Plate Count (APC) <1,000 CFU/g. After baking: <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.	
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*	Purpose of this study. All three components (crust, filling, meringue) have heat inactivation	

	the microbial reduction step? Are there different microbial reduction steps for different components?	steps, but the crust gets heat treated twice, the filling gets heat treated twice and there is an added inactivation due to the pH, the meringue gets heat treated once.	
2.b.3	*Is there a potential for recontamination?*	Very unlikely, controlled through Good Manufacturing Practices 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/display?*	Crust cook – 85ºC (185ºF) final temp, 15 min total cook in 176.7ºC (350ºF) non-humidified oven. Filling set – 90.6ºC (195ºF) for 10 min. Meringue set – 15 min total in a pre-heated 176.7ºC (350ºF) 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*	N/A; purpose of this study is to	

	storage/display at temperatures greater than those listed above?	validate microbial reduction.	
2.c.4	*Are there other hazards that may be created by preparation/storage/display?*	No.	
2.c.5	*What is the estimated maximum time from production to consumption?*	N/A	
2.c.6	*What is the time to spoilage or unacceptable quality?*	N/A	
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.***	N/A	
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.	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.

		Salmonella has been associated with numerous products containing undercooked egg ingredients including meringue pie (73).	
4.c	***What pathogens are likely to recontaminate the product after the inactivation step?***	N/A	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?***	From risk assessments conducted by FSIS regarding eggs and egg products (http://www.fsis.usda.gov/PDF/SE_Risk_Assess_Oct2005.pdf), for unpasteurized liquid whole egg product, estimates of 0 and 100 *Salmonella* spp. cells/ml, on average, are present in pooled product from multiple eggs (see Figure 3-45 from the risk assessment). In addition, for unpasteurized liquid egg white product, estimates of less than 10 *Salmonella* spp. cells/ml, on average, are present in pooled product from multiple eggs (see Figure 3-44 from the risk assessment). Finally, from the FSIS risk assessments, there are occasions when MPN levels of *Salmonella* exceed 1,000 CFU/ml for both unpasteurized liquid whole egg and liquid egg white products, but these appear to be rare events. The FSIS	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.

		data used in the risk assessments identified that *Salmonella* Enteritidis was present in some, but not all, samples collected and analyzed by FSIS. Thus, the number of *Salmonella* Enteritidis used in this inactivation study described below would represent a worst case.	
4.e	***For growth inhibition (TCS studies):***	N/A	
4.e.1	*Which pathogen(s) will grow the fastest? Consider Gram positive vs. Gram negative; vegetative microorganisms vs. 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 shelf-life, if appropriate.*	N/A	
4.e.2	*Predictive Model*	N/A	
4.e.3	*Compare choice with literature*	N/A	
4.e.4	*Any further information on growth/survival?*	N/A	
4.e.5	*Based on the above analysis, what challenge organisms are chosen for growth inhibition studies?*	N/A	
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	

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		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 that may contribute to lethality/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, see NACMCF (9)*	Using log 2 as worst case and building in a 2 log margin of safety, the target reduction is 4 logs.	
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 "shelf-life" as testing time***	N/A	
5.a.1	*Maximum time from production to*		

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	consumption		
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 5-7 (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*	Time 0 and 15 minutes.	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 (9).*	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***	Not necessary for this study	

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	for inoculum preparation		
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 the 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 or CFU/package, percentage of inoculum v/w or v/v.)***	A final target level of at least 4 log CFU/pie.	
6.e	***Determine packaging to be used.***	N/A	
6.f	***Determine the incubation temperature for growth inhibition studies or temperature(s) for thermal inactivation studies.***		
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 (95). 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.	

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6.h	***How many replicates are needed to ensure confidence in data****? Does variability in proximate analysis/production warrant >2-3 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 twelve pies will be needed for the study.	
7	**Determine other controls**		
7.a	***Are use of surrogates appropriate or necessary? If so, justify.***	No surrogates are appropriate.	
7.b	***Are uninoculated controls needed to assess spoilage, competitive microflora, or for other purposes?***	N/A	
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 176.7ºC (350ºF) oven.	Based on non-detection 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.	

Table 10: Evaluation of baked cheese pizza held out of refrigeration for up to 8 hours			
	Considerations	***Response***	***Additional comments***
1	**Determine the purpose of the study.**		
1.a	***Exempt from time/temperature control for safety (no refrigeration required)***	N/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***	N/A	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***	N/A	
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: 10% protein;	

	proximate analysis (moisture, salt, fat, protein, residual nitrite, etc) for product and/or individual components?	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?*	N/A	
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.	
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*	Yes there is potential for	Due to the large surface area, the pizza is

	recontamination?	recontamination once the pizza cools from the baking process and is handled by food service workers.	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 30ºC (86ºF).	
2.c.2	*What temperatures (and times) are expected during production, preparation, and storage/display?*	Only the baked product holding temperature is relevant – in this instance, 30ºC (86ºF) for up to 8 h at retail.	
2.c.3	*What potential is there for storage/display at temperatures greater than those listed above?*	There is the possibility that product will be held at temperatures as great as 40°C (104ºF), but quality deterioration would occur in less than 8 h.	
2.c.4	*Are there other hazards that may be created by*	*Listeria monocytogenes* contamination from the environment	

	preparation/storage?	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.	
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 Tables A and B). If yes, also answer 4.g 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.	Multi-component product. Crust has low a_w, but it 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.h 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 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, the organisms are the same 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*, *C. botulinum* spores survive baking; *L. monocytogenes*, *S. aureus* may be present from post-processing 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 (90).	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, 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.	

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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 vs. Gram negative; vegetative microorganisms vs. 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 shelf-life, if appropriate.*	No one organism was determined to grow faster. See 4.e.2.	
4.e.2	*Predictive Model*	Cheese surface: At pH 5.4, a_w 0.96, 27°C (80.6ºF): 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 lactic acid predicts a 1 log *L. monocytogenes* increase within 33 hours for the same conditions. PMP does not include *B. cereus* predictions at a_w = 0.96 but	

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		ComBase Predictor with 40% CO_2 predicts a 3 log *B. cereus* increase within 101 h. For the sauce/crust interface (pH 5.0, a_w 0.97) PMP predicts a 3 log *S. aureus* increase within 34 h (approximately 23 h without lag) under aerobic conditions; ComBase Predictor predicts a 3 log *S. aureus* increase 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 lactic acid predicts a 1 log *L. monocytogenes* 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 *B. cereus* increase 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 *S. aureus* increase within 22 h (approximately 16 h without lag)	

		under aerobic conditions; ComBase Predictor predicts a 3 log *S. aureus* increase 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 lactic acid predicts a 1 log *L. monocytogenes* 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 *B. cereus* increase within 42 h (21 h without lag).	
4.e.3	*Compare choice with literature*		
4.e.4	*Any further information on growth/survival?*		
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 that none could be completely excluded from consideration based on modeling alone.
4. f	***If inactivation studies***	N/A	
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 that may contribute to lethality/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, see (7).*		
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 "shelf-life" as testing time.***	10 h x 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*	N/A	

5.a.3	*For growth inhibition studies, determine appropriate sampling intervals for microbial analysis; use 5-7 (preferred) sampling intervals; fewer sampling intervals should be justified, e.g., using results from similar products.*	Sample 0, 4, 8, 10, 15 h.	
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*	N/A	
5.b.1	*When inactivation treatments may result in sublethal injury, repair and growth of microorganisms during product shelf-life should be considered (7).*		
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)***	Multi-strain 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

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			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 1200 g.) Individual slices of pizza will be inoculated on the surface and the sliced edge with either *S. aureus*, *L. monocytogenes* or *B. cereus.*	Each replicate will require 10 inoculated slices (two for each sampling time interval) and five control slices for each organism tested.
6.d	***Determine size of inoculum (populations e.g., log CFU/g or CFU/package, percentage of inoculum v/w or v/v)***	Not less than 2 logs of *L. monocytogenes*, *S. aureus* or *B. cereus* per g, 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 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 (86ºF).	Maximum temperature product will be exposed without adverse changes in product quality that would deter purchase and consumption.
6.g	***Determine sampling method and***	Analyze an entire slice of pizza	Slices will be tested for *S. aureus*, *B. cereus*

	sample size	(approximately 75 g).	and *L. monocytogenes* according to methods provided in Appendix A.
6.h	***How many replicates are needed to ensure confidence in data***? *Does variability in proximate analysis/production warrant > 2-3 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	***Are use of surrogates appropriate or necessary? If so, justify.***	No surrogates used.	
7.b	***Are uninoculated controls needed to assess spoilage, 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 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 (10). 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 only applicable 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.

Agence Française de Sécurité Santitaire des Aliments. 2008. Technical Guidance Document on shelf-life studies for *Listeria monocytogenes* in ready-to-eat foods. Available at http://ec.europa.eu/food/food/biosafety/salmonella/docs/shelflife_listeria_monocytogenes_en.pdf. Accessed December 1, 2008.